

# The Caspase-like Sites of Proteasomes, Their Substrate Specificity, New Inhibitors and Substrates, and Allosteric Interactions with the Trypsin-like Sites\*

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**Proteasomes are the primary sites for protein degradation in mammalian cells. Each proteasome particle contains two chymotrypsin-like, two trypsin-like, and two caspase-like proteolytic sites. Previous studies suggest a complex network of allosteric interactions between these catalytic and multiple regulatory sites. We used positional scanning combinatorial substrate libraries to determine the extended substrate specificity of the caspase-like sites. Based on this analysis, several new substrates were synthesized, the use of which confirmed earlier observations that caspase-like sites (often termed postglutamyl peptide hydrolase) cleave after aspartates better than after glutamates. Highly selective inhibitors of the caspase-like sites were also generated. They stimulated trypsin-like activity of yeast 20 S proteasomes up to 3-fold but not when binding of the inhibitor to the caspase-like sites was prevented in a mutant carrying an uncleaved propeptide. Although substrates of the caspase-like sites allosterically inhibit the chymotrypsin-like activity, inhibitors of the caspase-like sites do not affect the chymotrypsin-like sites. Furthermore, when caspase-like sites were occupied by the uncleaved propeptide or inhibitor, their substrates still inhibited the chymotrypsin-like activity. Thus, occupancy of the caspase-like sites stimulates the trypsin-like activity of proteasomes, but substrates of the caspase-like sites inhibit the chymotrypsin-like activity by binding to a distinct noncatalytic site.**

The majority of proteins in mammalian cells are degraded by the 26 S proteasome (1). This large (2.4-MDa) ATP-dependent complex consists of the 20 S proteasome and one or two 19 S regulatory complexes (2, 3). The 20 S particle is a hollow cylinder composed of four stacked rings. Two inner  $\beta$ -rings are identical in subunit composition, and each  $\beta$ -ring contains three different proteolytic sites, which differ in their specificity (4). The chymotrypsin-like site cleaves peptide bonds after hy-

drophobic residues, and the trypsin-like site cuts after basic residues, whereas the third site cuts preferentially after acidic residues (see Ref. 5 for references). This last “postacidic” site had been generally termed postglutamyl peptide hydrolase (PGPH)<sup>1</sup> (6), but we found that it cleaves after aspartates in fluorogenic substrates of caspases and therefore suggested the name “caspase-like” (5). All of these active sites cleave peptide bonds via a nucleophilic attack of the hydroxyl group of the N-terminal threonine of the catalytic  $\beta$ -subunit (7–9). The active subunits are generated from larger precursors that contain a propeptide at their N terminus, which block the active sites until removed autocatalytically during assembly of the 20 S particle (10). If the catalytic threonine is mutated to an alanine, this autoprocesing does not occur, and both substrate binding and catalysis are impossible, since the active sites are occupied by the 8–9-residue-long propeptides (11, 12).

The catalytic sites are located on the inner surface of this cylindrical particle, within the central chamber, and access of substrates to them is controlled by the narrow, gated channels in the  $\alpha$ -rings (13, 14). When these channels are closed, 20 S proteasomes are in a latent state, and peptides added exogenously are hydrolyzed only very slowly. The rate of peptide hydrolysis dramatically increases upon opening of these gated channels. Such opening can be triggered by the ATPases of the 19 S complexes (14) or by PA28/PA26 proteasome activator complexes (15), by mutation (13), or by various chemical treatments, such as low concentrations of SDS (16, 17).

We previously found that hydrophobic peptide substrates of the chymotrypsin-like sites can stimulate peptide hydrolysis by all active sites of the latent 20 S proteasome (5, 16). These effects were shown to involve binding of hydrophobic peptides to a distinct noncatalytic regulatory site and to result from peptide-induced opening of the channel in the  $\alpha$ -rings, because opening of the channels in the  $\alpha$ -ring by a variety of treatments (*e.g.* deletions of N-terminal tails of  $\alpha$ -subunits, treatment with SDS, and association with PA26 activators or 19 S regulatory complexes) abolished this activation (16). On the other hand,

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<sup>1</sup> The abbreviations used are: PGPH, postglutamyl peptide hydrolase; AEBBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; al, aldehyde; amc, 7-amido-4-methylcoumarinamide; AMC, 7-amino-4-methylcoumarin; Boc, *tert*-butyloxycarbonyl; BrAAP, branched chain amino acid-prefering; ex, epoxyketone; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; na, 2-naphthylamide; NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-vinyl sulfone; PS-SCL, positional scanning substrate combinatorial libraries; Z, benzyloxycarbonyl; AMP-PNP, 5'-adenylyl- $\beta$ , $\gamma$ -imidotriphosphate; HPLC, high pressure liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; OtBu, *tert*-butoxy; nL, norleucine; PyBOP, benzotriazol-1-yl-oxo-tris-pyrrolidinophosphonium hexafluorophosphate; mna, 4-methoxy-2-naphthylamide.

activation of proteolysis by hydrophobic peptides was reduced by potassium ions at concentrations similar to those found inside cells, through inhibition of spontaneous opening of these channels (16).

