The Human 26 S and 20 S Proteasomes Generate Overlapping but Different Sets of Peptide Fragments from a Model Protein Substrate*

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Intracellular protein degradation is a major source of short antigenic peptides that can be presented on the cell surface in the context of major histocompatibility class I molecules for recognition by cytotoxic T lymphocytes. The capacity of the most important cytosolic protease, the 20 S proteasome, to generate peptide fragments with an average length of 7–8 amino acid residues has been thoroughly investigated. It has been shown that the cleavage products are not randomly generated, but originate from the commitment of the catalytically active subunits to complex recognition motifs in the primary amino acid sequence. The role of the even larger 26 S proteasome is less well defined, however. It has been demonstrated that the 26 S proteasome can bind and degrade ubiquitin-tagged proteins and minigene translation products in vivo and in vitro, but the nature of the degradation products remains elusive. In this study, we present the first analysis of cleavage products from in vitro digestion of the unmodified model substrate β-casein with both the 26 S and 20 S proteasomes. The data we obtained show that 26 S and 20 S proteasomes generate overlapping, but at the same time substantially different, sets of fragments by following very similar instructions.

Cells present foreign, altered self, and self antigens to cytotoxic T lymphocytes (CTL) in the cell surface in the context of major histocompatibility (MHC) class I molecules. Following recognition and activation, the CTL then initiate target cell destruction. The antigenic peptides essential for target cell recognition are generated in the cytosol by proteolytic degradation of predominantly endogenous proteins. The resulting pool of peptides is a possible source for translocation of 7–15-mers by the transporter associated with antigen processing into the lumen of the endoplasmic reticulum. Translocated peptides that fulfill the criteria for binding to the available MHC class I allelic products stabilize membrane-bound, empty MHC class I heavy chains in association with β2-microglobulin on the luminal side of the ER. After release of chaperones and the binding of a suitable peptide to the binding groove, the trimeric complex is shuttled to the cell surface (1–3).

Evidence for the significant contribution of the proteasome to the generation of antigenic peptides in the cytosol has accumulated over the past decade. Treatment of cells with proteasome inhibitors caused a significant reduction not only of the in vitro activity of the proteasome, but also of the surface expression of MHC class I molecules and the corresponding ligands (4). Furthermore, in many independent cases, it has been demonstrated that the proteasome is capable of generating MHC class I restricted CTL epitopes and potential MHC class I binding peptides from short synthetic precursors in vivo (5, 6) and in vitro (7). The implication of the 11 S proteasome regulator PA28/20 S proteasome complex in antigen processing (8, 9), as well as the targeted disruption of the loci coding for the interferon-γ-inducible, catalytically active β-subunits LMP2 and LMP7 (10), verified the fundamental importance of the proteasome for antigen processing.

The 20 S proteasome is a cylindrical particle consisting of 28 subunits in four stacked, heptameric rings with a C2 axis of symmetry. The two outer rings comprise α-subunits, and the inner rings form the central cavity. They consist of β-subunits harboring the catalytically active β5 (X, LMP7), β1 (Y, LMP2), and β2 (Z, MECL-1) subunits, which belong to the family of N-terminal nucleophile hydrolases (11, 12). Substrates probably enter the channel leading to the interior chamber through a narrow constriction in the central portion of the α-ring, which is governed by the N-terminal extensions of the α-subunits. In the absence of SDS, access is restricted to unfolded proteins and short peptides. It has been suggested that this channel gating effect can be overcome by regulatory proteins in vivo and in vitro, e.g. by association of the 20 S proteasome with the 19 S cap regulatory particle (RP) (13).

The 26 S proteasome is a complex of the 20 S core and either one or two regulatory particles (14). The RP consists of at least 17 subunits forming two subcomplexes, the “base” and the “lid” (15, 13). The RP confers ATP dependence and recognition of polyubiquitinated protein substrates, leading to substrate unfolding, deubiquitination, and translocation of the substrate into the 20 S core. It has been shown that transcription from vectors coding for fusion proteins with an N-terminal monoubiquitin ligated either to the influenza nucleoprotein or to a human immunodeficiency virus Nef construct with a destabilizing arginine in position 1 results in enhanced specific lysis of transduced target cells. This is in contrast to the lysis of cells transfected with the same vector without the ubiquitin moiety.

