Contribution of Proteasomal $\beta$-Subunits to the Cleavage of Peptide Substrates Analyzed with Yeast Mutants*

(Received for publication, May 28, 1998, and in revised form, July 10, 1998)

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Proteasomes generate peptides that can be presented by major histocompatibility complex (MHC) class I molecules in vertebrate cells. Using yeast 20 S proteasomes carrying different inactivated $\beta$-subunits, we investigated the specificities and contributions of the different $\beta$-subunits to the degradation of polypeptide substrates containing MHC class I ligands and addressed the question of additional proteolytically active sites apart from the active $\beta$-subunits. We found a clear correlation between the contribution of the different subunits to the cleavage of fluorogenic and long peptide substrates, with $\beta$5/Pre2 cleaving after hydrophobic, $\beta$2/Pup1 after basic, and $\beta$1/Pre3 after acidic residues, but with the exception that $\beta$2/Pup1 and $\beta$1/Pre3 can also cleave after some hydrophobic residues. All proteolytic activities including the "branched chain amino acid-prefering" component are associated with $\beta$5/Pre2, $\beta$1/Pre3, or $\beta$2/Pup1, arguing against additional proteolytic sites. Because of the high homology between yeast and mammalian 20 S proteasomes in sequence and subunit topology and the conservation of cleavage specificity between mammalian and yeast proteasomes, our results can be expected to also describe most of the proteolytic activity of mammalian 20 S proteasomes leading to the generation of MHC class I ligands.

The 20 S proteasome, a threonine protease (1) and member of the N-terminal nucleophile-amidohydrolase family (2), is the central component of the non-lysosomal proteolytic system in eukaryotes. It is not only involved in general protein catabolism, but also in major regulatory processes such as cell cycle control and signal transduction pathways (3–5). In vertebrates, the proteasome additionally caters to the immune system: peptides generated as degradation products are delivered to MHC class I molecules for presentation on the cell surface (6). Although localized in the cytosol and nucleoplasm, the proteasome appears to be responsible for endoplasmic reticulum-associated protein degradation as well (7–9).

The cylindrical 20 S proteasome is composed of four longitudinally stacked heptameric rings. The two outer rings are made up of $\alpha$-subunits and connect the 20 S particle to supplementary complexes, most notably the 19 S cap and the interferon-$\gamma$-inducible PA28$\alpha$β complex. The 19 S cap complex confers specificity for ubiquitinated substrates (5). The PA28$\alpha$β complex appears to be an immunological adaptation, modulating the cleavage mechanism and improving the yield of antigenic peptides (10, 11).

The two inner rings, composed of $\beta$-subunits, harbor the catalytically active subunits, displaying their active sites on the inner surface of the central tunnel. Most $\beta$-subunits are generated from precursors that undergo N-terminal processing during proteasome assembly (12). Three of the mature subunits (MB1, $\delta$, and Z in human; $\beta$5/Pre2, $\beta$1/Pre3, and $\beta$2/Pup1 in yeast) are proteolytically active and carry an amino-terminal threonine residue as the catalytic nucleophile (13–16). Some of these critical Thr residues can be covalently modified by the proteasome-specific inhibitor lactacystin (13). In higher vertebrates, the cytokine interferon-$\gamma$ induces the expression of three additional active proteasomal subunits, LMP7, LMP2, and MECL1, which replace their constitutive counterparts MB1, $\delta$, and Z, respectively (17–19). However, how these exchanges modulate proteasome function and specificity is still poorly understood.

Biological studies on the specificity of the 20 S proteasome led to the description of three distinct proteolytic components, designated as the chymotrypsin-like, trypsin-like, and peptidyl-dlytaminylpeptide hydrolyzing (PGPH) activities. Recent studies confirmed that the yeast homologues of MB1, Z, and $\delta$ are the only active subunits with a functional N-terminal Thr nucleophile (15, 16) and represent the above-mentioned three classical activities, as defined with certain fluorogenic model substrates.

However, some biochemical studies also pointed toward the existence of additional proteolytic components within the proteasome (20); one of those, the so-called "branched chain amino acid-prefering" (BrAAP) component, cleaving after Leu in cer-
tain model substrates, was also assumed to represent the major protein-degrading activity of the proteasome (21). The existence of such additional activities was concluded from inhibitory studies. Whereas the cleavage of fluorogenic substrates represents the three classical activities could be inhibited with the general serine protease inhibitor 3,4-dichloroisocoumarin (DCI), the same treatment led to an increased turnover of certain other substrates, defined to represent the BrAAP component (20, 22). Using more specific inhibitors, evidence was provided that the BrAAP and PGPH activities might be related to each other (23, 24). More recently, the arrangement of the intermediate processed $\beta$7/Pre4 and $\beta$6/C5 proteptides in the crystal structure of the yeast 20 S proteasome led to the postulation of an additional catalytic activity, not related to the Thr sites and possibly involved in the processing of the $\beta$-subunit proteptides (14).

