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The Sizes of Peptides Generated from Protein by Mammalian 26 and 20 S Proteasomes

IMPLICATIONS FOR UNDERSTANDING THE DEGRADATIVE MECHANISM AND ANTIGEN PRESENTATION*

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Knowledge about the sizes of peptides generated by proteasomes during protein degradation is essential to fully understand their degradative mechanisms and the subsequent steps in protein turnover and generation of major histocompatibility complex class I antigenic peptides. We demonstrate here that 26 S and activated 20 S proteasomes from rabbit muscle degrade denatured, nonubiquitinated proteins in a highly processive fashion but generate different patterns of peptides (despite their containing identical proteolytic sites). With both enzymes, products range in length from 3 to 22 residues, and their abundance decreases with increasing length according to a log-normal distribution. Less than 15% of the products are the length of class I presented peptides (8 or 9 residues), and two-thirds are too short to function in antigen presentation. Surprisingly, these mammalian proteasomes, which contain two "chymotryptic," two "tryptic," and two "post-acidic" active sites, generate peptides with a similar size distribution as do archaeal 20 S proteasomes, which have 14 identical sites. Furthermore, inactivation of the "tryptic" sites altered the peptides produced without significantly affecting their size distribution. Therefore, this distribution is not determined by the number, specificity, or arrangement of the active sites (as proposed by the "molecular ruler" model); instead, we propose that proteolysis continues until products are small enough to diffuse out of the proteasomes.

The ubiquitin-proteasome pathway is the major proteolytic system in the cytosol of eukaryotic cells, where it catalyzes the selective degradation of short lived regulatory proteins and the rapid elimination of proteins with abnormal conformation (1, 2). In mammalian cells, this system also seems to be responsible for the breakdown of the bulk of cell proteins (3, 4). The critical protease in this pathway is the 26 S proteasome, an ATP-dependent proteolytic complex, which is formed by the association of the barrel-shaped 20 S proteasome (700-kDa) and two 19 S (700-kDa) regulatory complexes (5, 6). The 19 S complexes activate peptide hydrolysis within the 20 S proteasome (7) and are responsible for the recognition of ubiquitinated proteins (8). It contains six different ATPases, which probably unfold protein substrates and facilitate their entry into the 20 S particle (9–11). This cylindrical structure is composed of four stacked rings (5, 6). Each of the outer two rings contains seven different α-subunits, which surround a narrow opening through which substrates appear to enter (12). Each of the inner two rings is composed of seven different β-subunits, which enclose the central chamber where proteolysis occurs. On three of these β-subunits are found the active sites (13), one of which is "chymotrypsin-like" in specificity, one of which is "trypsin-like," and one that cleaves after acidic residues (14–17). *In vivo*, 20 S proteasomes exist not only as a part of the 26 S complexes, but also as free particles (18); however, it is not clear whether this free form ever functions in protein degradation *in vivo*.

As part of the continuous turnover of cell proteins, the great majority of peptides generated by proteasomes must be rapidly degraded into amino acids by cytosolic peptidases. In mammalian cells, some of the proteasomal products escape complete degradation and are presented to the immune system on the cell surface in complexes with MHC[1] class I molecules (3–5, 19, 20). These antigenic peptides are 8 or 9 residues long (21). Proteasomes are essential for the formation of the C terminus of most antigenic peptides but may not be required for the generation of their N termini (22). Thus, if proteasomes generate N-terminally extended versions of antigenic peptides, they can be trimmed by cytosolic peptidases to the presented epitopes (22, 23). Obviously, information on the sizes and nature of the products of protein breakdown by mammalian proteasomes is essential for a full understanding of both MHC class I antigen presentation and the postproteasomal steps in the complete degradation of proteins to amino acids.

Homologous 20 S proteasomes are also found in archaea and certain eubacteria (5, 6, 24, 25) that do not possess the ubiquitin system or 26 S complexes. In the archaeal particle, there is only one type of α-subunit and one type of β-subunit, and thus this 20 S particle contains 14 identical chymotrypsin-like active sites, which are positioned at equal distances around the β-rings (26). Unlike traditional proteases, which release the substrate after each cleavage event, proteasomes from the archaebacterium *Thermoplasma acidophilum* degrade proteins in a highly processive fashion into small peptides and do not dissociate from the substrate between cleavage events (27). It

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[1] The abbreviations used are: MHC, major histocompatibility complex; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; Amc, 7-amino-4-methylcoumarin; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; FITC, fluorescein isothiocyanate; IGF, insulin-like growth factor 1; Suc, succinyl; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.

has been proposed that this complex digests proteins according to a "molecular ruler" mechanism, in which the length of peptides produced would correspond to the distance between active sites (7 or 8 residues) (26, 28, 29). However, we have recently found that archaeal proteasomes generate products that range from 3 to 30 residues in length. The abundance of these peptides decreases as their size increases, and this relationship follows a log-normal distribution (30).

These findings on the archaeal proteasome cannot be automatically applied to their mammalian counterparts for several reasons. Eukaryotic proteasomes have fewer active sites (6 *versus* 14) (13, 26). These sites are asymmetrically distributed in the eukaryotic particle (13). Eukaryotic proteasomes have three different types of active sites, and therefore they cleave a much larger range of peptide bonds. The sensitivities of mammalian and archaeal proteasomes to inhibitors are different (27). In fact, with oligopeptide substrates (11–44 residues), eukaryotic and archaeal proteasomes generate different products (31, 32). Therefore, it is important to analyze systematically the products of protein degradation by 26 S proteasomes from mammalian tissues.

Most prior biochemical studies of proteasome activity have focused on 20 S particles because of the difficulties in purification of the 26 S complexes, their instability (5, 33), and the inability to obtain ubiquitinated proteins in amounts necessary for chemical studies. However, the physiological relevance of findings on the 20 S particles is uncertain. In fact, when isolated rapidly in the presence of glycerol, 20 S proteasomes exhibit little or no activity against protein substrates (5, 33). These latent 20 S particles can be activated *in vitro* by a variety of treatments (*e.g.* by the addition of detergents, such as 0.02% SDS, or by removal of glycerol), but it is unclear if, after such treatments, the 20 S proteasomes function in the same way as when they are associated with 19 S regulatory complexes as part of the 26 S particle. Therefore, an important goal of this work was to compare the nature of the products generated by 26 S proteasomes and activated 20 S proteasomes during the degradation of full-length proteins. One potential complication in interpreting results of such studies is the heterogeneity of 26 and 20 S proteasome subunits in many mammalian tissues (5). Therefore, rabbit skeletal muscle was chosen as the source of the proteasomes because of their homogeneous composition in this tissue (34).[2] Unlike other tissues (*e.g.* liver), muscles express exclusively X, Y, and Z catalytic subunits and do not contain the γ-interferon-inducible homologs (*i.e.* the immunoproteasomes). In these studies, four proteins of different lengths (casein, lactalbumin, insulin-like growth factor 1 (IGF), and ovalbumin) were used as substrates after denaturation, which appears to be necessary for proteins to traverse the narrow opening in the α-rings of the 20 S proteasomes (12). The present studies of 26 S function were made possible by the finding that in the presence of ATP, such denatured proteins are also degraded by the 26 S particles.