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‡The abbreviations used are: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; ER, endoplasmic reticulum; RP, regulatory particle; ND, non-denaturing; PAGE, polyacylamide gel electrophoresis; TPP2, tripeptidylpeptidase 2; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; H-AAP-CMK, H-alanyl-alanyl-phenylalanylalanine-chloromethylketone; H-AAF-AMC, H-alanyl-alanyl-phenylalanine-7-amino-4-methylcoumarin; succ-LVY-AMC, succinyl-leucyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin; AMC, 7-amino-4-methylcoumarin.
The rapid turnover of short-lived proteins and the presentation of resulting fragments on MHC class I molecules depends substantially on conjugation to ubiquitin, as demonstrated with cell-free as well as in vivo model systems (20, 21). Some proteins such as casein (22–24) and chemically modified ovalbumin (25), are degraded via the 26 S pathway without ubiquitin tagging. In the presence of antizyme, recombinant ornithine decarboxylase enharmonizing the H2-RK-restricted CTL epitope SIINFEKL from ovalbumin is a suitable substrate for the 26 S proteasome. The latter regulates the production of the intact SIINFEKL epitope with the correct C terminus (26). These findings support the assumption that the proteasome is responsible for the generation of the proper C termini for MHC class I ligands, whereas the generation of the N terminus is more likely to be the result of MHC class I-dependent trimming activities by ER-resident (27) and cytosolic aminopeptidases (28).

Biochemical studies on the specificity of the proteasome revealed three distinct proteolytic components, which are involved in chymotryptic, tryptic, and peptidylglutamylpeptide hydrolyzing activities, respectively. Analysis of the contribution of the individual β-subunits with yeast mutants has demonstrated a clear correlation between the individual subunits and the cleavage after preferred amino acids (29). Furthermore, the analysis of the degradation of the entire protein yeast enolase 1 with yeast 20 S proteasome revealed a processive mechanism of degradation that yielded peptide fragments with an average length of 7–8 amino acids. The digestion of enolase 1 generated diverse fragments that enabled a detailed analysis of the influence of flanking sequences surrounding the cleavage sites. This revealed the significance of the primary sequence composition next to the site of peptide bond hydrolysis (30). The influence of proline in position 4 (P4) upstream from the cleavage site seems to be particularly important. In contrast, only limited data are available for assessing the contribution of the 26 S proteasome to hydrolytic destruction of peptides and entire proteins (24, 31) with regard to the nature of degradation products.

This study represents the first detailed characterization of the proteolytic specificity of the 26 S proteasome. Our data demonstrate that 26 S and 20 S proteasomes cleave β-casein in such a way that peptide diversity by differential cleavage site usage is guaranteed, whereby fragments with hydrophobic amino acids, especially leucine, at the C terminus, are preferred in both cases. 26 S and 20 S proteasomes appear to operate on the basis of a conserved, underlying matrix for specificity that is distinct from random cleavage of the substrate proteins and is not only common to both conformations from human source, but to the 20 S proteasome from bakers' yeast, too. The mapping of β-casein fragments provides the basis for more detailed knowledge of the integration of the 26 S proteasome into the protein-degrading and antigen-supplying machinery of the cytosol.