In this study, we investigated whether the specificities defined with small fluorogenic substrates reflect truly distinct catalytic sites and how these specificities relate to the cleavages that can be observed in peptide substrates containing known MHC class I ligands. In addition, we addressed the question of additional proteolytically active sites associated with the yeast 20 S proteasome by analysis of the BrAAP component.

Using highly purified wild-type and mutant yeast proteasomes and several inhibitors of the proteasomal activities, we examined the cleavage specificity of the three active subunits for short fluorogenic peptides as well as peptide substrates derived from the murine JAK1 tyrosine kinase, the pp99 protein from murine cytomegalovirus, and the nucleoprotein from influenza A/PR/8/34. We found that all observed cleavages could be correlated to the three known active sites, showing that no additional proteolytic activity exists within the yeast 20 S proteasome for the given set of substrates. Furthermore, we found that the specificities of the different subunits for fluorogenic substrates are mainly identical to those observed for peptide substrates, with few, but notable exceptions. This allows now the clear association of certain cleavage events within peptide substrates with certain $\beta$-subunits and therefore an assessment of the contribution of the different $\beta$-subunits to the generation of proteasomal degradation products.

The 20 S proteasomes from yeast and higher eukaryotes are highly homologous. As shown in this paper and recently by Niedermann et al. (25), most cleavage sites in peptides are with argon atoms in MS-MS experiments. For relative quantitation of cleavage products, an azo dye was generated from free $p$-aminobenzoic acid (Z-GPALA-pAB) formation reaction, an azo dye was generated from free $p$-aminobenzoic acid. The extinction of the dye was measured at 545 nm.

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant Yeast 20 S Proteasomes**—The generation of mutant strains YUS4 (pup1-T1A), YUS1 (pre3-T1A), YUS5 (pup1-T1A pre3-T1A), and YWH23 (pre2-K33A) (numbering refers to amino acid positions in mature subunits) has been described recently (15). Wild-type yeast cells were as described (14).

**Purification of Proteasomes**—The purification of 20 S proteasomes from Saccharomyces cerevisiae has been described recently (14). The same procedure was applied to the purification of the various mutant proteasomes.

**Measurement of Proteasomal Activities against Substrates with Fluorogenic Leaving Group**—The fluorogenic substrates benzoyloxycarbonyl-Gly-Leu-7-amino-4-methylcoumarin (Z-GGL-AMC), succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC), benzoyloxycarbonyl-Ala-Arg-Arg-7-amino-4-methylcoumarin (Z-ARR-AMC), and benzoyloxycarbonyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Z-LLET-NA) (all purchased from Bachem, Heidelberg, Germany) were pre pared from 10 mM stocks in MeSO. Typically, 1 $\mu$g of proteasome (with and without inhibitors) was incubated in a 100 $\mu$l substrate solution. Fluorescence of the leaving group was determined after incubation times of 1–5 h with a Millipore spectrophotometer at 380 nm excitation and 440 nm emission for AMC and at 330 nm excitation and 410 nm emission for $\beta$-NA.

**Inhibitors**—Lactacystin was purchased from E. J. Corey (Harvard University); DCI and calpain inhibitor I (LLnL) (Sigma) were a gift from Dr. M. Orlowski (Mount Sinai, New York). It was incubated in 100 mM Tris (pH 8.0) at a final concentration of 1 mM together with 1 $\mu$g of proteasome, in the presence or absence of 0.01 unit of aminopeptidase M (Sigma). The reaction was stopped by the addition of 10% trichloroacetic acid and developed by the sequential addition of 2 volumes of 0.1% NaNO$_3$ (1 in 2 HCl), 2 volumes of 0.5% ammonium sulfate in 1 N HCl, and 4 volumes of 0.05% N-naphthylthioldendiamine (in 95% ethanol). In the course of the development reaction, an azo dye was generated from free $p$-aminobenzoic acid. The extinction of the dye was measured at 545 nm.

**Determination of Proteasomal Cleavages in Unmodified 19–25-mer Peptides**—Peptides were synthesized on an Applied Biosystems 432A automated peptide synthesizer applying Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry and purified by reversed-phase HPLC (Beck man Instruments, System Gold). Incubations of 1 $\mu$g of proteasome with peptide substrates at a final concentration of 50 $\mu$m were performed at 37 $^\circ$C in a total volume of 300 $\mu$l of assay buffer (20 mM HEPES/ROH (pH 7.8), 2 mM MgOAc, and 1 mM dithiothreitol).

**Separation of Cleavage Products and On-Line Analysis by Mass Spectrometry**—Proteasomal digests were analyzed with two different LC-MS systems. In the case of the first system, aliquots of 40 $\mu$l were loaded by autosampler (CTC A2005) and separated by a HPLC apparatus (Sykam, Gilsching, Germany) equipped with a $\mu$RPC C2/C18 SC 2/1/10 column (Pharmacia, Freiburg, Germany). Eluent A consisted of 0.05% trifluoroacetic acid; eluent B consisted of 80% acetonitrile containing 0.055% trifluoroacetic acid. The gradient was 23–63% eluent B in 20 min; the flow rate was 50 $\mu$l/min, with the gradient started 5 min after sample injection. Analysis was performed on-line by a tandem quadrupole mass spectrometer (TSQ 700, Finnigan MAT, Bremen, Germany) equipped with an ESI-F electrospray ion source. Each scan was acquired in centroid mode over the range m/z 300–25638.