EXPERIMENTAL PROCEDURES

Purification of 20 and 26 S Proteasomes—20 and 26 S proteasomes were simultaneously purified to homogeneity from rabbit psoas muscle. After the muscles were minced to small pieces, they were homogenized in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.25 M sucrose, 5 mM $MgCl_2$, 2 mM ATP. The homogenate was centrifuged for 15 min at 10,000 × *g* to remove cell debris and then was centrifuged for 1 h at 100,000 × *g*. The supernatants were spun for 6 h at 150,000 × *g*. The resulting proteasome-containing pellets were dissolved in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 1 mM ATP) and loaded onto a DEAE AffiBlue (Bio-Rad) column. After washing with 40 mM NaCl (in buffer A), proteasomes were eluted with buffer A containing 0.15 M NaCl and directly loaded on an Uno Q-12 column (Bio-Rad). Fractions containing proteasomal activity were identified by their ability to hydrolyze Suc-LLVY-Amc. Complete separation of the 26 and 20 S proteasomes was achieved by a gradient of 0.15–0.45 M NaCl in 200 ml (Fig. 1). The peak of the activity of 20 S proteasomes was stimulated by 0.02% SDS, while the 26 S activity was markedly inhibited by this concentration of SDS and by removal of ATP. Fractions containing 20 S proteasomes were dialyzed against 50 mM HEPES, 1 mM DTT, 10% glycerol, pH 7.5, and finally purified by chromatography on a heparin-Sepharose HiTrap column (Amersham Pharmacia Biotech). The 20 S proteasome was eluted by a 0–0.3 M gradient of KCl in 10 column volumes and stored at −70 °C. Fractions from the Uno Q-12 column containing 26 S proteasomes (*i.e.* peptidase activity that was inhibited by 0.02% SDS) were concentrated to 1 ml and loaded on a 38-ml glycerol gradient (23–37% glycerol in 25 mM HEPES, pH 7.5, 1 mM DTT, 0.5 mM ATP, 5 mM $MgCl_2$). After centrifugation for 22 h at 100,000 × *g*, the gradient was fractionated, and the active fractions were pooled and concentrated. The resulting preparations showed one major band on the native gel and were not cross-contaminated (Fig. 2*B*).

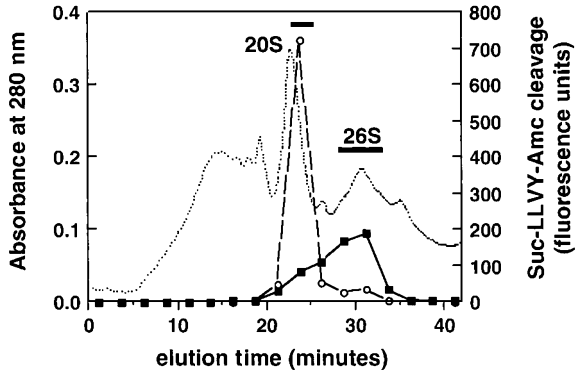

FIG. 1. **Separation of 20 and 26 S proteasomes on the Uno Q ion-exchange column.** After chromatography on DEAE AffiBlue column, the proteasome-rich fractions were loaded onto an Uno Q-12 (Bio-Rad) column, equilibrated with buffer A, containing 0.15 M NaCl. Elution was performed with a linear gradient of NaCl from 0.15 to 0.45 M in 200 ml at a flow rate of 2 ml/min, beginning at the zero time point. 5-ml fractions were collected and assayed for proteasomal activity using Suc-LLVY-Amc as the substrate. *Dotted line*, UV absorbance (total protein); *open circles* and *dashed line*, activity in the presence of 0.02% SDS; *closed squares* and *solid line*, activity in the presence of 1 mM ATP and 5 mM $MgCl_2$. *Horizontal bars* indicate fractions, used for final purification of 20 and 26 S particles.

Peptidase Assay for Proteasome Activity—Each sample (1–10 μl) was added to 100 μl of 100 μM Suc-LLVY-Amc in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1% Me_2SO, 5 mM $MgCl_2$, 1 mM ATP. After a 20-min incubation at 37 °C, the reaction was stopped by the addition of 900 μl of 1% SDS, and fluorescence of released Amc was measured (excitation, 380 nm; emission, 460 nm). In order to distinguish between 20 and 26 S proteasomes, the same incubation was repeated in the presence of 0.02% SDS instead of ATP. Peptide substrate incubated without proteasomes served as control.

Alternatively, proteasomal activity was measured in a continuous assay. The proteasome sample (0.02–1 μg) was added into the cuvette containing 500 μl of substrate preincubated at 37 °C. Fluorescence of released Amc was monitored continuously for 10–20 min, and the reaction velocity was calculated from the slopes of the resulting reaction progress curves. Consumption of substrate at the end of incubation never exceeded 1%.

Protein Substrates—Ovalbumin, bovine α-lactalbumin, and bovine β-casein were from Sigma, and recombinant human IGF was a kind gift of Dr. W. Prouty (Lilly). Fluorescein isothiocyanate (FITC)-casein was prepared as described (27); IGF and lactalbumin were denatured by reduction of disulfide bonds and carboxymethylation of the cysteins; and ovalbumin was treated with performic acid (27). Casein, which has little tertiary structure, does not require denaturation in order to become a substrate of the proteasome. Finally, in order to reduce the background in the reaction with fluorescamine, lysine residues and N-terminal amino groups on all protein substrates were blocked by reductive methylation (27).

Protein Degradation by Proteasomes—Denatured IGF, lactalbumin, casein, and ovalbumin were incubated with 20 or 26 S proteasomes at

[2] K. M. Woo, unpublished observations.