**EXPERIMENTAL PROCEDURES**

**Purification of 26 S Proteasome**

All steps were carried out at 4 °C. Approximately 250 ml of human red blood cells (erythocyte concentrate, Bloodbank Universitätsklinikum Tübingen, Tübingen, Germany) were washed three times with phosphate-buffered saline, pH 7.2, lysed for 30 min in 1.6-fold starting volume of buffer A (30 mM Tris-HCl, pH 7.6, 1.6 mM dithiothreitol (Sigma-Aldrich), 3.25 mM ATP (Sigma-Aldrich), 5 mM MgCl₂). Debris was spun down for 30 min at 10,000 × g (Sorvall RC-5C plus, SS-34) and the supernatant was adsorbed to 75 g DEAE-52-Servacel (Serva, Heidelberg, Germany). The batch material was washed three times with 500 ml of buffer B (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 10% (v/v) glyc erol), 125 mM KCl. Bound material was eluted with buffer B, 250 mM KCl in a total volume of 600 ml (Fig. 2A). Eluted material containing the 26 S proteasome was precipitated with 40% ammonium sulfate and collected by centrifugation (22,000 × g, 30 min, Sorvall RC-5C plus, SS-34) and the pellet was resuspended in 10 ml of buffer B, 125 mM KCl, cleared by centrifugation and subjected to FPLC size exclusion chromatography (350 ml of HiPrep Sephacryl S-400 in an XK26/70 column (Amersham Pharmacia Biotech) and eluted under isocratic conditions (buffer B, 125 mM KCl). Peak fractions displaying half-maximal proteolytic activity against the fluorogenic peptide substrates succinyl-Leu-Val-Thr-Arg-7-amino-4-methylcoumarin (suc-LLVY-AMC) (Bachem, Heidelberg, Germany) were collected (Fig. 2D) and the ionic strength slowly adjusted to 75 mM KCl with buffer B. The pooled peak fractions were subjected to FPLC anion exchange chromatography with 75 ml of TSK-DEAE-650S Toyopearl resin (Tosohs Ha GmbH, Stuttgart, Germany) in a XK16/40 column (Amersham Pharmacia Biotech). Bound material was eluted with a gradient ranging from buffer B, 75 mM KCl to buffer B, 500 mM KCl. The proteolytic activity corresponding to the 26 S proteasome eluted in a single peak around 195 mM KCl (Fig. 2B). Material displaying at least half-maximal proteolytic activity was slowly adjusted to 75 mM KCl with buffer B and subsequently loaded onto 8 ml of arginine-Sepharose-4B resin in a XK16/20 column (Amersham Pharmacia Biotech). Bound proteasome was eluted with a gradient ranging from buffer B, 75 mM KCl to buffer B, 300 mM KCl (Fig. 2E).

The 26 S proteasome peak fraction eluted around 169 mM KCl and was collected and concentrated (BioMax 50-kDa ultrafiltration device, Milipore, Bedford, MA) and stored at 4 °C. Both 26 S proteasome from glycerol density gradient centrifugation as well as concentrated material from the arginine-Sepharose-4B eluate were used in this study.

**Purification of 20 S Proteasome**

Human 20 S proteasome was purified from material eluting at 250 mM KCl (buffer B) of the crude extract adsorbed by DEAE-52-Servacel (see above). The eluent was chromatographed over gel filtration and anion exchange columns without prior ammonium sulfate precipitation (Fig. 2C). Subsequently, 20 S proteasome was further purified by chromatography with hydroxyapatite, mono Q, and phenyl-Sepharose columns as described (32).

**Measurement of Proteolytic Activities against Peptide Substrates with Fluorogenic Leaving Groups**

The fluorogenic peptide substrates suc-LLVY-AMC and H-α-LAlanyl-glycyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin (H-AAMC) were prepared from 10 mM stock solutions in MeSO and used in a final concentration of 100 μM. 50 μM of sample were incubated with 150 μM of buffer C (30 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 1 mM ATP, 0.5 mM dithiothreitol, 10 mM KCl) including the fluorogenic substrate in Ultra-Low 96-well plates (Corning Costar Europe, Badhoevedorp, The Netherlands). Fluorescence of the released AMC was determined after 1 h incubation at 37 °C with a Tecan spectrophotometer (Tecan, Crailsheim, Germany) at 360 nm excitation and 430 nm emission. Fluorescence readings of released AMC were recorded as arbitrary (fluorescence) units.

**Protease Inhibitors**

Lactacystin was purchased from E. J. Corey (Harvard University, Cambridge, MA), and β-L-acetyl-phenylalanine-cloroethylketone (H-AF-CMK) was from Sigma-Aldrich. Both inhibitors were used at final concentrations of 50 μM.

**Protein Concentration**

The amount of protein in pooled fractions and the final concentrate was determined by a variation of the Lowry method (Bio-Rad DC Protein Assay, Bio-Rad) and bovine serum albumin as a standard. Absorption was measured at 650 nm with an spectrophotometer (Ultram spec 3000, Amersham Pharmacia Biotech).

