Importance of the Different Proteolytic Sites of the Proteasome and the Efficacy of Inhibitors Varies with the Protein Substrate*

The relative importance of the different proteolytic sites in mammalian proteasomes in protein degradation has not been studied systematically. Nevertheless, it is widely assumed that inhibition of the chymotrypsin-like site, the primary target of the proteasome inhibitors used in research and cancer therapy, reflects the degree of inhibition of protein breakdown. Here we demonstrate that selective inactivation of the chymotrypsin-like site reduced degradation of model proteins by pure 26 S proteasomes by only 11–50% and decreased only slightly the breakdown of proteins in HeLa cells. Inactivation of the caspase-like site decreased breakdown of model proteins by 12–22% and of the trypsin-like site by 3–35%. The relative contributions of these different sites depended on the protein substrate, and the importance of the trypsin-like sites depended on the substrate’s content of basic residues. Simultaneous inhibition of the chymotrypsin-like and the caspase- or trypsin-like sites was needed to reduce degradation by > 50%. Thus, 1) all three types of active sites contribute significantly to protein breakdown, 2) their relative importance varies widely with the substrate, 3) assaying the chymotrypsin-like activity overestimates the actual reduction in protein degradation, and 4) inhibition of multiple sites is required to markedly decrease proteolysis.

The 26 S proteasome catalyzes the degradation of most proteins in eukaryotic cells (1, 2). This large ATP-dependent complex consists of the 20 S proteasome, in which proteins are degraded, plus one or two 19 S regulatory complexes (3–5). A variety of low molecular weight inhibitors of the proteolytic sites of the 20 S particles have been identified that can enter cells and block degradation by the ubiquitin-proteasome pathway (6). These agents have proven to be very valuable tools for studying the intracellular functions of the proteasome and have facilitated the discovery of numerous regulatory functions of this pathway. For example, proteasome inhibitors can block cell division, inflammation through inhibition of NF-κB activation, and the generation of antigenic peptides. Because these inhibitors also induce stress responses (the heat-shock and unfolded protein responses) and apoptosis, especially of tumor cells (6), they represent a promising new type of anticancer drug. In fact, the peptide boronate inhibitor, bortezomib (Velcade®), has been approved by the FDA for the treatment of multiple myeloma and is now undergoing clinical trials for many other types of cancer (7–9).

The 20 S proteasome contains six active sites. Two of the sites are chymotrypsin-like in specificity and cleave preferentially after hydrophobic residues, two are trypsin-like in specificity and cut after basic residues, and two are caspase-like and cleave preferentially after acidic residues (10–12). The relative contributions of these different types of active sites to the breakdown of different proteins have not been studied systematically by rigorous biochemical or pharmacological approaches. In yeast, inactivation of the chymotrypsin-like site, by mutation of the catalytic threonine residue of the chymotrypsin-like site, resulted in a significant growth retardation, increased sensitivity to heat and canavanine (an arginine analog whose incorporation causes production of misfolded proteins), and a large reduction in the degradation rate of the MATα2 transcription factor and several short-lived versions of β-galactosidase (13, 14). Similar mutations of the catalytic threonine in the caspase-like site caused no phenotypic defects and did not alter the rates of degradation of these substrates (14, 15). Inactivation of the trypsin-like sites reduced growth rates slightly, decreased sensitivity to cadmium (which also causes production of misfolded proteins), and surprisingly reduced the degradation rate of two model substrates (Leu-β-galactosidase and Arg-β-galactosidase), but not of two others (MATα2 and Ub-Pro-β-galactosidase). A strain lacking both the caspase-like and trypsin-like sites had a slightly stronger phenotype than strains lacking either site alone but fewer phenotypic defects than the strain lacking the chymotrypsin-like activity (14). Consequently, it was concluded that the chymotrypsin-like site is rate-limiting in protein breakdown by the proteasomes. These studies also suggest that the trypsin-like and especially the caspase-like sites play little, if any, role in protein degradation, a surprising conclusion, because these active sites have evolved specifically in eukaryotes. Bacterial and archaeal proteasomes have multiple identical active sites of broad specificity (16).

Even less is known about the contributions of different proteasomal sites to protein breakdown by mammalian proteasomes. Because the studies in yeast had suggested a primary role of the chymotrypsin-like sites, and because of the ability of hydrophobic peptides to enter cells, the various synthetic proteasome inhibitors were optimized according to their capacity to block the chymotrypsin-like sites (17). Little attention was paid to their ability to inhibit the caspase- or trypsin-like sites (6). It is therefore unclear whether the inhibition of protein breakdown results from the inhibition of the chymotrypsin-like sites alone, or if a reduction in proteolysis also requires inhibition of other activities. Nevertheless, the ability of inhibitors to reduce the hydrolysis of a model fluorogenic substrate by the chymotrypsin-like sites has often been assumed to represent the degree of inhibition of protein degradation (8, 18), despite several studies demonstrating that the trypsin-like and caspase-like sites also contribute to the degradation of model proteins.

* This work was supported in part by Grant GM046147-10 from the NIGMS, National Institutes of Health and from the National Space Biomedical Research Institute funded by NASA (to A. L. G.), and by a special fellowship from the Leukemia and Lymphoma Society (to A. F. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors contributed equally to this work.

2 Present address: Norris Cotton Cancer Center, and the Dept. of Pharmacology and Toxicology, Dartmouth Medical School, 1 Medical Center Dr., Lebanon, NH 03756.

3 A Senior Fellow of the Ellison Foundation. To whom correspondence should be addressed: Dept. of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1655; Fax: 617-432-1144; E-mail: alfred_goldberg@hms.harvard.edu.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Proteasome Active Sites Role in Protein Breakdown

by purified 20 S proteasomes (19, 20) and to the degradation of cystic fibrosis transmembrane conductance regulator protein in reticulocyte lysates (20, 21).

The relative contributions of the different active sites of proteasome to protein breakdown in mammalian cells are of particular interest to understand the physiological and therapeutic impact of these inhibitors. In the blood of multiple myeloma patients, bortezomib at maximal tolerated doses blocked the chymotrypsin-like sites by 80% and did not affect the trypsin-like sites, and its effects on the caspase-like sites or total protein degradation have not been analyzed (8, 22). In biochemical or cell biological studies, the failure of a proteasome inhibitor to block a process has often been interpreted as evidence of a nonproteasomal degradative system (reviewed in Ref. 23), on the untested assumption that protein degradation by proteasomes is completely blocked.

In this study, we have used site-specific inhibitors of the different active sites to systematically investigate the roles of these sites in the degradation of several different model unfolded proteins by purified mammalian 26 S proteasomes. These proteins (casein, histones, aged calmodulin, and denatured ovalbumin) are degraded in an ATP-dependent linear manner in the absence of ubiquitination (24 –26), and the rates of peptide bond cleavage in these polypeptides can be readily and accurately measured. On this basis, we subsequently investigated the effects of different cell-permeable proteasome inhibitors with known active site specificities on the breakdown of long-lived proteins in HeLa cells.

We demonstrate here that inactivation of one type of site is not sufficient to markedly block protein degradation, that the chymotrypsin-like site is not rate-limiting for many proteins, and that significant inhibition of protein breakdown is observed only when the chymotrypsin-like sites and either the trypsin-like or caspase-like sites are also inhibited. Thus, in mammalian cells, the caspase-like and trypsin-like sites play a significant, generally unappreciated role in protein degradation, but the relative importance of the three active sites depends on the protein being degraded.