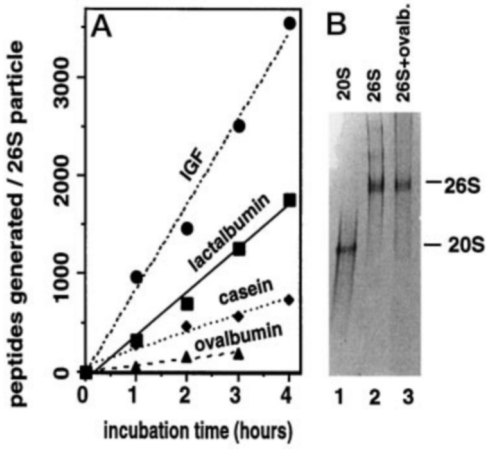

FIG. 2. **26 S proteasomes degrade nonubiquitinated, denatured proteins.** *A*, denatured IGF (510 μM), lactalbumin (320 μM), casein (90 μM), and ovalbumin (14 μM) were incubated with 26 S proteasomes at 37 °C in 50 mM Bis-tris propane, 0.5 mM ATP, 5 mM $MgCl_2$, and 2.5% glycerol. In order to ensure that 26 S proteasome functions at V_{max} in these assays, substrate concentrations exceeded by severalfold the K_m values for each protein (A. F. Kisselev, unpublished observations). Aliquots were analyzed for new amino groups using fluorescamine (27), which forms a fluorescent adduct with N termini of peptides, generated by proteasomal cleavage. A mixture of standard peptides was used to calibrate the assay (27). *B*, a native 5% PAGE was run using the system of Ornstein and Davis (52, 55). *Lane 1*, purified 20 S proteasomes; *lane 2*, purified 26 S proteasomes; *lane 3*, a mixture of ovalbumin and 26 S proteasome (from Fig. 2*A*) after a 3-h incubation. Ovalbumin (43 kDa) migrated out of the gel and was not detected.

37 °C in 50 mM Bis-tris propane, 1 mM DTT, 2–5% glycerol (30). In addition, for experiments with the 26 S particles, reaction buffer contained 0.5 mM ATP, 5 mM $MgCl_2$, and, for 20 S proteasomes, 0.02% SDS was added to stimulate the particles unless stated otherwise (as in Fig. 4). Aliquots were analyzed for new amino groups using fluorescamine (27), which forms a fluorescent adduct with N termini of peptides, generated by proteasomal cleavage. A mixture of standard peptides was used to calibrate the assay (27).

Inactivation of the Trypsin-like Sites in 20 S Proteasomes—Pure 20 S proteasomes (~0.8 μM) were incubated with 0.5 mM AEBSF (Pefablock SC, Boehringer Mannheim) for 1.5 h at room temperature, and all three peptidase activities were measured in continuous assay, using as substrates Suc-LLVY-Amc for the chymotrypsin-like activity, *tert*-butyloxycarbonyl-LRR-Amc for the trypsin-like activity, and Ac-YVAD-Amc for the postacidic activity. All substrates were obtained from Bachem (Bubendorf, Switzerland). The covalently modified enzyme was used in degradation reactions with casein and IGF as substrates.

RESULTS

Degradation of Nonubiquitinated Proteins by 26 S Proteasomes—It is well established that 26 S proteasomes catalyze the degradation of proteins conjugated to ubiquitin (1, 5), but they also have been reported to degrade a few nonubiquitinated proteins in an ATP-dependent manner (35–38). Because ubiquitinated substrates cannot be generated in homogeneous form in quantities necessary for analysis of the products, we tested whether 26 S proteasomes from rabbit muscle can degrade other proteins without ubiquitination. Indeed, in the presence of ATP, the highly purified 26 S particles degraded several denatured proteins (IGF, lactalbumin, ovalbumin) and casein at linear rates (Fig. 2*A*). Interestingly, the absolute rates of peptide bond cleavage (measured by the appearance of new amino groups) were faster with the smaller substrates (IGF and lactalbumin) than with casein or ovalbumin. To confirm that this degradation (Fig. 2*A*) was indeed due to 26 S proteasomes, we analyzed the reaction mixtures by native polyacrylamide gel electrophoresis (Fig. 2*B*). At the outset and after 3 h of incubation (*lane 3*), only 26 S proteasomes were detectable. Thus, the hydrolysis of these nonubiquitinated proteins was not due to contaminating 20 S particles or to 20 S generated by breakdown of 26 S proteasomes. Clearly, *in vitro*, and presumably *in vivo*, 26 S particles, like activated 20 S proteasomes, can hydrolyze some denatured proteins without ubiquitination.

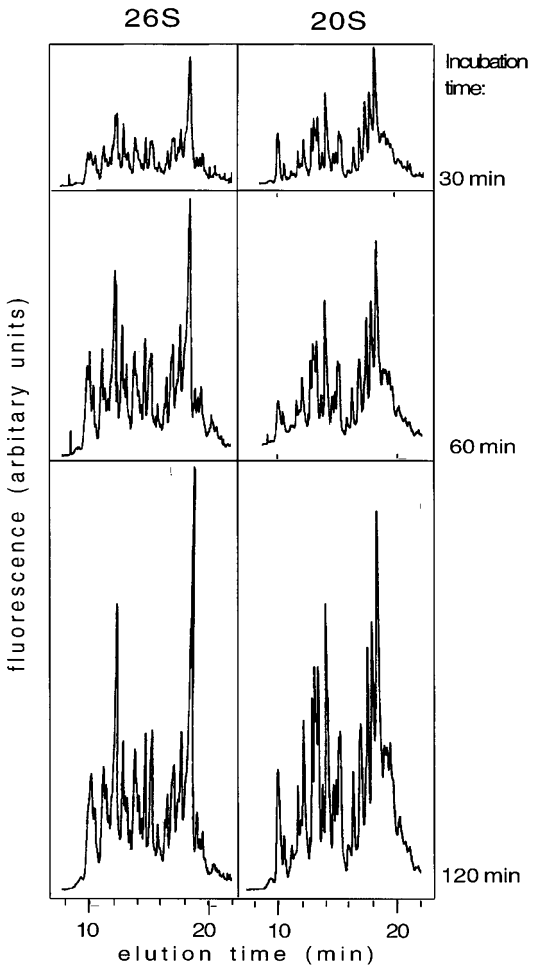

FIG. 3. **Both 20 and 26 S proteasomes degrade FITC-casein processively but generate different patterns of peptides.** FITC-casein (8 μM) was incubated with 26 S proteasomes, as described under "Experimental Procedures," except that the buffer contained 50 mM Tris-HCl, pH 7.5, instead of Bis-tris propane. At indicated times, aliquots were analyzed by HPLC on a C_{18} column (27). Fluorescent peptides were detected at an excitation wavelength of 492 nm and emission of 521 nm. The relative amounts of the peptides generated did not change with time, although their absolute amounts increased. Less than 20% of the FITC-casein was consumed during the incubation, as calculated by integration of its peak, which was eluted at 25 min (not shown). Proteasomes used in these experiments were prepared as described in Ref. 4.