**Denaturing PAGE**

5 μg of purified proteasome polypeptides were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) by standard techniques (33) and transferred to polyvinylidene difluoride (DuPont) or nitrocellulose (Sartorius AG, Göttingen, Germany) with a semidry transfer system (CTI).
Detection of human α2 and human LMP-7 was performed with mouse monoclonal antibody MCP-21 (34) α2- and rabbit polyclonal antiserum anti-LMP-7 (PW8200, Affiniti Research Products Ltd., Mannhead, United Kingdom).

Nondenaturing PAGE

Protein samples (5 μg/lane) were resolved by nondenaturing PAGE essentially as described by Glickman et al. (13). Nondenaturing minigels were run at 150 V for 2 h at 4 °C. Protein complexes displaying proteolytic activity were visualized by incubation of the gels for 10 min at 37 °C with suc-LLVY-AMC or H-AAF-AMC and subsequent exposure to UV light (360 nm). Following the fluorescence overlay assay, nondenaturing PAGE gels were stained with Coomassie Brilliant Blue.

In Vitro Degradation of β-Casein

For degradation of β-casein, 10 μg of proteasome were incubated for 3, 6, and 18 h at 37 °C with 100 μg of β-casein in buffer C in a final volume of 0.5 ml. Data are from incubations with a molar ratio of 1:300 to 1:800 (20 S or 26 S proteasome:β-casein), depending on the relative amounts of CP, RP, CP, and RP. Digests were stopped by freezing the samples at −80 °C.

The influence of ATP on β-casein degradation by the 20 S proteasome was tested with 25 μg of 20 S proteasome and 100 μg of β-casein in buffer C with or without ATP (1 mM). Reactions were stopped after 3 and 6 h.

Separation and Analysis of Cleavage Products

Peptide fragments resulting from in vitro degradation of β-casein were separated by reversed phase HPLC (SMART system, Amersham Pharmacia Biotech) over a μRP C18 SC2/1/10 column. Eluent A consisted of 0.1% trifluoroacetic acid; eluent B consisted of 0.081% trifluoroacetic acid, 80% acetonitrile. The gradient was 0–70% eluent B in 62.5 min with a flow rate of 150 μl/min. Fractions were collected by peak fractionation with a maximal volume of 500 μl. Peak fractions were dried and redissolved in 40% methanol, 1% formic acid and subsequently analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry (G2025A, Hewlett Packard, Waldbronn, Germany) and automated Edman N-terminal sequencing (Procius 494A pulsed liquid protein sequencer; Applied Biosystems, Weiterstadt, Germany).

Statistical Analysis

Computations were performed using Maple V Release 4 (Waterloo Maple, Inc.).

 Frequencies of Individual Amino Acids at Certain Positions Relative to Cleavage Sites—The probability \( q(k) \) for finding a given amino acid exactly \( k \) times in a given position by random selection of cleavage sites (considering that each single peptide bond can be randomly selected only once for each endoase molecule) can be calculated according to the hypergeometric distribution below.

\[
q(k) = \frac{\binom{K}{k} \binom{N-K}{n-k}}{\binom{N}{n}}
\]

\( n \) is the number of observed cleavages under consideration, \( N \) is the total number of potential cleavages in β-casein, \( k \) is the observed frequency of a given amino acid in a given position relative to the cleavage site, and \( K \) is the total number of a given amino acid in β-casein. \( q(k) \) values were used to calculate two-sided tail probabilities \( p \) to indicate deviation of observed frequencies from a random selection of certain amino acids at a given position. Because we considered positions P6 to P0 surrounding a cleavage site, the total number of potential cleavage sites \( (N) \) in β-casein was reduced from 209 to 199.

Comparison of Amino Acid Characteristics—To compare the characteristics of amino acids at certain positions for significant differences, the two-sided Student’s \( t \) test for two independent data sets was performed. Hydrophaticity parameters were taken from Kyte and Doolittle (35), Bulkiness parameters from Zimmerman et al. (36), and normalized frequency parameters for β-turn from Levitt (37). Results are shown as \( p \) values and mean value differences including the 95% confidence limit. Only differences with \( p \) values < 0.05 are reported.