In the case of the second LC-MS system, aliquots of 5 $\mu$l were separated by a HPLC apparatus (Applied Biosystems 140D solvent delivery system) equipped with a 300-$\mu$m C18 RP column (Gromsal, Gron, Herrenberg, Germany). Eluent A contained 4 mM NH$_4$Ac, adjusted to pH 3 with formic acid; eluent B contained 2 mM NH$_4$Ac in 70% acetonitrile, adjusted to pH 3 with formic acid. The gradient was 20–75% eluent B in 25 min; the flow rate after the pre-column split was 6 $\mu$l/min. Analysis was performed on-line by a hybrid quadrupole orthogonal acceleration time of flight mass spectrometer (Q-TOF, Micromass, Manchester, United Kingdom) equipped with an electrospray ion source. Each scan was acquired over the range m/z 400–1500 in 3 s.

Peptides were identified by their molecular mass. Additionally, the identity of main cleavage products was confirmed by fragmentation with argon atoms in MS-MS experiments. For relative quantitation of single molecular species, integrated ion currents were calculated and compared.

**RESULTS**

**Cleavage of Small Fluorogenic Substrates**—Cleavage of the standard fluorogenic substrates GGL, ARR, and LLE has been shown recently to be subunit-specific (15). Using for the first time yeast proteasome preparations, we repeated the cleavage of fluorogenic peptides to test the reproducibility of the results in our experimental setup. As expected, the GGL cleaving activity, defined to represent the chymotrypsin-like activity, is clearly correlated with the $\beta$5/Pre2 site: cleavage of the Z-GGL-AMC substrate was only present in those mutants that retain a functional $\beta$5/Pre2 subunit and was absent in pre2-K33A mutant proteasomes (Fig. 1A). Identical results were obtained with the Suc-LLVY-AMC substrate (data not shown). The ARR cleaving activity, defined to represent the trypsin-like activity, depends on a functional $\beta$2/Pup1 site: the cleavage of Z-ARR-AMC was not observed in the $\beta$2/Pup1-mutated proteasomes from the pup1-T1A and pup1-T1A pre3-T1A strains, but was observed in all other mutants (Fig. 1B). Correspondingly, $\beta$1/Pre3 is responsible for the PGPH-like activity: the Z-LLET-NA substrate was not cleaved in the double mutant pup1-T1A pre2-K33A.
**Pre3-T1A** and was only cleaved weakly in the **pre3-T1A** single mutant (Fig. 1C). The increased activities of some mutant proteasomes compared with wild-type particles are hard to explain, but might be caused by the lack of competition from inactivated subunits for substrate molecules or by an allosteric activation of the remaining active β-subunits by the presence of propeptide remnants still associated with the inactivated β-subunits. In conclusion, the three standard fluorogenic substrates can be used as subunit-specific functional markers. We therefore decided to test the influence of several inhibitors on the proteolytic activity of wild-type and mutant yeast 20 S proteasomes.

**Proteasomal Subunits Are Differentially Inhibited by Lactacystin, LLnL, and DCI**—It has been reported previously that the bovine proteasomal subunits corresponding to yeast β5/Pre2 and β2/Pup1 (13) or all six human active β-subunits (26) are inhibited by lactacystin due to covalent binding to the N-terminal threonine. Soaking of yeast 20 S proteasome crystals resulted only in a covalent modification of the β5/Pre2 subunit (14). In our hands, pretreatment of proteasomes with 1 mM lactacystin for 45 min led to absolute inhibition of GGL and ARR cleavage by 90% (Fig. 1G). As mentioned before, we observed similar results in other experiments (data not shown). Possible explanations for the apparent difference from the crystal structure data, which showed occupation of all active sites by LLnL and occupation of only β5/Pre2 by lactacystin, will be discussed.

DCI has been reported to inhibit proteasomal activities for lactacystin and LLnL was also observed in inhibitor titration experiments (data not shown). Possible explanations for the apparent difference from the crystal structure data, which showed occupation of all active sites by LLnL and occupation of only β5/Pre2 by lactacystin, will be discussed.

The third inhibitor tested in our study was DCI. Whereas yeast β5/Pre2 was clearly sensitive to inactivation by DCI after a 45-min incubation, β1/Pre3 was only weakly affected by DCI concentrations of 1 mM, and β1/Pup1 was not affected at all (Fig. 2). This result is in line with the observation that the three activities in the bovine pituitary proteasome are inactivated with widely differing rate constants (chymotrypsin-like > PGPH-like > trypsin-like) (27). However, it has also been reported for the bovine pituitary proteasome that all three classical activities can be inactivated with micromolar concentrations of DCI and short incubation times (20). This cannot be confirmed in our experiments with wild-type and mutant yeast proteasomes. To inhibit β1/Pre3 by 50%, the yeast proteasomes had to be preincubated in 100 μM DCI for 90 min. Lower DCI concentrations or shorter preincubation times did not significantly affect the β1/Pre3 activity (data not shown).