MATERIALS AND METHODS

Proteasome Substrates and Inhibitors—All fluorogenic substrates were purchased from Bachem. 4-Hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-vinylsulfone (NLVS)4 was a kind gift of Dr. Huib Ovaa (Department of Pathology, Harvard Medical School); leupeptin (Ac-Leu-Leu-Arg-al) was purchased from Roche Applied Science; Ac-APnLD-al was synthesized as described previously (12). Epoxomicin and clasto-lactacystin–β-lactone were purchased from Millenium Pharmaceuticals. Bovine β-casein, ovalbumin, and calmodulin were purchased from Sigma. A natural mixture of histones H1, H2A, H2B, H3, and H4 from calf thymus was obtained from Roche Applied Science.

Proteasome Purification and Peptidase Assays—26 S proteasomes were purified to homogeneity from rabbit muscles as described previously (27). The activity of the chymotrypsin-like sites was assayed using Suc-LLVY-amc (100 μM), of the caspase-like sites with Ac-nLPnLD-amc (100 μM) and of the trypsin-like sites with Boc-LRR-amc (100 μM or 1 μM). These fluorogenic substrates were incubated with proteasomes in assay buffer (50 mM Tris-HCl, pH 7.4, 40 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, and 0.05 mM/ml bovine serum albumin), and the release of 7-amino-4-methylcoumarin (amc) was monitored contin-uously (excitation, 380 nm; emission, 460 nm). The rate of cleavage was determined from the slope of the reaction progress curves.

Inhibition of Purified 26 S Proteasome by NLVS—To inactivate the chymotrypsin-like sites, 26 S proteasomes were incubated with either NLVS (4 μM) or Me2SO control (1%) at 37 °C for 30 min. After 30 min, the extent of inhibition of each active site was assayed, and the proteasomes were dialyzed overnight with dialysis buffer containing 20 mM HEPES, 40% glycerol, 5 mM MgCl2, 1 mM dithiothreitol, and 0.5 mM ATP prior to storage at −20 °C. Inactivation of the chymotrypsin-like activity was confirmed after dialysis by repeating the peptidase assay.

Degradation of Model Proteins by Purified 26 S Proteasomes—In order for calmodulin to be degraded rapidly in vitro by 26 S proteasomes, it was “aged” by incubation for 2 weeks at 37 °C in 50 mM HEPES containing 1 mM EGTA (25). Ovalbumin was denatured by treatment with performic acid as described previously (24). β-Casein and histones were degraded by 26 S proteasomes without prior denaturation. NLVS-treated or control 26 S proteasomes were preincubated with Ac-APnLD-al (25 μM) to inhibit the caspase-like sites, leupeptin (40 μM) to inhibit the trypsin-like sites, or Me2SO controls (1%) for 20 min at 37 °C to allow these slow binding inhibitors to bind to the active sites. Denatured ovalbumin (5 μM), casein (10 μM), histones (10 μM), and aged calmodulin (10 μM) were then added to these pre-treated proteasomes and incubated at 37 °C in the presence or absence of Ac-APnLD-al (25 μM) or leupeptin (40 μM). The final concentration of proteasomes in the assay was 56 μg/ml (or 28 μM assuming a molecular mass of 2 MDa). Rates of peptide bond cleavages in these proteins were measured by determining the appearance of new amino groups with fluorescamine (28). This assay was chosen because it does not require the modification of any of the amino acids in the proteins, as do other assays that involve labeling of proteins with fluorescent dyes or radiolabeling. Aliquots were taken at different times up to 120 min, and proteins were separated from peptides by precipitation with 5% perchloric acid. After centrifugation for 15 min at 20,000 × g, the supernatant was then neutralized with KOH. The fluorescamine assay was performed (in duplicate) at pH 6.8, which minimizes the reaction of side-chain amino groups with fluorescamine. A mixture of standard peptides was used to calibrate the assay and determine the amount of products generated (29). Assuming that proteasomes cut on average every eighth peptide bond in a polypeptide (24), consumption of the protein substrates at the end of incubation did not ever exceed 10%. The values of fluorescence were plotted against time, and rates of degradation were determined from the slopes of the resulting reaction progress curves.

Isolation and Assay of 26 S Proteasomes from HeLa Cells—HeLa cells were grown in 10-cm plates up to 50 – 80% confluency. These cells were then incubated for 1 h at 37 °C with media containing proteasome inhibitors, after which they were either harvested or incubated for an additional 3 h without inhibitors before harvesting. After washing three times with ice-cold phosphate-buffered saline, the cells were resuspended in the homogenization buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM ATP). Cells were permeabilized by the addition of 0.025% digitonin and incubation on ice for 5 min. The cytosol was “squeezed out” by centrifugation for 10 min at 20,000 × g (30) and then centrifuged for a further 2 h at 300,000 × g (31). This approach was found to yield similar results as centrifugation for 5 h at 100,000 × g to pellet 26 S proteasomes (32). After dissolving the pellet in the homogenization buffer, the peptidase activities of the proteasome were measured using Suc-LLVY-amc (100 μM) for the chymotrypsin-like activity, Ac-nLPnLD-amc (100 μM) for the caspase-like activity (12), and Boc-LRR-amc (1 μM) for the trypsin-
Proteasome Active Sites Role in Protein Breakdown

like site in the proteasome assay buffer as described above for purified proteasomes.

Assays of Protein Degradation in Cells—Degradation of long-lived proteins was measured by metabolic labeling of proteins with [3H]tyrosine, and following the release of radioactive tyrosine in the media during the chase period. HeLa cells were grown in DMEM in 6-well plates to ~60 – 80% confluency. The cells were washed three times with warm, serum-free DMEM, and incubated for 16 h with [3H]tyrosine (5 μCi/ml) in medium (2 ml) containing 10% dialyzed fetal bovine serum. The cells were then washed three times with DMEM containing unlabeled tyrosine (2 mM) and chased at 37 °C with DMEM (10% fetal bovine serum) containing 2 mM unlabeled tyrosine (to prevent re-incorporation of [3H]tyrosine into proteins) for 1 h to allow for the degradation of short-lived proteins. The media was then replaced with fresh media containing the proteasome inhibitor being tested (dissolved in 1% Me2SO) or 1% Me2SO for the control. Each inhibitor was added to 6 wells in parallel, and the cells were incubated with the inhibitors for 1 h at 37 °C. The inhibitor-treated cells were then washed once with DMEM containing unlabeled tyrosine (2 mM) and incubated for a further 3 h at 37 °C in a final volume of 2 ml of DMEM containing 10% fetal bovine serum and unlabeled tyrosine (2 mM). 200-μl aliquots were taken at different times, and trichloroacetic acid (final concentration, 10%) was added. These aliquots were incubated on ice for 30 min, centrifuged for 15 min at 20,000 × g, and the amount of trichloroacetic acid-soluble radioactivity determined by liquid scintillation counting.