RESULTS

Purification and Assessment of Purity—Human 26 S proteasome was enriched from erythrocytes to a >95% degree of purity with respect to unspecific background protein content of the sample (Fig. 1, A and B). The 20 S and 26 S proteasomes from erythrocytes do not have divergent subunit composition with respect to the interferon γ-inducible subunits. In contrast to the 20 S immunoproteasome isolated from interferon γ-induced cells, neither 20 S nor 26 S show incorporation of LMP-7, which is crucial for the formation of immunoproteasomes (34) (Fig. 1C). The process of purification (Fig. 2) was based on the recovery of fractions displaying proteolytic activity toward the chymotryptic substrate suc-LLVY-AMC. The increase in specific activity is at least 5,000-fold, not taking into account the fact that a substantial amount of measured activity at early points in the purification procedure can be attributed to 20 S proteasomes (Table I). In fluorescence overlay assays, the glyceral density gradient-purified 26 S fraction displays only minimal background activity against the fluorogenic substrate H-AAF-AMC. This is the preferred substrate among a large variety of short fluorogenic model peptides for tripeptidylpeptidase 2 (TPP2), a high molecular weight amino- and endopeptidase from the cytosol (39). The serine protease TPP2 has been associated with the processing pathway for MHC class I peptide ligands, where it could serve as a cytosolic trimming peptidase (39). To elucidate the influence of TPP2, which coelutes with the 26 S, but not the 20 S proteasome from size exclusion as well as anion exchange chromatography columns on substrate degradation in vitro, we performed ND-PAGE analysis together with fluorescent substrate overlay of samples from purified 20 S and 26 S proteasomes. In addition, we looked at intermediate products of purification. These and purified proteasomes were preincubated with the proteasome inhibitor lactacystin or the serine protease inhibitor H-AAF-CMK. The concentrated 26 S proteasome-containing fraction from the ar-
ginine-Sepharose-4B chromatography step displays a distinct proteolytic complex that migrates even slower than the 26 S proteasome complex with two regulatory caps (RP2CP). It also reveals a more improved activity against the fluorogenic substrate H-AAF-AMC than the 26 S proteasome (Fig. 3), which can be completely abolished by preincubation of the sample with H-AAF-CMK. The weak activity of the 26 S proteasome complexes toward H-AAF-AMC, on the other hand, does not change upon treatment with H-AAF-CMK (Fig. 3). The glycerol density gradient-purified 26 S proteasome (Figs. 1 (A and B) and 4 (A–D)) shows very weak activity toward H-AAF-AMC in the presence or absence of H-AAF-CMK (Fig. 4A). Both 20 S and 26 S proteasomes displayed complete inhibition of H-AAF-AMC degradation following preincubation with lactacystin (Fig. 4C). The same analysis of purified proteasomes by the fluorescence overlay assay was conducted in the presence of the reference fluorogenic substrate for the chymotryptic activity of the proteasome, suc-LLVY-AMC. The preincubation of 20 S and 26 S proteasome with H-AAF-CMK did not yield any reduction of sample activity (Fig. 4B), whereas lactacystin inhibited the degradation of suc-LLVY-AMC (Fig. 4D). The results allow us to conclude that our 20 S and 26 S proteasome preparations were of high purity and suitable for the in vitro degradation of peptides and proteins. However, to eliminate the possibility that trace amounts of TPP2 contribute to peptide generation, all in vitro digests were performed in the

![Fig. 2 Method of purification.](http://www.jbc.org/)