Another allosteric effect that we uncovered was that the substrates of the caspase-like sites cause a noncompetitive inhibition of the chymotrypsin-like activity (5). Because the  $K_i$  value for this allosteric inhibition was similar to the  $K_m$  of these acidic peptides for the caspase-like sites, we concluded that the inhibition of the chymotrypsin-like activity was caused by the binding of these acidic peptides to the caspase-like sites (5). However, Myung *et al.* (19) and Schmidtke *et al.* (18) were unable to prevent these allosteric effects by blocking the caspase-like site and therefore concluded that acidic peptides inhibit the chymotrypsin-like activity by binding to a distinct regulatory site. On the other hand, these investigators used inhibitors of the caspase-like sites that either were weak (18) or showed only moderate selectivity (19). Thus, it remains unclear whether substrates of the caspase-like sites inhibit the chymotrypsin-like activity by binding to the caspase-like or a distinct regulatory site(s). In order to study the mechanism of this allosteric inhibition, we here characterize the extended substrate specificity of the caspase-like site and design a peptide analogue inhibitor that matches the sequence of the best substrate. Such an inhibitor could be used to probe interactions between the caspase-like and trypsin-like sites.

Positional scanning substrate libraries have proven to be useful tools to define the specificity of "classical" proteolytic enzymes including caspases (20) and many serine and cysteine proteases (21–23). These studies led to the design of selective substrates and inhibitors of different caspases (20). Recently, these libraries have been used to study the specificity of proteasomes and its modulation by different PA28 activators (24), but without assigning cleavage patterns to individual active sites. To address the specificity of the individual active sites, Nazif and Bogoy (25) used positional scanning irreversible inhibitor libraries with the vinyl sulfone pharmacophore. This approach, although it led to the development of selective inhibitors of the trypsin-like sites, is limited from a synthetic point of view, because it can use only 2 amino acids, glutamine and asparagine, in the P1 position.

In this study, we use positional scanning libraries, in combination with subunit-selective inhibitors, to define further the specificity of the individual active sites of proteasome. Specifically, we defined substrate-binding preferences of the caspase-like sites in proteasomes and then designed several improved substrates and inhibitors of the caspase-like sites. Application of these inhibitors led to the discovery of novel allosteric interactions between the caspase- and trypsin-like sites and resolved whether the inhibition of the chymotrypsin-like activity by acidic peptides involves their binding to the caspase-like sites.

#### EXPERIMENTAL PROCEDURES

**Enzymes and Inhibitors**—20 and 26 S proteasomes from rabbit muscle and 20 S particles (wild type and  $\beta$ 1T1A mutant) from yeast *Saccharomyces cerevisiae* (strains SUB61 and MHY1157, respectively) were purified as described previously (16). Recombinant human caspase-1, -3, and -8 were produced and purified as described (26). Recombinant PA26 was a kind gift of Drs. C. Hill and A. Duff (University of Utah). YU102 (Ac-GPFL-ex) was purchased from Affinity Research Products (Exeter, UK). NLVS was a kind gift of Dr. Matt Bogoy (University of California San Francisco), and AEBSF (Pefablock SC) was purchased from Roche Applied Science. Fluorogenic substrates of proteasome (except those synthesized in this study) and caspases were from Bachem (King of Prussia, PA). Protected amino acids used in substrate and inhibitor synthesis were from Calbiochem-Novabiochem (La Jolla, CA) or Advanced ChemTech (Louisville, KY).

*Analysis of Proteasome Specificity with Positional Scanning Libraries*—A positional scanning synthetic combinatorial library (PS-SCL) with the general structure Ac-X-X-X-Asp-amc designed to determine the specificities of the human caspase family and Granzyme B (20) has been used in this study to characterize the specificity of the caspase-like sites in proteasomes. 20 S proteasome samples (3  $\mu$ g/well) were added to reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 100  $\mu$ M substrate mix in a total volume of 100  $\mu$ l. Under these conditions, the final concentration of each individual compound was  $\sim$ 0.25  $\mu$ M. Production of 7-amino-4-methylcoumarin (AMC) was monitored as described (20). In some experiments, proteasomes were preincubated for 30 min at room temperature with 1  $\mu$ M NLVS and 0.5 mM AEBSF to inactivate the chymotrypsin-like and trypsin-like sites, respectively.