To determine the total amount of radioactivity initially incorporated into cell proteins, the media remaining after the last time point was measured by metabolic labeling of proteins with [3H]tyrosine, and following the release of radioactive tyrosine in the media during the chase period. HeLa cells were grown in DMEM in 6-well plates to ~60 – 80% confluency. The cells were washed three times with warm, serum-free DMEM, and incubated for 16 h with [3H]tyrosine (5 μCi/ml) in medium (2 ml) containing 10% dialyzed fetal bovine serum. The cells were then washed three times with DMEM containing unlabeled tyrosine (2 mM) and chased at 37 °C with DMEM (10% fetal bovine serum) containing 2 mM unlabeled tyrosine (to prevent re-incorporation of [3H]tyrosine into proteins) for 1 h to allow for the degradation of short-lived proteins. The media was then replaced with fresh media containing the proteasome inhibitor being tested (dissolved in 1% Me2SO) or 1% Me2SO for the control. Each inhibitor was added to 6 wells in parallel, and the cells were incubated with the inhibitors for 1 h at 37 °C. The inhibitor-treated cells were then washed once with DMEM containing unlabeled tyrosine (2 mM) and incubated for a further 3 h at 37 °C in a final volume of 2 ml of DMEM containing 10% fetal bovine serum and unlabeled tyrosine (2 mM). 200-μl aliquots were taken at different times, and trichloroacetic acid (final concentration, 10%) was added. These aliquots were incubated on ice for 30 min, centrifuged for 15 min at 20,000 × g, and the amount of trichloroacetic acid-soluble radioactivity determined by liquid scintillation counting.

To determine the total amount of radioactivity initially incorporated into cell proteins, the media remaining after the last time point was removed, and the cells dissolved in 2 ml of NaOH (0.2 N). Aliquots of these dissolved cells were then counted, and the radioactivity released into the media at each time point was calculated as a percentage of total radioactive protein. The rates of protein breakdown (~1%/h in the control cells) were calculated from the slopes of the plot. The degree of inhibition of protein breakdown was determined by dividing the rate in the presence of the inhibitor by the rate in the controls.

### TABLE 1
Effect of inhibitors on peptidase activities of the 26 S rabbit proteasomes

| Substrate                  | Inhibitors            | % inhibition of active sites |
|----------------------------|-----------------------|----------------------------|
| NLVS                       | Ac-APnLD-al           | 95 ± 0.5                   |
| Ac-APnLD-al                | Leupeptin             | 11.5 ± 9.5                 |
| Caspase-like site          |                       | 15.5 ± 7.5                 |
| Trypsin-like site          |                       | 7 ± 2                      |

### TABLE 2
Effect of inhibition of individual active sites on the degradation of different model proteins by purified 26 S rabbit proteasomes

| Sites inactivated | Inhibitor                  | Protein substrate | % inhibition of protein degradation |
|------------------|----------------------------|-------------------|------------------------------------|
| Chymotrypsinlike | NLVS                       | Casein            | 50 ± 1                             |
|                  |                            | Ovalbumin         | 11 ± 8                             |
|                  |                            | Histones          | 35 ± 2                             |
|                  |                            | Calmodulin        | 17 ± 5                             |
| Caspase-like     | Ac-APnLD-al               | Casein            | 12 ± 7                             |
|                  |                            | Ovalbumin         | 9 ± 7                              |
|                  |                            | Histones          | 19 ± 8                             |
|                  |                            | Calmodulin        | 22 ± 7                             |
| Trypsin-like     | Leupeptin                  | Casein            | 3 ± 2                              |
|                  |                            | Ovalbumin         | 14 ± 1                             |
|                  |                            | Histones          | 30 ± 13                            |
|                  |                            | Calmodulin        | 13 ± 1                             |

* NLVS (4 μM), Ac-APnLD-al (25 μM), and leupeptin (40 μM) inhibited each active site by >95% (see Table 1). These numbers correspond to means ± range of two observations.

RESULTS

Effect of Inhibiting Individual Active Sites on Degradation of Different Proteins—We initially studied the relative contributions of the three types of active sites of the proteasome to the breakdown of several model proteins by purified rabbit muscle 26 S particles. Casein, oxidized ovalbumin, and aged calmodulin were chosen as substrates, because they are degraded by purified 26 S proteasomes without ubiquitylation at significant rates that are linear for several hours in the presence of ATP (24, 25). We also studied the degradation of bovine histones (a mixture of H1, H2A, H2B, H3, and H4) because of their high content of basic amino acids. The histones were hydrolyzed by 26 S proteasomes at about half of the rate of casein (not shown) also without ubiquitylation, presumably because these proteins by themselves have little tertiary structure. Each of these proteins was incubated at 37 °C with 26 S proteasomes in the presence of ATP. Degradation was followed using fluororescine to measure the rate of cleavage of peptide bonds (i.e. the production of new amino groups) (28, 33). The appearance of amino groups was previously shown to be proportional to the rates of disappearance of full-length proteins (28). The degradation of these model substrates by this assay occurred at linear rates for >120 min (data not shown). Prior work had demonstrated that, on the average, proteasomes cleave every eighth peptide bond in a protein (although peptide products actually range from 2–24 residues in length) (24). We therefore estimate that not >10% of the substrate was consumed at the end of the incubation.

To assess the contributions of different active sites to protein degradation, the proteasomes were preincubated with specific inhibitors of individual active sites. NLVS was used to inactivate the chymotrypsin-like sites, leupeptin to block the trypsin-like sites, and Ac-APnLD-al (12) to inhibit the caspase-like sites. NLVS is an irreversible inhibitor, and at the concentration used it reproducibly caused a 95% inhibition of the chymotrypsin-like sites without significant effects on the two other activities (Table 1). Irreversible inhibitors of the trypsin-like and caspase-like sites, which could achieve similar specific inhibition of their targets, are not available. Therefore, we used the reversible inhibitors of these sites, leupeptin and Ac-APnLD-al (12). Leupeptin was used at a concentration (40 μM), which exceeds its Ki value for the trypsin-like site (1 μM) by 40-fold. Ac-APnLD-al was used at a concentration (25 μM), which exceeds its Ki value for the caspase-like activity (0.4 μM) (12) by >50-fold. A large excess of inhibitor was used to minimize the possibility that stretches of a protein with higher affinity to the active site would be cleaved by these sites even in the presence of the competitive inhibitors. As shown in Table 1, these agents achieved a 95% inhibition of the targeted sites at these concentrations without significantly inhibiting the other two sites (using fluorogenic substrates specific for each site to assay inhibition).

We then compared the rates of degradation of different proteins by control and inhibitor-treated proteasomes. Surprisingly, a 95% inhibition of the chymotrypsin-like site decreased the degradation of casein by only 50 ± 1% (Table 2) and decreased reproducibly the hydrolysis of other proteins to a lesser extent: the histones by 35 ± 2%, calmodulin by...
17 ± 5%, and ovalbumin by only 11 ± 8%. Thus, the contribution of the chymotrypsin-like sites to degradation depends on the protein being hydrolyzed.