**A**. batch adsorption. The major proteolytic peak elutes at 250 m M to 300 m M KCl in buffer B. The eluate was diluted and loaded onto the anion exchange column directly (C), or following prior ammonium sulfate precipitation of the 26 S proteasome containing fraction (B). The 26 S proteasome elutes with a maximum at 195 m M KCl in buffer B, the 20 S proteasome with a maximum at 137 m M KCl, respectively. **D**, size exclusion chromatography of the 26 S-containing fraction shown in **B**. **E**, affinity chromatography of the 26 S-containing peak fraction shown in **D**. The 26 S-containing fraction elutes with a maximum at 169 m M KCl in buffer B. **F**, final purification of 26 S proteasome from the peak fraction shown in **E** by density gradient centrifugation.
presence or absence of the TPP2 inhibitor H-AAF-CMK. 26 S proteasomes remained stable for more than 18 h at 37 °C, as judged from fluorescence overlay of ND-PAGE gels (Fig. 3B) and Coomassie staining of ND-PAGE gels (data not shown). No active 20 S proteasome could be detected in 26 S fractions after incubation at 37 °C for up to 48 h (data not shown), excluding a contribution of 20 S proteasomes to the fragments generated by 26 S proteasomes.

Analysis of Degradation Products—The primary analysis by reversed phase HPLC and SDS-PAGE of degradation products from β-casein generated in incubations for up to 6 h revealed no detectable difference for peptide products, whether H-AAF-CMK had been added or not (Fig. 5). In contrast, β-casein digests using 20 S or 26 S proteasomes resulted in clearly divergent product patterns (Fig. 5, A and B). To exclude an influence of ATP on the 20 S proteasome and on the substrate, control digests in the presence and absence of ATP were included (Fig. 5, C and D), resulting in identical patterns of peptide peaks. Therefore, ATP has no effect on the degradation of β-casein by 20 S proteasomes. To allow for an accurate assessment of the differences in the enzymatic activity of those closely related proteolytic complexes, peak fractions from digests up to 6 h were subjected to mass spectrometric analysis (matrix-assisted laser desorption ionization/time of flight) and quantitative Edman degradation. 74 different fragments for the 20 S and 66 for the 26 S proteasome were characterized.

The efficiency of purification was monitored by measuring the amount of suc-LLVY-AMC hydrolysis per milligram of recovered protein from respective steps in the process of purification (left column). The relative amount of recovered enzymatic activity was calculated and is mirrored in the right column. Both columns do thus not show the amount of recovered 26 S proteasome per se, but of all suc-LLVY-AMC hydrolyzing activity.

| Step in purification | Arbitrary fluorescence units/mg | Recovered total activity |
|----------------------|---------------------------------|--------------------------|
| Crude lysate         | <200                            | 100                      |
| Eluate batch adsorption | 3.9 × 10^5                      | 68                      |
| (NH₄)₂SO₄ precipitate |                               | --                      |
| Pooled peak gel filtration | 4.2 × 10^5                      | 18                      |
| Pooled peak ion exchange chromatography | 6.3 × 10^5 | 16 |
| Pooled peak affinity chromatography | 1.1 × 10⁶ | 9 |
| Pooled peak density gradient | 1.1 × 10⁶ | 4 |

*— not determined.

The fragments and their C and N termini are projected onto the map that represents the entire amino acid sequence of β-casein (Fig. 6). Different fragments from the same digest were detected in varying amounts, ranging from less than 1 pmol to 170 pmol (from an input of 4 nmol). As previously observed in digests of enolase-1 by yeast 20 S proteasome (30), overlapping fragments are generated, indicating the alternating use of certain cleavage sites. Taken together, these fragments fit into a pattern that gives the first clue for the assessment of proteasomal activity and specific differences in enzymatic activity.