**BrAAP Activity as Measured with the Z-GPALA-pAB Substrate Is Catalyzed by the β1/Pre3 Active Site**—Micromolar concentrations of DCI have been shown to activate an additional proteolytic component of bovine 20 S proteasomes, the BrAAP component (20). We therefore tested whether this activity is also present in yeast 20 S proteasomes. The existence of mutant yeast proteasomes gave us the unique possibility to test whether or not this component, if present, can be associated with one of the three active β-subunits or whether it represents an additional, so far unrecognized, active site.
Using the assay introduced by Orłowski et al. (20) to measure the BrAAP activity of the proteasome, the Z-GPALA-pAB substate was incubated with the different yeast proteasome preparations. Generation of the leaving group (pAB) in the absence of aminopeptidase M reflects direct cleavage after the C-terminal alanine, whereas the liberation of additional pAB in the presence of aminopeptidase M is described as cleavage after the penultimate leucine residue, generating Ala-pAB in the first place. The cleavage after Leu is thought to represent the so-called BrAAP activity. Significant BrAAP activity was found in all proteasome preparations, except the one with an inactivated β1/Pre3 subunit. Here, the addition of aminopeptidase M did not lead to release of additional pAB (Fig. 3C), indicating that cleavage after leucine in the GPALA substrate is catalyzed by the β1/Pre3 subunit. On the other hand, cleavage after alanine was clearly independent of β1/Pre3 (Fig. 3C) and could be attributed to β3/Pre2 or β2/Pup1. The finding that the BrAAP activity was performed by β1/Pre3 was also supported by the fact that this activity could not be inhibited by lactacystin, found previously not to influence β1/Pre3 activity against LLE (Figs. 1F and 3, A, B, and D (+ aminopeptidase M (APM), +lactacystin (LC))).

Analysis of the Degradation of Peptide Substrates—Most cleavages in long peptide substrates occur after hydrophobic (Phe, Tyr, Ile, Leu), basic (Arg, Lys), and acidic (Asp, Glu) residues (see below), a situation similar to the cleavage of fluorogenic substrates, which have been related to chymotrypsin-like, trypsin-like, and PPGH-like activities in the proteasome. To investigate whether these cleavages are catalyzed by the four different mutant yeast proteasome preparations containing the inactivated β-subunits β2/Pup1, β1/Pre3, β2/ Pup1β1/Pre3−, and β5/Pre2−. Products were separated and analyzed by LC-MS. Three typical sets of HPLC profiles displaying integrated ion currents for the masses of particular degradation products are shown in Fig. 4.

We limited ourselves to the interpretation of only those cleavages showing a dramatic reduction or complete absence in digestions using mutant proteasomes and avoided to compare relative amounts of fragments generated under the influence of different proteasome mutants. The reason for this is that many peptide fragments generated are intermediate products and subject to further degradation. For quantitative analysis, one would have to consider consecutive cleavages, stabilizing or destabilizing the fragment under consideration. This kind of analysis would go far beyond the aim of this study. However, it is possible to recognize a strong requirement for a certain subunit in those cases where corresponding mutants exhibit no or greatly reduced product formation (compare Fig. 4).

Degradation of the JAK1 21-mer Peptide—The JAK1 21-mer peptide is part of JAK1 tyrosine kinase and contains the MHC class I ligand SYFPEITHI (28). Incubation of this peptide with mammalian proteasomes leads to efficient generation of the MHC I ligand (10, 25), which is flanked by two dominant cleavage sites after Phe7 and Ile16, respectively. The same two cleavages are efficiently catalyzed by the yeast proteasome, in addition to minor cleavages that are also conserved between the mammalian and yeast systems. However, the yeast enzyme additionally cleaves after His15, a cleavage not observed in digests with the mammalian proteasome (Fig. 5).

Cleavage after Phe7 is β5/Pre2-dependent since generation of fragments 1–7 and 8–21 was nearly absent in β5/Pre2-defective proteasomes (Fig. 5, A and B). Cleavage after Ile16 was also catalyzed by the β5/Pre2 subunit: generation of fragment 17–21 was nearly missing in digests with β5/Pre2-deficient proteasomes (Fig. 5C). Correspondingly, a functional β5/Pre2 subunit was necessary and sufficient for the generation of SYFPEITHI (positions 8–16): β2/Pup1 β1/Pre3 double mutants (which carry only functional β5/Pre2 subunits) efficiently generated SYFPEITHI, whereas β5/Pre2 mutants did not generate any detectable amount (Fig. 5D). The observation that the formation of fragments 1–7, 8–16, and 8–21 can be completely inhibited with lactacystin (Fig. 5, G–I) is in agreement with an essential role of β5/Pre2 and indicates that β1/Pre3 does not perform this cleavage.