20 and 26 S Proteasomes Degrade Proteins Processively—In order to test whether mammalian 26 and 20 S proteasomes degrade proteins processively, we incubated these enzymes with casein and analyzed at different times the nature of the products of the reaction by SDS-PAGE. 20 S proteasomes were purified in the latent state in the presence of glycerol but then were activated by the addition of 0.02% SDS. Despite the disappearance of casein, no Coomassie-stainable polypeptide fragments were detected in the gel (not shown), indicating that the substrate was degraded all the way to oligopeptides. To increase the sensitivity of the detection of individual peptide products, we initially used casein as a substrate that was covalently modified with FITC at multiple sites (27). Incubation conditions were chosen to ensure a linear rate of breakdown of the FITC-casein and the presence of the substrate in large excess. At different times, the fluorescent products released by proteasomes were analyzed by HPLC on a reverse-phase column (Fig. 3). With both the 26 and 20 S particles, a large number of products were

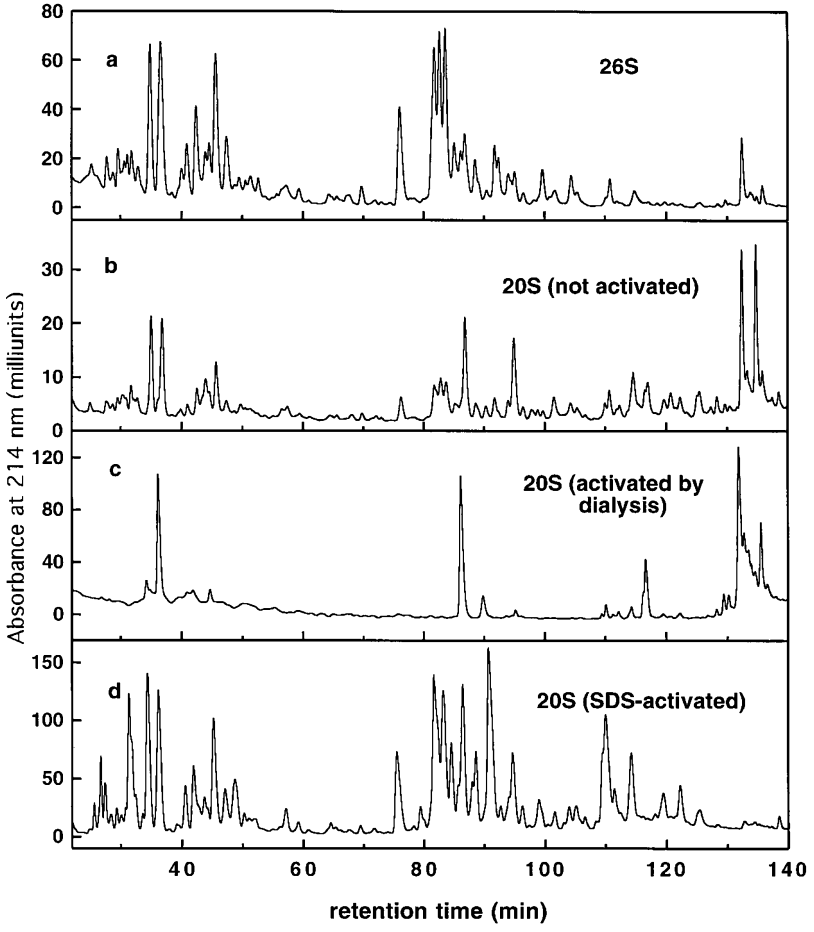

FIG. 4. **20 and 26 S proteasomes generate different patterns of products from IGF.** IGF (360 μM) was incubated at 37 °C in 50 mM Bis-tris propane, pH 7.5, 1 mM DTT, 5% glycerol, 5 mM $MgCl_2$, and 0.5 mM ATP with 26 S proteasomes (50 nM) for 2 h (*a*), latent 20 S proteasomes (250 nM) for 4 h (*b*), activated 20 S proteasomes (10 nM) for 2 h (*c*), or latent 20 S proteasomes (250 nM) in the presence of 0.02% SDS for 3 h (*d*). 20 S proteasomes in *c* were activated prior to incubation by an overnight dialysis against 50 mM Bis-tris propane, pH 7.5, 1 mM DTT at +4 °C. Digests were loaded on a C_8 Vydac column (0.2 × 25 cm) equilibrated with 0.06% trifluoroacetic acid. Peptides were eluted by a gradient of acetonitrile from 0 to 8% in 20 min, to 28% within the next 100 min, to 36% within the subsequent 20 min, and to 44% in the last 10 min at a flow rate of 0.15 ml/min. The large peak of undegraded excess IGF, which was eluted after 150 min, is not evident.

generated, indicating that the protein was cleaved at multiple sites. As the reaction proceeded, the areas under the individual product peaks increased in parallel with each other, but the relative amounts of these fluorescent products did not change, and no new peaks appeared. Thus, no peptides were generated that were degraded in subsequent proteolytic rounds.

These results together demonstrate that activated 20 S proteasomes and the ATP-dependent 26 S particles degrade proteins in a highly processive manner into oligopeptides without dissociation of the substrate. A similar mechanism of protein breakdown was found earlier for the archaeal 20 S proteasomes (27). By contrast, when chymotrypsin, a typical nonprocessive protease, was incubated under similar conditions with casein (24 kDa), polypeptide fragments ranging from 14 to 20 kDa were generated (not shown), and with FITC-casein as the substrate, the pattern of fluorescent products varied with incubation time. Thus, unlike the proteasome, chymotrypsin released large products that were subsequently cleaved further (27).