Even smaller reductions in proteolysis were seen upon inhibition of the other sites. Inhibition of the trypsin-like sites by 95% with leupeptin decreased the degradation of histones by 30 ± 13% but calmodulin by only 13 ± 1% and ovalbumin by 14 ± 1%. Thus, the contributions of the trypsin-like sites to the breakdown of these proteins are quite similar to that of the chymotrypsin-like sites. However, loss of the trypsin-like activity did not reduce significantly the degradation of casein, in sharp contrast to the 50% inhibition of its degradation upon inactivation of the chymotrypsin-like site. Thus, the importance of the trypsin-like sites varies widely depending on the protein being degraded and, apparently, depends on amino acid composition. With four substrates, the degree of inhibition of protein degradation by leupeptin correlated very strongly (r = 0.950) with their content of basic residues (Fig. 1). By contrast, no correlation was found between the percentage of hydrophobic residues and the degree of inhibition seen upon inactivation of the chymotrypsin-like site with NLVS, presumably because other properties influence susceptibility of sequences to cleavage by this active site.

Inhibition of the caspase-like sites by 95% with Ac-APnLD-al also resulted in only small reductions (9–22%) in the degradation rates of these four proteins. The inhibitor of the caspase-like sites reduced degradation of casein by 12 ± 7% and histones by 19 ± 8%, which were much smaller than the inhibitions seen upon inactivation of the chymotrypsin-like site. However, inhibition of the caspase-like sites caused a similarly small reduction in the degradation of ovalbumin (9 ± 7%) and calmodulin (22 ± 7%), as did inhibition of the chymotrypsin-like or trypsin-like sites. No correlation was found between the content of acidic residues of these proteins and the extent of inhibition of their degradation by Ac-APnLD-al, presumably because the caspase-like sites can also cleave readily after certain hydrophobic residues (12, 34).

**Effects of Inhibition of Two or All Three Types of Active Sites**—Thus, the individual active sites of the proteasome appear largely redundant for the degradation of these model proteins, although the importance of these specific active sites varied markedly with different proteins. These findings imply that after one type of site is inactivated (Table 2), the four residual sites can catalyze protein breakdown, although at slower rates. We therefore examined the effects on proteolysis of simultaneous inhibition of two types of active sites (Table 3). As expected, loss of two types of sites consistently reduced protein breakdown much more than inhibition of a single type of site. In fact, two site-specific inhibitors together consistently had at least additive effects in reducing protein degradation, but generally (Table 3 and Fig. 2), they caused a greater inhibition than what would be predicted based on adding the effects of each inhibitor alone (Table 2). For example, blocking both the caspase- and trypsin-like sites was expected to cause only a 15% reduction in casein hydrolysis (based on the 12% plus 3% effects alone) but instead caused a 45% decrease, whereas blocking both the chymotrypsin- and trypsin-like sites caused a 43% inhibition of ovalbumin degradation, instead of the expected 20% (based on the 11% and 9% effects alone).

With ten of the twelve mixtures of inhibitors and substrates, the effects were more than additive (Fig. 2), and this trend was highly significant (p = 0.002) when differences were analyzed by Student’s t test. Thus, upon inactivation of one type of site, the other active sites can become increasingly important in digesting proteins to peptides. Consequently, the nature of the cleavages made must differ, and the rates of proteolysis measured upon inactivation of one site (Table 1) do not simply reflect the contribution of that site but also the capacity of the residual sites to catalyze the degradation of the specific substrate.

If these inhibitors were equally effective against sequences in proteins as for fluorogenic tetrapeptide substrates (Table 1), inhibition of all three types of active sites by 95% would be expected to reduce protein cleavages between 95 and 86% (0.95 × 0.95 × 0.95). In fact, with all three inhibitors present, breakdown of casein, ovalbumin, and histones fell, as predicted, by 87–91% (Table 3). However, the three inhibitors together reduced calmodulin degradation by 73 ± 9%, which probably also does not differ from the anticipated low value of 86% plus experimental error. Considering the potential errors involved in such determinations, the agreement is very strong and clearly supports the validity of this approach and the assumption that the inhibition of activity against the fluorogenic substrates (even by the competitive inhibitors, leupeptin and Ac-APnLD-al) approximates the degree of inhibition of peptide bond cleavages in proteins.

**Inhibition of Active Sites of Proteasome Inside Cells**—We then set out to test whether the different active sites of proteasome play similar roles in protein degradation in mammalian cells, as suggested by these findings on pure proteasomes. Our ability to study the contributions of individual active sites to protein breakdown inside cells is limited by the lack of cell-permeable-specific inhibitors of the trypsin-like and caspase-like sites. Leupeptin is cell-permeable, but its primary targets are lysosomal proteases. Peptide vinylsulfones specific to the trypsin-like sites (35) and the inhibitor of caspase-like sites used in this study (Ac-APnLD-al) are not cell-permeable. The only cell-permeable inhib-

---

**TABLE 3**

Effect of inhibition of two active sites on the degradation of different proteins by purified 26 S proteasomes

| Active sites inactivated | Inhibitors | Protein substrate | Casein % inhibition of protein degradation | Ovalbumin | Histones | Calmodulin |
|-------------------------|------------|------------------|------------------------------------------|-----------|---------|-----------|
| Chymotrypsin- and caspase-like | NLVS and Ac-APnLD-al | 70 ± 7 | 31 ± 5 | 50 ± 2 | 53 ± 1 |
| Chymotrypsin- and trypsin-like | NLVS and leupeptin | 68 ± 8 | 43 ± 6 | 63 ± 5 | 40 ± 8 |
| Caspase- and trypsin-like | Ac-APnLD-al and leupeptin | 45 ± 6 | 30 ± 16 | 56 ± 14 | 43 ± 4 |
| Chymotrypsin-caspase- and trypsin-like | NLVS and Ac-APnLD-al and leupeptin | 91 ± 0 | 89 ± 4 | 87 ± 5 | 73 ± 9 |

* NLVS (4 μM), Ac-APnLD-al (25 μM), and leupeptin (40 μM) inhibited each active site by >95% (see Table 1). These numbers correspond to means ± range of two observations.
Proteasome Active Sites Role in Protein Breakdown

TABLE 4
Extent of inhibition of different proteasomal activities after 1-h treatment of HeLa cells with different inhibitors

| Inhibitor | Concentration µM | Chymotrypsin-like | Caspase-like | Trypsin-like | % inhibition of active sites |
|-----------|------------------|-------------------|--------------|--------------|----------------------------|
| Bortezomib | 0.05             | 68.5 ± 7.5        | 20 ± 10      | 10 ± 4       |                             |
|           | 0.15             | 81 ± 1.5          | 24 ± 6       | 3 ± 3        |                             |
|           | 0.5              | 92.3 ± 1.5        | 90 ± 2       | 19 ± 6       |                             |
| Epoxomicin | 0.15             | 85 ± 6            | 1 ± 1        | 28 ± 6.5     |                             |
| Clasto-lactacystin-β-lactone | 2.10 | 97.5 ± 0.6 | 3 ± 3 | 87 ± 4 |                             |
| NLVS      | 50               | 96 ± 1            | 36 ± 2       | 43 ± 4       |                             |
| MG-262    | 1                | 99.5 ± 0.3        | 54 ± 4       | 83 ± 5       |                             |
|           | 4                | 86 ± 5            | 13 ± 7       | 7 ± 6        |                             |

Results are means ± S.E. of three to six independent experiments.