Cleavage after His15 was detected only in mutants with a functional β2/Pup1 subunit, as demonstrated for fragment 16–21 (Fig. 5E). Cleavage after Tyr9, leading to FPEITHI (positions 10–16) in the mammalian system (10), also took place in the yeast system, albeit much more weakly. The minor product FPEITH (positions 10–15) is an example of a fragment that depends on two different functional subunits: β5/Pre2 for the N-terminal cleavage and β2/Pup1 for the C-terminal cleavage. Correspondingly, fragment 10–15 was only formed in digests with proteasomes that contain both β5/Pre2 and β2/Pup1 (Fig. 5F).

Degradation of the pp89 25-mer Peptide—The pp89 25-mer peptide is derived from the murine cytomegalovirus pp89 IE-protein and contains the L1-presented CTL epitope YPH-FMPTNL (29). In digests with the mammalian proteasome, two main products were generated: fragments 8–15 and 5–15, the latter one being a candidate precursor peptide for the CTL epitope (10). Again, the same dominant cleavage sites and many of the subdominant ones were also used by the yeast enzyme (Fig. 6).

Cleavage after Tyr4 was essentially β5/Pre2-dependent since formation of fragments 1–4 and 5–25 was present only in
minimal amounts in β5/Pre2-defective proteasomes (Fig. 6, A and B), and the generation of fragment 1–4 could be inhibited by lactacystin (Fig. 6O). However, the situation was more complicated for the cleavage after Tyr5, which took place in all mutants tested (Fig. 6, C and D). This observation can be explained only if two different subunits effectively catalyze the same cleavage. The β5/Pre2 subunit was clearly capable of performing the cleavage since it took place efficiently in the β2/Pup1β1/Pre3 double mutant. This leads to the question which subunit catalyzes the same cleavage in the β5/Pre2-defective mutant. Since formation of fragment 8–15 was only weakly inhibited by lactacystin (Fig. 6P), which affected only β5/Pre2 and β2/Pup1, we conclude that β1/Pre3 contributes to this cleavage. It should be noted that cleavage after Tyr4 was also, but to a much lesser extent, catalyzable by β1/Pre3, explaining the residual activity observed with β5/Pre2− mutants.

Cleavage after Leu3 was strictly β5/Pre2-dependent since all products that terminate in Leu15 were observed only in the presence of proteasomes with active β5/Pre2 subunits. Prominent examples are the main fragments 5–15 and 8–15 (Fig. 6, E and F) as well as fragment 16–25 (Fig. 6G). A rather weak cleavage after Met11 was also observed and was mainly β5/Pre2-dependent (Fig. 6, H and I). Cleavage after Asp9 was almost absolutely β1/Pre3-dependent (Fig. 6, J and K) and therefore not sensitive to lactacystin (Fig. 6, M and N). Cleavage after Lys32 was absolutely β2/Pup1-dependent: fragment 21–25 appeared only when proteasomes with a functional β2/Pup1 subunit were used (Fig. 6L).

Degradation of the Influenza Nucleoprotein 24-mer Peptide—The 24-mer peptide from influenza nucleoprotein contains the Kd-restricted ligand and CTL epitope TYQRTRALV (positions 147–155) (30). Digests with the mammalian proteasome confirmed a strong conservation of preferred cleavage sites with the yeast 20 S proteasome. Most dominant cleavage sites were clustered around the C terminus of the epitope, some of them destroying the MHC ligand (Fig. 7).

The overall degradation of the influenza peptide was dominated by cleavages after arginine residues, catalyzed by the
β2/Pup1 subunit. Degradation of the substrate peptide (positions 1–24) was significantly retarded in digests with β2/Pup1-defective proteasomes (Fig. 7A). The most dominant cleavages after Arg13 and Arg17 were absolutely β2/Pup1-dependent (Fig. 7, B–E), and the same was true for the less pronounced cleavage after Arg 11 (Fig. 7F). Interestingly, cleavage after Ala 14 was also catalyzed by β2/Pup1, as shown for fragments 1–14 and 15–24, which were missing in digests using β2/Pup1-defective proteasomes (Fig. 7, G and H).

Cleavage after Leu15 was a more complicated matter. It was performed by all the mutants; the data shown in Fig. 7I favor the involvement of both β2/Pup1 and β5/Pre2 over β1/Pre3. Fragment 1–6 was generated by a β1/Pre3-dependent activity cleaving after Asp (Fig. 7J). The cleavage after Leu4 was the most dominant site in the vicinity of the N terminus of the CTL epitope. Because of the dominant cleavages mediated by the β2/Pup1 subunit (especially Arg13), fragment 5–24 was usually not observed in digests using proteasomes with active β1/Pup1 subunits. Only in digests with β2/Pup1-defective proteasomes could this fragment be detected (Fig. 7K). A similar situation applied to peptide NDATYQRTRALV (positions 5–16) (Fig. 7L), which is a potential precursor of the CTL epitope TYQRTRALV (positions 8–16). The formation of this peptide was strongly enhanced in digests with 20 S mutants lacking functional β2/Pup1. The finding that this fragment was generated when mutants with inactive β2/Pup1, but active β5/Pre2 were used argues strongly for the involvement of β5/Pre2 in this cleavage event. This is also supported by the result that cleavage after Leu4 was inhibited by lactacystin (data not shown).