20 and 26 S Proteasomes Generate Different Patterns of Peptides—Because proteolysis within the 26 S complex is catalyzed by its core 20 S proteasome, it has been widely assumed that the pattern of peptide bond cleavage by the 20 S proteasome reflects proteolysis by the larger 26 S particle. However, careful analysis of the spectra of peptides produced by the 20 and 26 S proteasomes showed the unexpected result that they generated different patterns of products. (See, for example, peptides eluted between 12 and 14 min in Fig. 3.) These differences in product patterns suggest that 20 and 26 S proteasomes can cleave proteins at different sites. However, it was also possible that these differences were an artifact due to 0.02% SDS, which was used to activate 20 S proteasomes and might also be altering the substrate. In addition, these differences might possibly be due to some conformational difference in the substrate due to the Mg^{2+} and ATP used to stabilize the 26 S particles. Alternatively, the covalent modification of casein by very hydrophobic FITC residue might lead to a differential binding to these different proteasome particles.

To exclude these possibilities, we compared the pattern of peptides generated by 20 and 26 S proteasomes from IGF (which had not been modified with FITC) during incubation under the exact same conditions (*i.e.* in the presence of Mg^{2+} and ATP). As was found with FITC-casein, the patterns of peptides generated by the 26 S and latent 20 S proteasomes from IGF were different (Fig. 4, *a* and *b*). Moreover, when the 20 S proteasomes prior to incubation were activated by dialysis against buffer lacking glycerol, the pattern of the products differed to an even greater extent than with latent particles (Fig. 4*c*). Finally, when the 20 S proteasomes were activated by the addition of 0.02% SDS (Fig. 4*d*), they generated a distinct pattern of the products from those generated by the 26 or 20 S proteasomes activated in other ways. These distinct patterns were reproducible with different enzyme preparations. Thus, the observed differences in the pattern of the products are due to differences in the behavior of 20 and 26 S particles. Since these two particles have the same active sites, the associated 19 S complexes in the 26 S proteasomes as well as the nature of the treatment used to activate 20 S particles must influence how proteins are cut within the 20 S proteasome.

Product Sizes—To analyze the lengths of peptides generated by mammalian proteasomes, we used a size-exclusion chromatography method developed earlier to study the products of the archaeal proteasomes (30). Methylated casein, IGF, and ovalbumin were incubated for 2–4 h with SDS-activated 20 S proteasomes or 26 S particles (in the presence of ATP). Under

these conditions, degradation rates (*i.e.* the generation of new amino groups) were linear, and usually less than 20% of the original substrate amount was consumed. The generated peptides were separated from the undegraded protein on a reverse-phase column and subsequently fractionated on a gel filtration column, which resolves peptides according to their molecular masses in a 500–10,000-Da (4–90-amino acid) range (30). The abundance of products in the different fractions (measured by the fluorescamine assay) was plotted against their elution times, which are proportional to the logarithms of their molecular weights. The resulting curves were reproducible and characteristic of each substrate and the type of proteasome (Fig. 5).

The overall size distribution of the peptide products from these different proteins was quite similar with the 20 and 26 S proteasomes. In each case, when plotted against the logarithms of the molecular weights, the curves resembled a normal distribution. In other words, the size distribution of proteasome products seemed to fit a log-normal distribution. This fit was stronger with the longest substrate (ovalbumin), probably because of the greater total number of peptide products generated. The sizes of the products covered a wide range, from less than 500 Da (*i.e.* 4–5 residues, which corresponds to the lower separation limit of the column) to about 22 residues with most protein substrates, but when casein was the substrate, few peptides of up to 3500 Da (30 residues) were generated. Although the peptides released by the 20 and 26 S particles fell within the same size range, they did not appear to be exactly the same sizes. With both IGF and ovalbumin as substrates, 26 S proteasomes consistently generated more products smaller than 1000 Da (8–9 residues) than did the 20 S proteasomes, and the mean size of the products of the 26 S proteasome was on the average 1 or 2 residues shorter (Fig. 5, Table I).

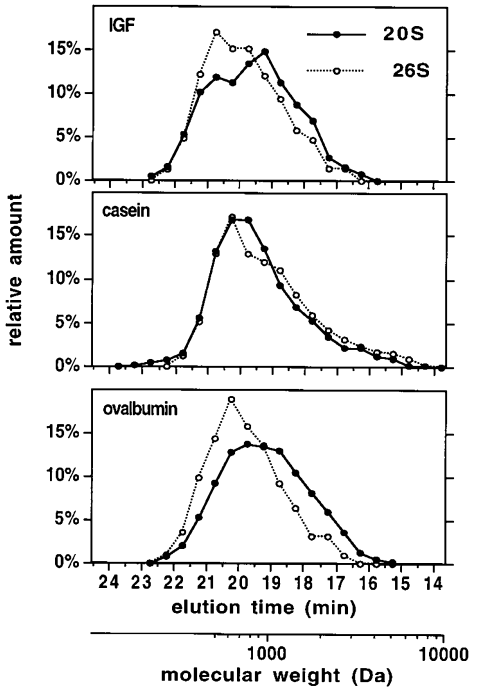

FIG. 5. **Size-exclusion chromatography of peptides generated by SDS-activated 20 and 26 S proteasomes (*dashed lines*) from different substrates (indicated in each *panel*).** After a 2- or 4-h incubation as described under "Experimental Procedures," the products were separated from the undegraded substrates on a C_{18} reverse phase column (30). At the end of the incubation, usually less than 20% of the protein was degraded. The pooled products were run on a polyhydroxyethyl aspartamide column as described (30), and the molar amounts of peptides in each fraction were determined by the fluorescamine assay. Each curve is an average of two experiments.

To analyze better the relative distributions of peptides of different sizes, we graphed these data in the form of cumulative frequency curves (Fig. 6). For each time point (Fig. 5), the fraction of peptides with molecular weights equal to or less than those of peptides eluting at this specific point was calculated, and then graphed against the molecular weights and apparent lengths on a linear scale (Fig. 6). On such an integral plot, the slope of the curve at each point equals the fraction of peptides of this particular size. The size distribution plots of peptides generated by 20 S proteasomes (Fig. 6*A*) from the three different proteins of very different lengths were indistinguishable. Similarly, the peptides generated by 26 S proteasomes (Fig. 6*B*) from ovalbumin and IGF had a very similar size distribution, but products of casein degradation were slightly longer, as had also been found with this substrate and archaeal 20 S proteasomes (30). Casein degradation may yield larger products because casein is phosphorylated at multiple sites or because every seventh residue in it is proline, both of which may retard cleavages.