Comparison of 26 S and 20 S Proteasome Specificity—The 26 S proteasome produces fragments with an average length of 10.1 amino acid residues, as judged from the number and individual length of the peptides. If the molar amount of fragments is taken into account, the average peptide length shrinks to 7.2 amino acid residues. In comparison, the 20 S proteasome generated peptides with average sizes of 18.3 and 15.8 amino acid residues, respectively. Only fragments that did not contain the original C or N terminus of the substrate were computed for the calculations, to avoid any bias on the outcome of size distribution patterns from fragments derived from cleavages of one peptide bond only. Fragments with less than 5 amino acids...
are difficult to detect and, if present in the digests, do not contribute to the analysis. Further statistical analysis revealed both divergent features and qualities common to the 20 S and 26 S proteasomes. The analysis of the cleavage sites and the hypergeometric (Student's t test) distribution is summarized in Table II. The most prominent features of human 26 S and 20 S proteasomes include a preference for hydrophobic amino acids (parameters: Kyte-Doolittle) and especially leucine in P1, so that fragments arising from the proteasome have a hydrophobic rather than a charged C terminus. The 20 S cleaves after 11 of the 22 available leucine residues, the 26 S cleaves after 14 of the 22. Turn-promoting (parameters: Levitt) and flexibility-providing amino acids are not favored (26 S) or even strongly disfavored in P1 (20 S). Turn-promoting and flexibility-providing amino acid residues are very rarely found in P2 for both 20 S and 26 S, but the 26 S favors cleavages if turn-promoting amino acids or especially proline are available in P1. Other amino acids enhance (proline in P4) or reduce (glutamine in P6) the probability of peptide bond hydrolysis by the 26 S. Those preferences did not seem to be significant for the human 20 S. Analogous data from the degradation of β-casein by wildtype yeast 20 S proteasome show preferences for hydrophobic and bulky amino acids in P1, proline, and other turn-promoting and flexibility-providing amino acids in P4. Amino acids that are significantly reduced are proline in P2 and flexibility-providing and turn-promoting residues in P1. The analysis of the cleavages made by both human 20 S and 26 S proteasomes underlines the importance of leucine in P1. Extension of this form of analysis to human 20 S and 26 S and yeast 20 S highlights the importance of leucine in P1 and proline in P4 and P1'. These preferences in specificity are

**FIG. 5.** Optical density at 214 nm of eluted digestion products from reversed phase HPLC. Black line, β-casein (control); light gray line, 20 S proteasome + β-casein; dark gray line, 26 S proteasome + β-casein. A and B, after 6 h. Digest in A had been preincubated for 1 h with 100 μM H-AAF-CMK before adding β-casein. C, 20 S proteasome + β-casein, 1 mM ATP added. D, 20 S proteasome + β-casein, no ATP added.

**FIG. 6.** Fragments detected from digestion of β-casein by 26 S and 20 S proteasome after 6 h. Map in A shows a projection of all fragments generated by the 20 S proteasome onto the β-casein sequence. Map in B shows a projection of all fragments generated by the 26 S proteasome onto the β-casein sequence.
TABLE II

| P4 | P3 | P2 | P1 | P1 |
|----|----|----|----|----|
| Yeast 20 S (see Footnote 2) | Proline | Turn-promoting | Leucine | No flexible |
| Human 20 S (this paper) | No turn-promoting | Leucine | No flexible |
| Human 26 S (this paper) | No turn-promoting | No flexible | Hydroporphic (Bulky) |
| Human 26 S (this paper) | No turn-promoting | No flexible | Turn-promoting | No bulky |
| Human 26 S (this paper) | Proline | Leucine | Proline | No glutamine |
| Human 26 S (this paper) | Proline | Leucine | Proline | No glutamine |

The relative preference of proteasomes from yeast and human source for specific amino acids or certain amino acids with shared qualities in defined positions is shown. The cleavage of the peptide bond occurs between P1 and P1 cleavages obtained from digests of \( \beta \)-casein by wild type 20 S proteasome from \textit{Saccharomyces cerevisiae} (see Footnote 2). Line 2, analysis of cleavages obtained from digests of \( \beta \)-casein by 26 S proteasome from human source. Line 3, analysis of cleavages obtained from digests of \( \beta \)-casein by 26 S proteasome from human source.

The degradation of c-Fos seems to be partially ubiquitin-independent (49) and ubiquitin-dependent (50). Whether 20 S and 26 S proteasomes contribute independently to the generation of MHC class I ligands or together is not known. Studies address the participation of proteasomes in antigen presentation is well established, the relative contribution of 20 S and 26 S subtypes is not so clear. Targeting of peptides from minigenes as well as whole proteins to the 26 S proteasome by covalent linkage to ubiquitin leads to epitope generation and enhanced MHC class I restricted CTL recognition (16, 18–20, 26, 45). Evidence for the indispensable role of ubiquitin is contradictory and will have to be analyzed in more detail (21, 46).