![TABLE 4](image)

FIGURE 2. The inhibition of the two active sites has synergistic effects in reducing protein degradation by 26 S proteasomes. For each protein substrate, the inhibition observed when inhibitors of two different active sites were used simultaneously (Table 3) was plotted against the sum of the inhibitions (Table 2) observed when these agents were used independently (% inhibition expected). NLVS was used to inactivate the chymotrypsin-like sites, Ac-APnLD-al the caspase-like sites, and leupeptin the trypsin-like sites, as shown in Table 1. To determine if the synergistic effect of two inhibitors is statistically significant, the distances of each data point from the line of identity (dashed line on the figure) were analyzed by the Student’s t test (using Kaleidagraph Software package). The mean distance of 10.2 ± 2.6% (mean ± S.E.) was found to be highly significant (p < 0.002).

These inhibitors were used to treat HeLa cells. To assay proteasomal activities in extracts of control and inhibitor-treated HeLa cells with the standard fluorogenic peptide substrates used against pure proteasomes, it is essential to show that these substrates are not digested by other proteolytic enzymes on these subcellular proteolytic enzymes. We have recently shown (31) that to eliminate the activity of other cellular proteolytic enzymes on these substrates, the cell extract can be prepared by “squeezing out” the cytosol from digitonin-permeabilized cells (expected to contain largely cytosolic proteases), the cell extract can be prepared by “squeezing out” the cytosol from digitonin-permeabilized cells (30), followed by partial purification of proteasomes from digitonin-permeabilized cells (30), followed by partial purification

To test whether the commonly used proteasome inhibitors (epoxomicin, bortezomib, clasto-lactacystin-β-lactone, NLVS, and MG262), in addition to the chymotrypsin-like sites, also inhibit the caspase-like and trypsin-like activities of the proteasome by ultracentrifugation of the cytosolic fraction. Out of five inhibitors tested, only NLVS was able to achieve an inhibition of >80% of the chymotrypsin-like sites without significantly inhibiting the other two activities (Tables 4 and 5). In addition to the chymotrypsin-like site, bortezomib inhibited the caspase-like sites in cells (Table 4), as was also noted recently by Berkers et al. (40) and Altun et al. (41). (We found that bortezomib blocks two sites in the purified 26 S proteasomes, where the Ki was 19 ± 4 nM for the chymotrypsin-like site and 240 ± 45 nM for the caspase-like site.) Epoxomicin inhibited the trypsin-like, in addition to the chymotrypsin-like, activities (Table 4). MG262 and clasto-lactacystin-β-lactone inhibited all three active sites (Table 4).

Proteasomes are present in both nuclear and cytosolic fractions, and a small amount is associated with the endoplasmic reticulum (42). To determine if the inhibitors reach and affect proteasomes in all these fractions similarly, the proteasome peptidase activities in extract from digitonin-permeabilized cells (expected to contain largely cytosolic proteasomes) were compared with those in the remaining cytosol-free cell pellet. The residual proteasomes in the pellet fraction must be derived primarily from nuclei and partially from the ER. These pellets were disrupted by sonication, and after centrifugation at 20,000 × g, their peptidase activities were measured. High concentrations of epoxomicin (31) blocked 95% of the cleavages of substrates of the chymotrypsin- and caspase-like sites in these extracts. Cleavage of these peptides was reduced by >10-fold upon ATP depletion. In addition, cleavage of Ac-nLPnLD-amc was not inhibited by pan-caspase inhibitor Z-VAD-chloromethyl ketone. Thus, the chymotrypsin-like and caspase-like activities of the proteasome can be measured in cytosolic and residual extracts of HeLa cells without further purification. In contrast, the cleavage of substrates of the tryptic-like sites in residual extracts prepared by sonication is not significantly inhibited by epoxomicin or upon ATP depletion and, therefore, must be carried out by other proteolytic enzymes. These enzymes were still present in the proteasome fraction after ultracentrifugation, and no trypsin-like activity could be measured in these extracts. Therefore, we compared the inhibition of the chymotrypsin-like and caspase-like activities in cytosolic and residual extracts of NLVS-, epoxomicin-, and bortezomib-treated cells and found that it was quite similar (Table 5). Thus, measuring inhibition of proteasomes in the material squeezed out from digiton-
Inhibition of proteasomal active sites in HeLa cells after 1h inhibitor-treatment and 3hrs after inhibitor removal

| Inhibitor | Concentration | Time without inhibitors | Chymotrypsin-like site | Caspase-like site | Trypsin-like site |
|-----------|---------------|-------------------------|------------------------|-------------------|------------------|
|           | µM            | h                       |                        |                   |                  |
| NLVS      | 50            | 0                       | 68 ± 7.5               | 20 ± 10           | 10 ± 4           |
| Bortezomib| 0.5           | 0                       | 47 ± 13                | 26 ± 13           | 0 ± 0            |
| Epoxomicin| 0.15          | 0                       | 81 ± 1.5               | 24 ± 6            | 3 ± 3            |
|           | 2             | 3                       | 68 ± 5                 | 33 ± 9            | 6.5 ± 5          |
|           | 5             | 3                       | 92.3 ± 1.5             | 90 ± 2            | 19 ± 6           |
|           | 15            | 0                       | 77 ± 5                 | 75 ± 7            | 11 ± 6           |
| Epoxomicin| 0.15          | 3                       | 85 ± 6                 | 1 ± 1             | 28 ± 6.5         |
|           | 2             | 3                       | 80 ± 5                 | 6 ± 6             | 26 ± 6           |
|           | 50            | 3                       | 97.5 ± 0.6             | 3 ± 3             | 87 ± 4           |
| NLVS      |               | 0                       | 94 ± 2                 | 8 ± 9             | 79 ± 6.5         |
|           |               | 3                       | 86 ± 10                | 13 ± 15           | 7 ± 12           |
|           |               | 3                       | 73 ± 6                 | 12 ± 5            | 11 ± 7           |

Results are means ± S.E. of three to six experiments.

Inhibitors affect similarly the activity of digitonin-extracted (cytosolic) and residual (nuclear-ER) proteasomes

After 1-h exposure to inhibitors, cells were either collected immediately (0 h) or incubated for an additional 3 h without inhibitors (see Table 4), and then collected. Inhibition was measured in crude extracts obtained by squeezing-out cytosol from digitonin-permeabilized cells and subsequent sonication of the cell pellet (residual). Inhibition of the trypsin-like sites in these fractions cannot be measured because of the presence of large amount of nonproteasomal proteolytic enzymes that cleave basic substrates.

One potential problem with such studies is that if inhibitors were present for prolonged periods, the degree of inhibition might increase with time due to continuing reaction of the inhibitors with the active sites, or to some indirect mechanism. Therefore, cells were treated with irreversible inhibitors for 1 h, the inhibitors were removed, and protein breakdown was measured during the 3-h chase. In a parallel experiment, to analyze the effects of inhibition of the active sites on protein breakdown, the degree of inhibition of each active site was measured with site-specific fluorogenic substrates immediately after the treatment with inhibitors and 3 h later. Although the degree of inhibition decreased only slightly (if at all) during the 3 h after removal of the inhibitors (Table 6), we routinely averaged the degree of inhibition at the start and end of the 3-h measurement period to give a more accurate value of percentage inhibition (Table 7).