DISCUSSION

Cleavage Specificity of the Proteasomal Subunits—Using short fluorogenic peptide substrates in combination with mutant proteasomes lacking specific subunit activities, we found a clear correlation between active subunits and cleavage specificity. This finding supports the conventional belief that the three “classical” fluorogenic substrates are cleaved by three different active sites in the proteasome (β5/Pre2 → hydrophobic; β2/Pup1 → basic, and β1/Pre3 → acidic) and can be used as subunit-specific probes. However, the study of longer peptide substrates (21–25 amino acids) in the same system led to more complex results: whereas the formula β5/Pre2 → hydrophobic, β2/Pup1 → basic, and β1/Pre3 → acidic can be applied to many of the cleavage sites, there is a certain class of hydrophobic and small residues that do not obey this simple rule. Evidence that the specificities of the different subunits might overlap was provided by Rivett and co-workers (17, 18) before proteasomes carrying different inactivated subunits were available. These experiments were performed with the help of inhibitors for the tryptic and chymotryptic activities (17, 18). A special case is the so-called BrAAP activity, cleaving after the hydrophobic residue leucine in the model substrate GPALA, which can now be clearly attributed to β1/Pre3 (Fig. 3), as suggested previously (23, 24). The observed specificities are summarized for the different subunits below.

β2/Pup1 proved to be responsible for all cleavages after basic residues (Arg, Lys) without any exception. This result is in line with the observed specificity for fluorogenic substrates. However, β2/Pup1 is not absolutely limited to cleavage after basic residues. A cleavage after alanine was clearly identified as...
\(b_2/Pup1\)-catalyzed (Fig. 7, G and H), and there are indications for \(b_2/Pup1\)-catalyzed cleavages after hydrophobic residues (Fig. 7I). Thus, \(b_2/Pup1\) is also (at least partially) responsible for cleavages after small and hydrophobic amino acids.

\(b_1/Pre3\) catalyzed all observed cleavages after acidic residues (Asp, Glu). In addition, \(b_1/Pre3\) was responsible for the cleavage after leucine in the GPALA substrate, as mentioned above (Fig. 3C), and was involved in the cleavage after Tyr 7 in the pp89 peptide substrate (Fig. 6, C and D). This indicates a broader role of \(b_1/Pre3\) in cleavages after hydrophobic residues.

\(b_5/Pre2\) is mainly responsible for cleaving after hydrophobic residues, as expected. However, some of these cleavages are, at the same time, also catalyzed by \(b_1/Pre3\) or \(b_2/Pup1\).

In conclusion, whereas cleavages after basic and acidic residues strictly correlate with subunits \(b_2/Pup1\) and \(b_1/Pre3\), respectively, cleavages after hydrophobic residues can be the result of \(b_5/Pre2\) alone or with contributions by the \(b_1/Pre3\) subunit or, apparently to a lesser extent, the \(b_2/Pup1\) subunit. Likewise, cleavage after small residues appears not to be restricted to a single subunit.

This overlap might be explained by the contribution of the sequence context around potential cleavage sites as a major factor in determining which subunit will preferentially cleave after a certain hydrophobic or small residue (and also whether there is cleavage at all after a suitable P1 residue). The number of cleavage sites analyzed in this study is too small to make detailed predictions about how flanking sequences guide the involvement of the three active subunits in cleavages after certain residues. Recent results, however, namely the extensive characterization of >400 degradation products from the 436-amino acid protein yeast enolase-1 after digestion with wild-type and mutant yeast proteasomes, support the assumption that the presence of certain amino acids in several positions around the P1 site strongly influences proteasomal cleavage activities.\(^3\)

**Influence of Inhibitors on Distinct Active Sites—Inhibition**

\(^3\) A. K. Nussbaum, T. P. Dick, W. Keilholz, M. Schirle, S. Stevanovic, K. Dietz, W. Heinemeyer, M. Groll, D. H. Wolf, R. Huber, H.-G. Rammensee, and H. Schild, manuscript in preparation.
Specificity of Proteasomal β-Subunits

![Diagram of cleavage sites in the influenza nucleoprotein 24-mer peptide as digested with the yeast 20 S proteasome (top) and data explaining the correlation between cleavages and proteasomal subunits (bottom).](Image)

No Additional Catalytic Sites in the Yeast Proteasome—Orłowski et al. (20) biochemically defined catalytic components distinct from the three classical activities (chymotrypsin-like, trypsin-like, and PGPH-like); one of them was called the BrAAP activity. This component was also assumed to represent the "caseinolytic" activity of the proteasome (21). Two other additional, "non-classical" activities were identified and called the "small neutral amino acid-preferring" (20) and acidic chymotrypsin-like (32) activities.