Although the products cover a wide size range (95% percent were between 3 and 22 residues) (Fig. 6), the median sizes of peptides were 5 residues for 26 S proteasome products and 6 residues for the 20 S proteasome. At least two-thirds of the products of both particles were shorter than 8 residues, and only 15% were 8 or 9 residues long, which corresponds to the length of MHC class I antigenic peptides. Thus, mammalian proteasomes do not preferentially generate peptides of an appropriate size to bind to MHC class I molecules. A small fraction of the peptides (15–25%) was longer than 10 residues. None of the peptides generated by 26 S proteasomes from IGF or ovalbumin was longer than 20 residues, but some of the 20 S products were up to 30 residues long. In addition, about 2% of the peptides generated from casein appeared to contain more than 30 residues, perhaps as a consequence of casein's unusual primary structure, as discussed above.

Product Sizes Are Not Consistent with the "Molecular Ruler" Model—Surprisingly, despite the very different number, arrangement, and specificities of their active sites, the size distribution of the products of the mammalian proteasomes (Fig. 5) had a similar shape, mean size (Table I), and size range as the peptides generated by proteasomes from archaea *T. acidophilum* (30). For example, with IGF as a substrate, the mean

TABLE I

Mean sizes of peptides generated and number of cuts made in a single polypeptide chain by proteasomes

Mean sizes were calculated from the distributions of products of rabbit muscle proteasomes (Fig. 6) and *Thermoplasma* proteasomes (from Ref. 30). The values for mean sizes are averages ± ranges in two experiments. The number of cuts was calculated as protein length divided by mean size minus one (30).

Substrate (number of residues)	Mean number of residues in peptides generated			Cuts per polypeptide by rabbit muscle	
	Thermoplasma (20 S)	Rabbit muscle			
		20 S	26 S	20 S	26 S
IGF (70)	7.0 ± 0.2	8.3 ± 0.8	6.5 ± 0.2	7	8
Casein (209)	11.2 ± 0.2	8.9 ± 1.0	9.0 ± 0.6	22	22
Ovalbumin (385)	ND[a]	9.5 ± 0.5	7.4 ± 0.0	40	51

[a] Not determined.

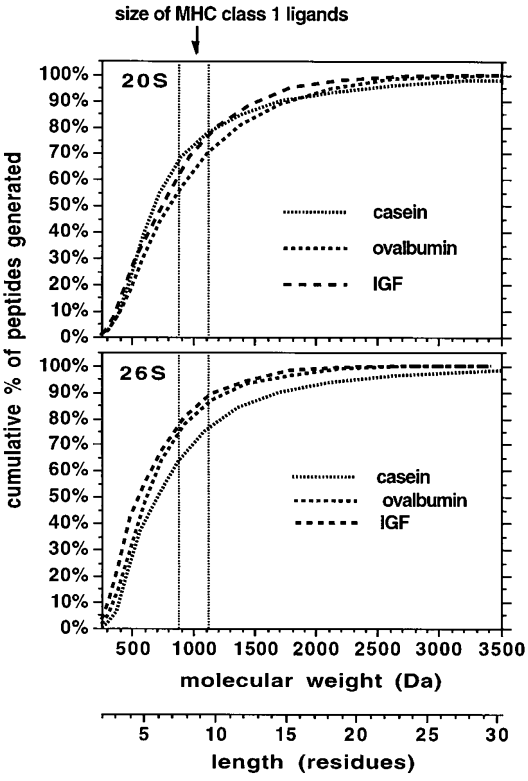

FIG. 6. **Cumulative frequency curves for 20 and 26 S proteasome products.** The curves were obtained by transformation of the size-exclusion chromatography data from Fig. 5. For each point, the fraction of peptides with this and lower molecular mass was calculated. The scale for peptide length was obtained by dividing the molecular mass scale by 115 Da, the average molecular mass of an amino acid residue in these substrates.

sizes of peptides released by rabbit 20 S proteasome were the same as those generated by its archaeal counterparts (Table I). These similarities in the size distributions of the products of eukaryotic and archaeal particles strongly suggest that this log-normal distribution is a fundamental feature of the 20 S proteasome that was conserved during evolution. Thus, the number, catalytic properties of the active sites, and distance between them have little or no influence on the sizes of the products.

To further test whether the nature of the individual active sites and the distances between them influence the size distribution of the products, we selectively inactivated the two trypsin-like active sites in 20 S proteasomes by reaction with the specific irreversible inhibitor of these sites, AEBSF, as was described recently (39). 20 S proteasomes were preincubated with AEBSF at room temperature, resulting in the inhibition of the trypsin-like activity by almost 90% (Table II). The two other activities were not affected by this treatment. This loss of trypsin-like activity did not affect the rate of cleavage of peptide bonds in the protein substrates (Table II), in agreement with observations on a yeast mutant affecting the homologous activity (15, 16). However, this modification did cause some changes in the pattern of peptides generated (Fig. 7, compare peaks eluting at 40–50 min and at 80–100 min). Nevertheless, this inactivation of the two trypsin-like sites did not affect the log normal distribution of the size of proteasome products. In fact, when these peptides were analyzed by size-exclusion chromatography, little or no difference was detected between proteasomes with inactivated trypsin-like sites and fully active proteasomes (Fig. 8). Thus, the number, specificity of individual active sites, or the distances between them do not significantly affect the general size distribution of the peptides generated by proteasomes.

TABLE II

Effect of AEBSF treatment on three peptidase activities of 20 S proteasomes and protein degradation by this particle

Specific activity against fluorogenic substrates (all at 100 μM) was measured continuously in the absence of SDS, magnesium, and ATP. Activity against IGF (490 μM) and casein (94 μM) was measured by fluorescamine assay as described under "Experimental Procedures."

Substrate	Specific activity		Inhibition
	Nontreated proteasome	AEBSF-treated proteasome	
	nmol Amc released/min·mg		*%*
Suc-LLVY-Amc (chymotrypsin-like)	159	156	0
Boc[a]-LRR-Amc (trypsin-like)	11.6	1.4	88
Ac-YVAD-Amc (postacidic)	1.02	1.16	0
	nmol products generated/mg·min		
IGF	183	175	4
Casein	9.0	9.5	0

[a] *tert*-Butyloxycarbonyl.