Some proteins can be degraded by the 26 S proteosome without prior attachment to mono- or polyubiquitin via the \( \alpha \)-or \( \epsilon \)-amino groups of the substrate. This has been shown for c-Jun (47), ornithine decarboxylase (23, 24, 48), ornithine decarboxylase containing a short stretch from ovalbumin (26), denatured insulin-like growth factor, lactalbumin, and \( \beta \)-casein as well as fluorescein isothiocyanate-labeled casein (31). The degradation of c-Fos seems to be partially ubiquitin-independent (49) and ubiquitin-dependent (50). Whether 20 S and 26 S proteasomes contribute independently to the generation of MHC class I ligands or together is not known. Studies addressing the specificities of 20 S proteasomes alone will therefore be of limited in \textit{vivo} relevance if 20 S and 26 S proteasomes differ in their cleavage site selection.

For this reason we have now accomplished an in-depth analysis of degradation products from \( \beta \)-casein. This protein was incubated with both 26 S and 20 S proteasomes for up to 6 h (Fig. 5). The recovered fragments were separated on reversed phase HPLC columns, and the elution profiles gave the first indication of a substantial difference in the nature of products (Fig. 5), as previously reported (31). Identification of fragments revealed that the 26 S proteasome generated shorter cleavage products, when compared with peptides derived from 20 S proteasome-mediated degradation (Fig. 7). This finding contradicts the assumption that the 26 S proteasome might be responsible for the initial destruction of ubiquitin-tagged protein substrates, followed by additional 20 S proteasome-mediated cleavages. Instead, our results suggest that the action of 20 S and 26 S proteasomes is carried out independently. However, we cannot rule out the possibility that the 20 S proteasome...
produces a higher number of fragments than the 26 S proteasome with less than 5 amino acids that escape detection.

It has previously been reported that the 20 S proteasomes from yeast (wild-type and mutant) and from human source do not cleave at random, but in a highly specific manner (29, 30), which is subunit-dependent for trypsin- and chymotrypsin-like cleavages and partially subunit-independent for chymotrypsin-like cleavages. A dominant feature of these studies was the preference for proline in position 4 (P4) and leucine in P1 upstream from the cleavage site in digests of yeast enolase 1 with wild-type yeast 20 S proteasome. A preference for \( \beta \)-turn-promoting amino acids in P1\(^*\) was observed, too. An effect of proline on the generation of two CTL epitopes from c-akt and pp89 from yeast (wild-type and mutant) and from human source do not demonstrate that the different conformations of the proteasome employ common and conserved parameters to yield peptide fragment sets that are only about 25% identical. Despite this small proportion, approximately 50% of the cleavage sites found were virtually identical. Thus, the 26 S and 20 S proteasomes together generate a diverse pool of peptides with hydrophobic C termini that could be eligible for binding to MHC class I heterodimers. This diversity cannot be caused by a different contribution of interferon-\( \gamma \)-inducible \( \beta \)-subunits because 20 S and 26 S proteasomes from erythrocytes lack LMP7, which is required for the maturation of immunoproteasomes (38). We speculate that the 19 S regulatory particle brings about a conformational change in the quaternary structure of the core particle that induces a change in specificity. Nevertheless, we cannot exclude the possibility that an undetectable imbalance in active \( \beta \)-subunit composition or modification between the 26 S and 20 S proteasomes is the cause of the specificity differences observed. Future studies will have to determine whether or not polyubiquitinated proteins are degraded by the employment of similar cleavage specificities in comparison to peptides and proteins without such a targeting tag. Finally, the manifestation of common rules for proteasomal degradation, regardless of the substrate, will hopefully improve the epitope prediction from artificial and endogenous peptide and protein substrates in its relevance to human disease.

Acknowledgments—We thank Prof. Dr. Nortoff from the blood bank of the University Hospital Tübingen for the donation of erythrocyte concentrate samples and Lynne Yakes for editing the manuscript. We also thank Tobias P. Dick and Klaus Dietz for their successful efforts to create a statistical evaluation program to serve our purposes.

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The Human 26 S and 20 S Proteasomes Generate Overlapping but Different Sets of Peptide Fragments from a Model Protein Substrate

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*J. Biol. Chem. 2000, 275:21140-21148.*
doi: 10.1074/jbc.M000740200 originally published online May 2, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000740200

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