Testing the wild-type and mutant yeast 20 S proteasomes for cleavage activity against the BrAAP substrate Z-GPALA-pAB (20), we observed that the cleavage after the penultimate leucine residue, defined to represent the BrAAP activity, is performed by β1/Pre3. This is evident from the missing BrAAP activity in the β1/Pre3 mutant (Fig. 3) and, in addition, from the remaining BrAAP activity of the β2/Pup1 and β5/Pre2 mutants in the presence of lactacystin (Fig. 3, B and C), which inhibits β5/Pre2 and β3/Pup1, but not β1/Pre3 under the applied conditions (Fig. 1F). The cleavage after the ultimate alanine residue is catalyzed by β5/Pre2 and, upon lack of β5/Pre2 or upon its preferential inhibition by LLnL, also by β2/Pup1 (data not shown). The cleavage after alanine is also lactacystinsensitive (Fig. 3, A, B, and D), supporting the view that either the β2/Pup1 or β5/Pre2 subunit is involved and not β1/Pre3, which is not inhibited by lactacystin under the applied conditions (Fig. 1F). The finding by Orłowski et al. (20) that the BrAAP component is not only insensitive to, but even activated by DCI might be explained by inhibition of the β5/Pre2 subunit,

Studies with wild-type and mutant proteasomes provided evidence, that under the conditions used in our experiments, lactacystin is an inhibitor of both the β5/Pre2 and β2/Pup1 catalytic subunits, but not of the β1/Pre3 subunit, which is only inhibited after longer preincubation times (>4 h) and at lactacystin concentrations >1 mM (data not shown). This preferential inhibition is in agreement with the results of Fenteany et al. (13), who described an irreversible inhibition of only chymotryptic and tryptic activities. In crystal structural data of the yeast proteasome, lactacystin was found covalently associated with only the β5/Pre2 subunit (14). However, the experimental conditions of crystal soaking are very different from those above, and this observation cannot exclude a weaker association with β2/Pup1, resulting in inhibition of the proteolytic activity of this subunit. The preferential interaction of lactacystin with β5/Pre2 and β2/Pup1 under the conditions used in our experiments proved to be very helpful because it provided an additional tool to discriminate catalytic contributions made by subunits β5/Pre2 and β2/Pup1 versus β1/Pre3. For example, the use of lactacystin confirmed the association of the BrAAP activity with β1/Pre3 (Fig. 3) and the contribution of β1/Pre3 to cleavage after Tyr^7 in the pp59 peptide substrate (Fig. 6P).

The finding that LLnL inhibits only β5/Pre2 in our hands, at concentrations where it covalently modifies all three active β-subunits in the yeast crystal structure (14), may be explained by the soaking condition of proteasome crystals. The incubation with the inhibitor solution was performed for a much longer period of time (6 h versus 45 min). Indeed, increasing the preincubation time of the inhibitor with proteasomes to 90 min resulted in partial inhibition of the β2/Pup1 and β1/Pre3 subunits at LLnL concentrations >1 mM (data not shown). In addition, hemiacetal formation between peptide aldehyde groups and the active-site Thr^1 O-γ may be favored under the conditions of crystallization. A similar observation was made for the inhibition by DCI. Preincubations for 45 min with DCI concentrations up to 1 mM resulted in the inhibition of only β5/Pre2, whereas a 90-min preincubation inhibited also β2/Pup1 and β1/Pre3 to 50% at 100 μM and to 90% at 1 mM (data not shown).

FIG. 7. Summary of cleavage sites in the influenza nucleoprotein 24-mer peptide as digested with the yeast 20 S proteasome (top) and data explaining the correlation between cleavages and proteasomal subunits (bottom).
otherwise competing with β1/Pre3 for the same substrate molecules. In summary, we conclude that in our system no additional active site apart from the three threonine protease catalytic centers is responsible for cleaving the GPALA substrate and that, as defined by the GPALA substrate, β1/Pre3 represents the BrAAP component. No evidence for additional activities could be found with any of the other substrates tested in this study. Since the BrAAP or caseinolytic activity has been described as the most significant among the additional activities, we regard it as unlikely that other putative activities (small neutral amino acid-prefering and acidic chymotrypsin) will turn out to be catalyzed by some unconventional, additional active site.

Since our evidence is based on wild-type and mutant proteasomes from yeast, we cannot definitely rule out that the bovine proteasomes used by Orlowski et al. (20) do harbor activities that are distinct from the known threonine-type proteolytic subunits. However, we regard this possibility as unlikely for the following reasons. (i) Proteasomes from yeast and mammals are conserved in subunit structure and topology (14, 33) as well as in cleavage specificity when tested with long peptide substrates (25). (ii) Yeast proteasomes were highly purified and homogeneous in subunit composition, whereas bovine proteasomes might represent a mixture of different species including inducible subunits. This subunit heterogeneity might lead to experimental complications. (iii) Fluorogenic substrates used in our experiments were shown to be cleaved by the yeast proteasome in a subunit-specific manner. It is still to be shown that these and other fluorogenic substrates are cleaved in a strictly subunit-specific manner by mammalian (bovine) proteasomes.