Surprisingly, inhibition of the chymotrypsin-like sites by 58% with 0.05 µM bortezomib caused little or no inhibition of protein breakdown (Table 7). Even at concentrations of bortezomib that caused a 75% inhibition of the chymotrypsin-like activity (0.15 µM), there was only a 14% inhibition of the degradation of long-lived proteins. It is noteworthy that this inhibition of the chymotrypsin-like site resembles that observed in patients treated with bortezomib and is sufficient to cause apoptosis of multiple myeloma cells (8). A similar small inhibition of protein breakdown (13%) was caused by NLVS, which inhibited the chymotrypsin-like activity by 80%. Clearly, the degree of inhibition of the chymotrypsin-like sites in cells greatly overestimates the inhibition of protein degradation, in accord with our findings on pure proteasomes. A somewhat larger (21%) inhibition of cellular protein breakdown was observed with 0.15 µM epoxomicin, which, in addition to decreasing the chymotrypsin-like activity by 90%, inhibited the trypsin-like activity by 27%.

A large (56%) reduction in proteolysis was achieved with 2 µM epoxomicin, which inhibited the trypsin-like sites by 83% and the chymotrypsin-like activity by 96%. When the chymotrypsin- and caspase-like sites were both inhibited by 85% with bortezomib (0.5 µM), the rate of protein breakdown was also reduced significantly by 40% (which is slightly less than the inhibition seen upon inactivation of these sites in pure particles). Thus, it is necessary to completely inhibit the chymotrypsin-like sites and either the caspase- or trypsin-like sites to reduce degradation of proteins in HeLa cells by ~50%, in accord with findings on pure proteasomes. The standard inhibitors, even at relatively high...
Proteasome Active Sites Role in Protein Breakdown

| Inhibitor | Concentration (µM) | Chymotrypsin-like site | Caspase-like site | Trypsin-like site | Protein breakdown (%) |
|-----------|-------------------|------------------------|------------------|------------------|----------------------|
| Bortezomib| 0.05              | 58 ± 15                | 23 ± 17          | 5 ± 4            | 6 ± 7                |
|           | 0.15              | 75 ± 5                 | 28 ± 11          | 5 ± 6            | 14 ± 7               |
| NLVS      | 50                | 80 ± 7                 | 12 ± 9           | 9 ± 9            | 13 ± 8               |
| Epoxomicin| 0.15              | 82 ± 8                 | 4 ± 6            | 27 ± 9           | 21 ± 8               |
|          | 0.2               | 96 ± 2                 | 5.5 ± 9.5        | 83 ± 8           | 56 ± 6               |
| Bortezomib| 0.5               | 85 ± 5                 | 83 ± 7           | 15 ± 9           | 40 ± 11              |

concentrations, only can inactivate two types of sites and can thus reduce intracellular proteolysis by 40–50% (Table 7). The remaining protein degradation must be catalyzed largely by the uninhibited caspase-like or trypsin-like sites or by lysosomal proteases through autophagic vacuole formation or endocytosis of membrane proteins.

DISCUSSION

The Advantages of the Present Methods for Analyzing Inhibitor Actions—Prior investigations of the roles of different active sites of mammalian proteasomes in protein degradation have yielded varying conclusions. Most studies have assumed that the degree of inhibition of the chymotrypsin-like sites reflects the degree of inhibition of protein degradation and that the contributions of other sites to this process are insignificant. However, several studies have demonstrated a significant role of the trypsin-like and caspase-like sites in the degradation of model substrates by activated 20 S proteasomes (19, 20) and by the 26 S particles in reticulocyte lysates (21). The assumption that the chymotrypsin-like sites are the only important sites in protein degradation is based on experiments in yeast where mutations that inactivate the chymotrypsin-like sites caused a strong inhibition of cell growth and degradation of certain substrates, whereas inactivation of the trypsin-like and caspase-like sites had little or no effect (13–15).

Such in vivo findings based on growth assays or even measurements of the levels of reporter proteins can be misleading, because they do not directly assay the rates of degradation of the polypeptide, may focus on an atypical substrate, and may be complicated by suppressor mutations or cellular adaptations to the partial loss of proteasomal function. For example, yeast respond to the loss of the chymotrypsin-like sites by doubling their content of proteasomes (14). The direct assays of proteolytic rates after metabolic labeling used in the present study and our exposure of cells to proteasome inhibitors for only 1 h minimized these possible complications.

Cell proteins are degraded by proteasomes into peptides 2–24 residues long (11, 24, 26), which are then hydrolyzed within seconds by peptidases to amino acids (44, 45). Proteasomes are the primary sites for degradation of both short-lived and long-lived proteins in mammalian cells (1, 2, 37). We chose to study the degradation of long-lived proteins (1, 2, 37), because they comprise the bulk of cell proteins, and their hydrolysis occurs at linear rates (in contrast to that of short-lived proteins). Therefore, measurement of their degradation is a highly reliable way to quantify the effects of inhibitors on the degradation of the average cell protein.

To measure the importance of the different active sites in 26 S proteasomes, we measured the generation of new amino groups in model polypeptides after denaturation, which enabled us to follow their degradation in the absence of ubiquitination (24–26). This approach allowed us to determine actual rates of peptide bond cleavage under linear conditions and to avoid chemical modification of substrates (e.g., methylation), which is likely to change their recognition by specific active sites. These assays allowed reliable and precise measurement of the initial degradation rates under conditions when not >10% of the substrate is degraded during the experiment. These rates could not be measured with the same precision by high-performance liquid chromatography- or gel-based assays often used by other investigators (20, 46).

With the fluorescamine assay, however, the rate of appearance of peptide products is proportional to the rate of disappearance of the protein, and the amount of products generated equals the number of substrate molecules degraded multiplied by the mean number of cuts made in a single polypeptide (24, 28). It is noteworthy that, when an active site is inhibited, protein degradation is still progressive, and the number of cuts made in the polypeptide does not change (11, 24). Thus, the catalytic sites that remain active assume increasing importance in polypeptide cleavages and the peptides generated, though different, still show the usual size distribution (11, 20, 24, 46). Consequently, the percent decrease in peptide generation measured here upon inhibition of the active sites reflects the percent decrease in both the rate of polypeptide cleavage and in substrate molecules consumed by the proteasome. Also, because similar results were obtained in our studies of 26 S particles and intact cells (see below), the behavior of the pure proteasomes appears to reflect their function in vivo.

The Importance of Different Active Sites Depends on the Protein Substrate—One of the striking findings made here is that the relative contributions of the active sites of proteasome depends on the protein being degraded. For example, inactivation of the chymotrypsin-like site decreased the breakdown of ovalbumin by purified proteasomes and overall proteolysis in HeLa cells only slightly (11–15%) but decreased the degradation of casein by 50% (Table 2). On the other hand, loss of the trypsin-like activity had no effect on casein degradation and little effect on that of ovalbumin or calmodulin (13%) but inhibited histone degradation by 35%. Another striking example is the degradation of the tau protein by 20 S proteasomes, which was reduced by 40% by an inhibitor of the trypsin-like sites, but was not affected by inhibition of the chymotrypsin-like sites (20).