DISCUSSION

Mechanism of Protein Breakdown by 20 and 26 S Proteasomes—The present study demonstrates that protein substrates within the mammalian 20 or 26 S particles are cut at many sites (Table I) to yield small oligopeptides, without the release of longer fragments. Such a highly processive mode of degradation seems to be a fundamental feature of intracellular proteases, including 20 S proteasomes from yeast (40), the simpler proteasomes from archaebacteria (27), and the bacterial ATP-dependent proteases, ClpAP (41), La (42), and HslUV (43). This processivity must help to ensure that cell proteins targeted for destruction are rapidly eliminated without the generation of large fragments that might retain some biological activity and be highly toxic to the cell. Since the 20 S proteasome by itself exhibits this highly processive behavior, this particle must have structural features and enzymatic mechanisms to ensure that the substrate once bound is not released until its degradation is completed. Once a polypeptide has entered the 20 S particle, its exit is probably only possible through the narrow openings in the α-rings, which are located at a significant distance from the central proteolytic chamber (12, 26). In addition, interactions of the substrate with the large inner surface of the particle may promote retention of longer polypeptides. Another possible mechanism that might reduce substrate dissociation could be that the polypeptide chain, while covalently attached to one active site in a transition state complex, is attacked in turn by other active sites until degradation to small products is completed (26).

Within the 26 S particle, the association of the 19 S regulatory complex with the 20 S proteasome enhances its peptidase activities (7) and confers the ability to digest ubiquitinated proteins (5). In addition, even in the absence of ubiquitination, the 26 S complexes can degrade a number of denatured polypeptides (Fig. 2), as had been reported for casein (38), ornithine decarboxylase (36), and c-Jun (35). Degradation of these nonubiquitinated proteins still requires ATP, which is necessary for the stability and function of the 26 S complex (5). Since this ubiquitin-independent process can occur *in vitro*, presumably it is also occurring to some extent *in vivo*, at least for unfolded substrates (44). The maximal rates of degradation of these different nonubiquitinated proteins varied widely (Fig. 2*A*), presumably because they retain some secondary structure or tend to aggregate, both of which should retard entrance into the proteolytic chamber. The actual influence of ubiquitination on the rate of degradation of these unfolded proteins will be interesting to study. Possibly, the attachment of multiple ubiquitin moieties to these proteins simply facilitates their binding to the 19 S complex.

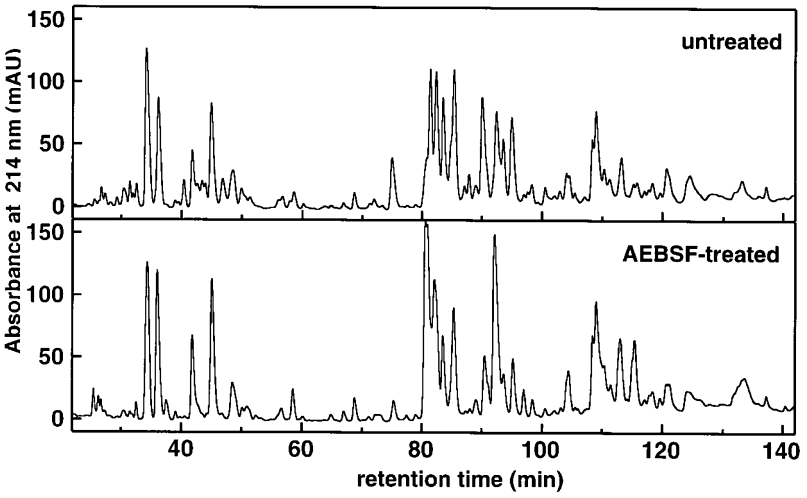

FIG. 7. **Pattern of peptides generated from IGF by control and by 20 S proteasomes (*top*) with inactivated trypsin-like site (*bottom*).** AEBSF-treated and control 20 S proteasomes were incubated with IGF in the presence of SDS as described under "Experimental Procedures." Digests were run on HPLC as described in Fig. 4.

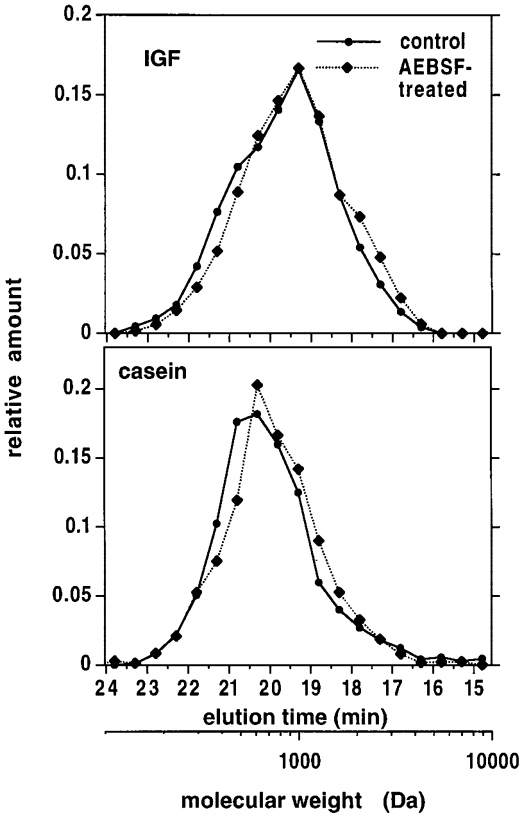

FIG. 8. **Inactivation of the trypsin-like sites in 20 S proteasomes does not change significantly the sizes of the products.** AEBSF-treated and control proteasomes were incubated with IGF and casein for 2 h as described under "Experimental Procedures," and the peptides generated were analyzed by size-exclusion chromatography, as in Fig. 5. *Closed circles* and *solid lines*, mock-treated proteasomes; *closed diamonds* and *dotted lines*, AEBSF-treated proteasomes (this treatment selectively inhibited trypsin-like activity, Table II).

Pattern of Peptides Generated by 20 and 26 S Proteasomes Differ—In most published studies of proteasome function, activated 20 S proteasomes have been used as models of proteasome function *in vivo*, and it has been widely assumed, especially in studies of the production of antigenic peptides (45–48), that activated 20 S proteasomes and 26 S particles generate identical spectra of peptides. Clearly, this assumption is not valid. The patterns of peptides released by 20 and 26 S proteasomes are not identical, and the spectrum of peptides generated by the 20 S particle depends on its mode of activation. In the present studies, 20 S proteasomes were isolated and maintained in a latent state by the presence of 10% glycerol, but their activity could only be studied after activation with 0.02% SDS, the addition of Mg^{2+} (5 mM), or dialysis against a buffer lacking glycerol. Since the patterns of peptides generated from IGF by the 20 S particles under these three different conditions differed from each other in reproducible ways and also differed from the peptides released by the 26 S particle (Fig. 4), the specific cleavages made in a protein substrate by the 20 S proteasome vary with the precise incubation conditions and whether the particle is free or part of the 26 S complex.