**Generation of Dual Cleavage Products**—In this study, we show that the cleavages generating the SYFPEITHI fragment from the JAK1 peptide and the DMYPHFMPNL fragment from the pp89 peptide are all performed by the β5/Pre2 subunits of yeast 20 S proteasomes. These results bear on previous experiments performed with mammalian 20 S proteasomes. There, we found that the generation of the same peptides is boosted by the presence of PA28 (10). Meanwhile, this effect was confirmed (25) and observed with additional peptide substrates. In the absence of PA28, the N- and C-terminal cleavages that generate an internal fragment occurred independently from each other and could be out-competed with excess substrate. In contrast, when PA28 was present, the second cleavage did not take place independently from the first cleavage (10). These observations led us to propose the model in which two concerted cleavages take place between two neighboring subunits.

However, the crystal structure of the yeast proteasome made clear that the two β5/Pre2 subunits are not direct neighbors in the 20 S particle and are also isolated from the β1/Pre3 and β2/Pup1 subunits. An identical subunit topology is expected for the mammalian proteasome (33). Consequently, the positive influence of PA28 on the formation of internal fragments is unlikely to be based on a simultaneous cleavage event performed by the β5/Pre2 corresponding subunits MB1/LMP7. Furthermore, the distance from MB1/LMP7 to either Z/MECL1 or δ/LMP2 is too long to generate peptides of 9–11 amino acids by a simultaneous cleavage event. Whether or not β2/Pup1 (Z, MECL1) and β1/Pre3 (δ, LMP2), which are neighbors in the same β-ring, might be involved in the generation of internal fragments from peptide substrates under the influence of PA28 cannot be tested in the yeast system. No PA28 homologous protein is present in the yeast genome, and we could not find significant activation or modulation of the yeast proteasome by human PA28 (data not shown). Studies with mammalian proteasomes and inhibitors under conditions that selectively affect the different proteasomal activities are currently being performed to address the involvement of the neighboring subunits Z/MECL1 and δ/LMP2 in the generation of internal fragments when PA28 is present. Of special interest here is whether PA28 is able to influence the specificity of subunits Z/MECL1 and δ/LMP2 to allow a participation in cleavages after hydrophobic amino acids that might now be able to generate the SYFPEITHI and DMYPHFMPTNL fragments by a concerted cleavage event. Our finding that β2/Pup1 and β1/Pre3 are able to cleave after hydrophobic amino acids is in favor of this possibility.

Because we used peptide substrates of up to only 25 amino acids in this study, we did not investigate the length of the fragments generated from wild-type or mutant yeast proteasomes in detail. It is interesting, however, to note that the size of internal fragments generated from synthetic peptide substrates centers around 7 amino acids and that the average fragment length does not seem to differ between wild-type and mutant proteasomes with fewer threonine active sites. This is of interest because one of us (34) suggested that the distance between the subunits carrying the threonine active sites corresponding to an octapeptide in extended conformation determines the length of fragments generated. The data here do not support this hypothesis. Whether or not this model is correct will be answered by the detailed analysis of proteasomal digestion products of the yeast enolase-1 protein, where >400 protein fragments, generated in digestions with yeast wild-type and mutant proteasomes, were characterized.

In addition, in the yeast proteasome, processing intermediates cleaved at residues –8 and –9 of subunits β7/Pre4 and β6/C5 were observed in well defined conformations (14). This led to the suggestion of the existence of an additional unspecific hydrolytic site at the inner β-annulus of the proteasome. The results presented previously (15) and here argue against this hypothesis. A reconsideration of the hydrolytic activity at the inner β-annulus must await the results of the analysis of processing intermediates in proteasomes with inactivated β-subunits, which is on the way.

The analysis of the proteolytic activity of the yeast proteasomes also revealed that the dominant cleavage activity was performed by the β5/Pre2 subunit, generating hydrophobic C termini. This effect is even more enhanced by the contribution of the β2/Pup1 and β1/Pre3 subunits to cleavages after hydrophobic residues as well. We are aware of the fact that the yeast 20 S proteasomes lack the interferon-inducible β-subunits of mammalian proteasomes. But there is no evidence that the presence of these subunits does alter the specificity of the proteasome in a way that will induce the generation of new cleavage sites. The only differences observed so far are changes in the quantity of peptide fragments generated when proteasomes from interferon-induced or LMP2/LMP7-transfected cells were analyzed (25, 35). Therefore, our results will describe most of the cleavages performed in mammalian proteasomes as well and provide an explanation for the dominance of hydrophobic residues at the C termini of MHC class I ligands. Because the proteasome does not cleave after every hydrophobic, basic, or acidic amino acid, however, there must be rules that govern the selection of cleavage sites. These rules are not evident in the limited set of data available here, but will be looked for in the >400 fragments generated from the yeast enolase-1 protein. If it is possible to identify rules that guide the interaction of substrate molecules with the proteasome and

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therefore allow a partial prediction of protein processing, the forecast of CTL epitopes, which so far is based on MHC ligand motifs only, will become much more accurate. The knowledge of subunit contribution to the generation of CTL epitopes will also be extremely useful for the development of subunit-specific proteasome inhibitors that suppress the generation of some CTL epitopes and enhance the production of others, but do not affect the entire proteasomal activity.

Acknowledgment—We thank Dr. M. Orlowski for providing the substrate Z-GFALA-pAB to measure the BrAAP activity.

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