The simplest possible explanation of these findings would be that the contribution of each site depends simply on the amino acid composition of the protein being degraded. However, no similar correlation was found between amino acid content and sensitivity to inhibition by leupeptin of the trypsin-like site, which prefers substrates with basic residues in both the P1 and P3 positions (35, 47, 48). However, no similar correlation was found between amino acid content and sensitivity to inhibitors of the chymotrypsin- or caspase-like sites. Pos-
Proteasome Active Sites Role in Protein Breakdown

sibly, these four model proteins are not a sufficiently large sampling to establish such a correlation. Also, although these active sites are defined by their preferred P1 residues, binding of these sites is also determined by the nature of the P2 and P3 residues (12, 35, 47, 48), and the caspase-like sites cleave rapidly after branched chain, as well as acidic amino acids (12, 34). It is also noteworthy that, although the proteasome tends to cut a polypeptide preferentially at specific sites, proteolysis appears to be a stochastic process in which the 20 S cleaves at many possible sites (e.g. typically 30 – 40 cuts per ovalbumin), and generates a very large number of different peptide products from the same protein (e.g. hundreds from ovalbumin) (11, 24). With the loss of an individual active site, the variety of cleavage sites and peptide products must decrease, perhaps even more than the overall rate of proteolysis.

The present results also indicate that the effects of inhibitors on the degradation of any specific cell protein could be quite atypical and may not reflect the functional status of the cell’s proteasomes. For example, NLVS caused a much greater inhibition of casein degradation than that of the other model proteins or of overall intracellular protein breakdown. Accordingly, it has been reported that the degradation of IκB (measured by the disappearance of the protein) was blocked at much lower concentrations of MG132 than the degradation of the bulk of short-lived proteins (49). By contrast, measurement of overall rates of protein breakdown provides a more accurate and representative measure of the cell’s degradative capacity and inhibitor potency.

A corollary of these observations is that a failure of even high concentrations of a proteasome inhibitor to block a cellular process or breakdown of a protein cannot rule out proteasome involvement, unless it is also established that all active sites were shut down by this inhibitor. Using multiple inhibitors together, this requirement can be met for pure proteasomes and probably in cell extracts; however, no equally effective mixture of inhibitors was found that blocks all three active sites in intact cells, due to poor penetrance of selective inhibitors of the trypsin- and caspase-like sites. Often the failure of an inhibitor of the chymotrypsin-like sites to block a cellular process, such as antigen presentation (23, 50), has been interpreted as evidence for the involvement of other proteolytic systems. However, such a result might simply indicate that the critical proteasomal sites were not inactivated under these experimental conditions.

Inhibition of Overall Protein Breakdown Requires Inhibition of Two Types of Active Sites—Another surprising finding was that the loss of the chymotrypsin-like activity causes only a 10 – 30% inhibition of the breakdown of most proteins (Table 7), and that inhibition of two active sites is necessary to reduce protein degradation by 50% or more by pure proteasomes and in cells (Table 3). In accordance with our observations, Bence et al. (51) noted that the extent of inhibition of the degradation of a specific protein by lactacystin differed considerably from the degree of inhibition of the chymotrypsin-like activity in cells. Also, Oberdorf et al. (21) reported that a 50% inhibition of cystic fibrosis transmembrane conductance regulator degradation in reticulocyte extracts required simultaneous inactivation of both the chymotrypsin- and caspase-like sites. Thus, protein degradation in vitro and in vivo is reduced significantly only when either the trypsin-like or caspase-like sites are inhibited, together with the chymotrypsin-like sites. Such inhibition of these second sites requires higher concentrations of the standard inhibitors (e.g. bortezomib) than are necessary to block the chymotrypsin-like site alone.

When two active sites were inactivated in the pure proteasomes, there was a significantly greater extent of inhibition of protein breakdown beyond that expected based upon simple additive effects of loss of two active sites (Fig. 2). In other words, when one type of active site is inactivated, the two others assume increasing importance in cleavage of the protein, and not surprisingly, inhibition of either of the residual sites has a greater impact in reducing protein degradation than when that site alone is blocked.

Implications for Understanding the Therapeutic Actions of Proteasome Inhibitors—The present findings can explain the surprising lack of toxicity of the clinically used inhibitor bortezomib for most cells. Development of this agent was almost stopped because of the recognition that proteasomes served many essential roles and the assumption that blocking proteasome function would be highly toxic to all cells (17). In fact, this agent, although a very potent inhibitor of the chymotrypsin-like site, is much less cytotoxic than most standard chemotherapy, probably because overall protein breakdown is inhibited only partially at the concentrations used therapeutically. During clinical trials of bortezomib (Velcade™), it was established that therapeutically effective concentrations of the drug caused a 75% inhibition of the chymotrypsin-like sites in red blood cells (which resembled the inhibition in other tissues) (8). This inhibition was observed within an hour of intravenous administration of bortezomib to patients and then steadily decreased leading to a complete recovery of proteolytic capacity in red cells before the next dose (22). The present findings demonstrate that inhibition of the chymotrypsin-like sites by this amount would reduce overall protein breakdown to a small extent (10 – 25%).

Assuming that proteasome inhibition in multiple myeloma cells is the same as in red blood cells, an obvious important question is how these concentrations of the inhibitors can block growth and cause apoptosis of myeloma cells at concentrations where overall proteolysis is reduced by only 10 – 25%. The most plausible explanation is that, in myeloma cells, proteasome function appears to be particularly important. In fact, 1) growth of these cells depends on the function of NFκB, whose production requires proteasomal processing and degradation of IκB; 2) these cells are continually degrading very large amounts of abnormal immunoglobulins, and thus are dependent on the ERAD process (52, 53). This special sensitivity of the myeloma cell to proteasome inhibition and the limited ability of bortezomib to block protein degradation, generally due to its sparing the trypsin-like sites and weak inhibition of the caspase sites, together with its intermittent mode of administration, appear to explain the relatively large therapeutic index of this promising new therapeutic agent (54).

Acknowledgments—We are grateful to Huib Ova for providing NLVS, to Millennium for providing bortezomib, and to Mary Dethavong for valuable assistance in the preparation of the manuscript.

REFERENCES

1. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761 – 771
2. Crau, A., Gaczyńska, M., Akopian, T., Gramm, C. F., Fenteany, G., Goldberg, A. L., and Rock, K. L. (1997) J. Biol. Chem. 272, 13437 – 13445
3. Baumeister, W., Walz, J., Zähringer, U., and Seemüller, E. (1998) Cell 92, 367 – 380
4. Voges, D., Zwickl, P., and Baumeister, W. (1999) Annu. Rev. Biochem. 68, 1015 – 1068
5. Pickart, C. M., and Cohen, R. E. (2004) Nat. Rev. Mol. Cell. Biol. 5, 177 – 187
6. Kisselev, A. F., and Goldberg, A. L. (2001) Chem. Biol. 8, 739 – 758
7. Voorhees, P. M., Dees, E. C., O’Neil, B., and Orlofski, R. Z. (2003) Clin. Cancer Res. 9, 6316 – 6325
8. Adams, J. (2002) Curr. Opin. Chem. Biol. 6, 493 – 500
9. Adams, J. (2004) Nat. Rev. Cancer 4, 349 – 360
10. Dick, T. P., Nussbaum, A. K., Deeg, M., Heinemeyer, W., Groll, M., Schirle, M., Keilholz, W., Stevanovic, S., Wolf, D. H., Huber, R., Ramnasse, H. G., and Schild, H. (1998) J. Biol. Chem. 273, 25637 – 25646
11. Nussbaum, A. K., Dick, T. P., Keilholz, W., Schirle, M., Stevanovic, S., Dietz, K., Heinemeyer, W., Groll, M., Wolf, D. H., Huber, R., Ramnasse, H. G., and Schild, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12504 – 12509
Proteasome Active Sites Role in Protein Breakdown