As shown in Figs. 3 and 4, the association of the 20 S proteasome with the 19 S complex alters the pattern of peptides it generates (Figs. 3 and 4). Because skeletal muscle contains only one type of 20 S particle (*i.e.* it lacks alternative interferon-inducible subunits) (34),[2] the differences between 20 and 26 S proteasomes are not due to the presence of different groups of β-subunits. Therefore, the 20 S proteasome, depending on the state of the particle, is capable of cleaving proteins in multiple ways, and the structural basis for these different modes of proteolysis will be important to understand. Possibly, the 19 S complex injects polypeptides into the 20 S particle in a highly specific manner and thus may influence the cleavages made. Alternatively, because the 19 S complex stimulates the peptidase activities of the 20 S proteasome, proteolysis may occur in a distinct manner and proceed further before the products are released. It is noteworthy that PA28, the interferon-induced activator of peptide hydrolysis by the 20 S proteasome, also changed the pattern of bonds cut in a 25-residue oligopeptide (49). In fact, with IGF and ovalbumin as substrates (Fig. 6 and Table I), the products of the 26 S proteasome appeared to be slightly shorter than those of the 20 S proteasome alone. Perhaps the 19 S particles at the ends of the 20 S proteasome retard the release of the peptide products, leading to additional cleavages.

Determinants of the Sizes of Peptide Products—The molecular ruler hypothesis has been proposed to explain the proteasome's mechanism for degrading proteins. Accordingly, peptide products are generated by the coordinate actions of two adjacent active sites (26, 28, 29), and the distance between these sites must be a major factor determining the products' sizes. However, a variety of evidence indicates that the proteasome is unlikely to function by such a mechanism. 1) The length of most peptides generated by the archaeal proteasomes does not equal the distance between its adjacent active sites, which corresponds to a peptide of 7 or 8 residues. In fact, the sizes of its products actually ranged in length from 3 to 30 residues and followed a log-normal distribution (30), although the mean size

was approximately 8 residues in length. 2) In place of the 14 chymotrypsin-like active sites in the archaeal proteasomes, the eukaryotic particles contain two chymotrypsin-like sites, two trypsin-like sites, and two active sites cleaving after acidic residues. Within a single β-ring, the trypsin-like and postacidic sites are located on adjacent subunits at similar distances apart as active sites of archaeal proteasome (30 Å), while the chymotrypsin-like site is on the opposite side of the ring (13). Despite having far fewer active sites and greater distances between them, the mammalian proteasomes do not generate longer products than the archaeal particles. A similar finding was noted recently by Niedermann *et al.* (31), who studied the products generated in the breakdown of short polypeptides (22–44 residues) by activated 20 S proteasomes (although this group analyzed the products after prolonged incubations when many peptides released by the proteasome were digested further in later catalytic rounds). 3) When the two trypsin-like sites of the muscle 20 S proteasome were irreversibly inhibited, the particles generated a pattern of peptide products distinct from those produced by control proteasomes (Fig. 7). However, their size distribution was not significantly different (Fig. 8). The products of both types of particles followed a log-normal frequency distribution, and the mean length of the peptides generated by the particle with four active sites was at most 1 residue longer than proteasome with six functional sites. In accord with these results, mutations that inactivate each of the active sites in the yeast 20 S proteasomes or even two of them did not cause production of peptides of increased lengths (40). 4) About two-thirds of the products are less than 8 residues long, which is the shortest distance between neighboring active sites. Such small products, after initially being cleaved from the substrate, must have undergone additional cleavages, as was suggested by Dick *et al.* (50).

It was most surprising to find that the archaeal and SDS-activated mammalian particles, despite their different catalytic properties and functional organizations, generate products with very similar mean sizes (Table I) and size distributions. It seems quite unlikely that the similarities in product size and in the log normal distributions of products found for archaeal and mammalian proteasomes (even after loss of trypsin-like sites) is coincidental. Instead, this common feature is likely to reflect some fundamental property of the particle that has been conserved through the evolution. These findings clearly indicate that the specificities of the different active sites and the distances between them are not major determinants of product size. Instead, it seems more likely that cleavage of peptide bonds continues randomly within the central chamber until peptides are small enough to escape further proteolysis by diffusing out of the 20 S particle. The rate of diffusion of a peptide should be inversely proportional to its hydrodynamic radius, and as the radius is approximately proportional to the logarithm of its molecular weight, a log-normal distribution of the products could result. Moreover, the small openings in the β- and α-rings and possibly the spaces between the subunits may act as a filter or sieve preventing or retarding the diffusion of larger fragments out of the particle.

Strong support for this type of mechanism was the finding that loss of trypsin-like sites did not alter significantly the sizes of peptide products. To test this model further, it would be interesting to analyze the effects on product size of inactivation of the postacidic site, but potent inhibitors of their activity are not known. Although potent irreversible inhibitors of the chymotrypsin-like site are available, their effects on product size would be difficult to interpret for several reasons (*e.g.* the chymotrypsin-like site appears to be rate-limiting in protein breakdown (3, 4, 15, 17), and occupancy of one such site allosterically activates the other (47, 51).

Fate of Peptides Generated by Proteasomes—In mammalian cells, a small fraction of the peptides generated by the proteasome are utilized for MHC class I antigen presentation (19, 20). Exactly why only certain peptides are selected and presented on the surface is uncertain. Only 8–9-mer peptides with hydrophobic or basic residues at their C termini can bind tightly to MHC class I molecules (21). It was therefore widely assumed that peptides of this size were the predominant products of the proteasome (53). However, only 15% of all peptide products fall within this size range (Fig. 6). Moreover, two-thirds of all peptides are shorter than 8 residues and therefore cannot be used in class I antigen presentation. Another 15% of the proteasomal products are longer than 8 residues and may be utilized for antigen presentation after subsequent trimming by cellular exopeptidases (22). In fact, this N-terminal trimming reaction can also be stimulated by γ-interferon, which induces leucine aminopeptidase when it promotes antigen presentation (23).

The great majority of the proteasome products *in vivo* are rapidly digested to amino acids. These peptides released by proteasomes have mean lengths of 6–9 residues (Table I). In other words, the proteasomes cleave only 10–15% of peptide bonds in proteins. In the conversion of cell proteins into amino acids, the remaining 85–90% of the peptide bonds must be quickly hydrolyzed by cellular endo- and exopeptidases, since free peptides are not found in cell extracts (54). These enzymes must be highly active in the cytosol, but their identity remains unclear.

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