12. Kisselev, A. F., Garcia-Calvo, M., Overkleeft, H. S., Peterson, E., Pennington, M. W., Ploegh, H. L., Thornberry, N. A., and Goldberg, A. L. (2003) J. Biol. Chem. 278, 35869–35877
13. Chen, P., and Hochstrasser, M. (1996) Cell 86, 961–972
14. Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., and Wolf, D. H. (1997) J. Biol. Chem. 272, 25200–25209
15. Arens, C. S., and Hochstrasser, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7156–7161
16. Zwickl, P., Goldberg, A. L., and Baumeister, W. (1999) in Proteasomes: The World of Regulatory Proteolysis (Wolf, D. H., and Hilt, W., eds) pp. 8–20, Landes Bioscience Publishing Co., Georgetown, TX
17. Goldberg, A. L. (2005) in Cancer Drug Discovery and Development: Proteasome Inhibitors in Cancer Therapy (Adams, J., ed) pp. 17–38, Humana Press, Totowa, NJ
18. Chauhan, D., Hideshima, T., and Anderson, K. C. (2005) Annu. Rev. Pharmacol. Toxicol. 45, 465–476
19. Vinitsky, A., Cardozo, C., Sepp-Lorenzino, L., Michaud, C., and Orlowski, M. (1994) J. Biol. Chem. 269, 29860–29866
20. Cardozo, C., and Michaud, C. (2002) Arch. Biochem. Biophys. 308, 103–110
21. Oberdorf, J., Carlson, E. J., and Skach, W. R. (2001) Cancer Res. 61, 1237–1238
22. Hamilton, A. L., Eder, J. P., Pavlick, A. C., Clark, J. W., Liebes, L., Garcia-Carbonero, R., Chachoua, A., Ryan, D. P., Soma, V., Farrell, K., Kinchla, N., Boyden, J., Yee, H., Zeleniuch-Jacquotte, A., Wright, J., Elliott, P., Adams, J., and Muggia, F. M. (2005) J. Clin. Oncol. 23, 6107–6116
23. Rock, K. L., and Goldberg, A. L. (1999) Annu. Rev. Immunol. 17, 739–779
24. Kisselev, A. F., Akopian, T. N., Woo, K. M., and Goldberg, A. L. (1999) J. Biol. Chem. 274, 3363–3371
25. Tarcsa, E., Szymanska, G., Lecker, S., O'Connor, C. M., and Goldberg, A. L. (2000) J. Biol. Chem. 275, 2487–2498
26. Cascio, P., Hilton, C., Kisselev, A., Rock, K., and Goldberg, A. (2001) EMBO J. 20, 2357–2366
27. Kisselev, A. F., Kaganovich, D., and Goldberg, A. L. (2002) J. Biol. Chem. 277, 22260–22270
28. Akopian, T. N., Kisselev, A. F., and Goldberg, A. L. (1997) J. Biol. Chem. 272, 1791–1798
29. Kisselev, A. F., Akopian, T. N., and Goldberg, A. L. (1998) J. Biol. Chem. 273, 1982–1989
30. Shamu, C. E., Story, C. M., Rapoport, T. A., and Ploegh, H. L. (1999) J. Cell Biol. 147, 45–58
31. Kisselev, A. F., and Goldberg, A. L. (2005) Methods Enzymol. 398, 364–378
32. Gaczynska, M., Rock, K. L., and Goldberg, A. L. (1993) Nature 365, 264–267
33. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimbacher, S., and Weigele, M. (1972) Science 178, 871–872
34. McCormack, T. A., Cruikshank, A. A., Grenier, L., Melandri, F. D., Nunes, S. L., Plamondon, L., Stein, R. L., and Dick, L. R. (1998) Biochemistry 37, 7792–7800
35. Nazif, T., and Bogov, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2967–2972
36. Myung, J., Kim, B. K., Lindsten, K., Dantuma, N. P., and Crews, C. M. (2001) Mol. Cell 7, 411–420
37. Fuertes, G., Martin De Llanos, J. J., Villarroya, A., Rivett, A. J., and Knecht, E. (2003) Biochem. J. 375, 75–86
38. Adams, J., Behnke, M., Chen, S., Cruickshank, A. A., Dick, L. R., Grenier, L., Klander, J. M., Ma, Y. T., Plamondon, L., and Stein, R. L. (1998) Bioorg. Med. Chem. Lett. 8, 333–338
39. Groll, M., Kisselev, A. F., Kaisers, N., Huber, R., and Crews, C. M. (2000) J. Am. Chem. Soc. 122, 1237–1238
40. Berkers, C. R., Verdoes, M., Lichtman, E., Fiebig, E., Kessler, B. M., Anderson, K. C., Ploegh, H. L., Ovaa, H., and Galardy, P. J. (2005) Nat. Methods 2, 357–362
41. Altun, M., Galardy, P. J., Shringarpure, R., Hideshima, T., Leblanc, R., Anderson, K. C., Ploegh, H. L., and Kessler, B. M. (2005) Cancer Res. 65, 7896–7901
42. Rivett, A. J. (1999) Curr. Opin. Immunol. 10, 110–114
43. Gronostajski, R. M., Pardee, A. B., and Goldberg, A. L. (1985) J. Biol. Chem. 260, 3344–3349
44. Saric, T., Beningen, J., Graef, C. I., Akopian, T. N., Rock, K. L., and Goldberg, A. L. (2001) J. Biol. Chem. 276, 36474–36481
45. Reits, E., Neijssen, J., Herberts, C., Benchkhuisen, W., Janssen, L., Drijfhout, J. W., and Neele, J. (2004) Immunity 20, 495–506
46. Mykles, D. L., and Haize, M. F. (1995) Biochem. J. 306, 285–291
47. Groll, M., Nazif, T., Huber, R., and Bogov, M. (2002) Chem. Biol. 9, 655–662
48. Harris, J. L., Alper, P. B., Li, J., Rechtstainer, M., and Bakes, R. J. (2001) Chem. Biol. 8, 1131–1141
49. Merini, A. B., Gabai, V. L., Yaglom, J., Shifrin, V. I., and Sherman, M. Y. (1998) J. Biol. Chem. 273, 6373–6379
50. Rock, K. L., York, I. A., Saric, T., and Goldberg, A. L. (2002) Adv. Immunol. 80, 1–70
51. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) Science 292, 1552–1555
52. Landowski, T. H., Esseltine, D. L., Porter, J. B., Schenkein, D., and Anderson, K. C. (2005) Cancer Res. 65, 3828–3836
53. Mitsiades, N., Mitsiades, C. S., Poulaki, V., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Treon, S. P., Munshi, N. C., Richardson, P. G., Hideshima, T., and Anderson, K. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14374–14379
54. Richardson, P. G., Sonneveld, P., Schuster, M. W., Irwin, D., Stadtmauer, E. A., Facon, T., Harousseau, J. L., Ben-Yehuda, D., Lonia, S., Goldschmidt, H., Reece, D., San-Miguel, J. F., Blade, J., Boccadoro, M., Cavenagh, J., Dalton, W. S., Boral, A. L., Esselte, D. L., Porter, J. B., Schenkein, D., and Anderson, K. C. (2005) N. Engl. J. Med. 352, 2487–2498