*Synthesis of Substrates*—The P4-P2 portion of all substrates was synthesized using Fmoc-based solid phase peptide synthesis on the Wang resin. After acidic cleavage (trifluoroacetic acid/ $\text{CH}_2\text{Cl}_2$ ), the P4-P2 portions were condensed with either Asp(OtBu)-amc, Glu(OtBu)-amc, or Leu-amc under the agency of PyBOP and diisopropylethylamine, followed by final acidic deprotection (in case of Asp and Glu) to give, after preparative HPLC, the desired fluorogenic substrates. Asp(OtBu)-amc, Glu(OtBu)-amc, and Leu-amc were prepared as described by Knight (27).

*Synthesis of Peptide Aldehyde Inhibitors*—Peptides Ac-APnL and Z-PnL were synthesized on a solid phase using standard Fmoc-based peptide chemistry (28). A fully protected semicarbazone derivative of Asp-al, Fmoc-Asp(OtBu)-semicarbazone, was prepared from Fmoc-Asp(OtBu)-COOH using Weinreb amide methodology (29), after which the Fmoc group was removed by a 1,8-diazabicyclo[5.4.0]undec-7-ene/octanethiol mixture as described (30). The P4-P2 peptide fragments were coupled to Asp(OtBu)-semicarbazone in solution as described (31). The tBu protecting group was removed by a 25% solution of trifluoroacetic acid in dichloromethane (32). The semicarbazone group was removed by a mixture of methanol, acetic acid, and formaldehyde (32). The resulting aldehydes were purified to homogeneity by preparative HPLC. The structure of all inhibitors synthesized was confirmed by NMR and mass spectrometry.

*Assays of Proteasome Activity*—The cleavage of fluorogenic peptide substrates was measured in 96-well plates by continuously monitoring the fluorescence of the reaction product (free AMC). The rates of reaction were determined from the slopes of the resulting reaction progress curves. 20 S proteasome activity was assayed in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol. The buffer used for assays of 26 S proteasomes also contained 40 mM KCl, 5 mM  $\text{MgCl}_2$ , 0.5 mM ATP or AMP-PNP, and 50  $\mu$ g/ml bovine serum albumin. Proteasomes from rabbit muscle were assayed at 37 °C, and yeast proteasomes were assayed at 30 °C. PA26 was always present at a 4-fold molar excess over 20 S proteasomes.

*Measuring  $K_i$  of Peptide Aldehyde Inhibitors*—Proteasomes were added to a 100  $\mu$ M solution of Ac-nLPnLD-amc containing different concentrations of inhibitors. Steady state rates in the presence ( $V_i$ ) or absence ( $V_o$ ) of inhibitor were determined from the slopes of the reaction progress curves. Apparent  $K_i$  ( $K_{i(\text{app})}$ ) was determined as the reciprocal slope of the plot of  $V_o/V_i$  against the concentration of the inhibitor according to the equation  $V_o/V_i = 1 + [S]/K_{i(\text{app})}$ . True  $K_i$  was then determined from the following equation:  $K_i = K_{i(\text{app})}/(1 + [S]/K_m)$ . Inhibition of caspase-1, -3, and -8 by Z-PnLD-al and Ac-APnLD-al was monitored using continuous fluorometric assays as described (33, 34). Briefly, appropriate dilutions of enzyme were combined with  $K_m$  concentrations of substrate in the absence or presence of several concentrations of the inhibitor of interest, in a total volume of 100  $\mu$ l. Liberation of AMC was monitored for up to 1 h at room temperature. The assay was conducted in a buffer containing 100 mM HEPES, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, pH 7.5. The substrates used were Ac-WEHD-amc for caspase-1 and Ac-DEVD-amc for caspase-3 and caspase-8. Reaction rates were fit by nonlinear regression to the Michaelis-Menten equation for competitive inhibition to obtain a value for the inhibition constant  $K_i$ .

#### RESULTS

*Characterization of the Specificity of the Caspase-like Site by Position-scanning Libraries*—Positional scanning synthetic combinatorial libraries (PS-SCL) of general structure Ac-X-X-X-Asp-amc (20, 35) were developed and successfully used to map the specificity of caspases in the P2–P4 positions. Since proteasomes can cleave standard caspase substrates (5), we used these libraries to characterize the specificity of the caspase-like sites in the proteasomes. This PS-SCL is composed

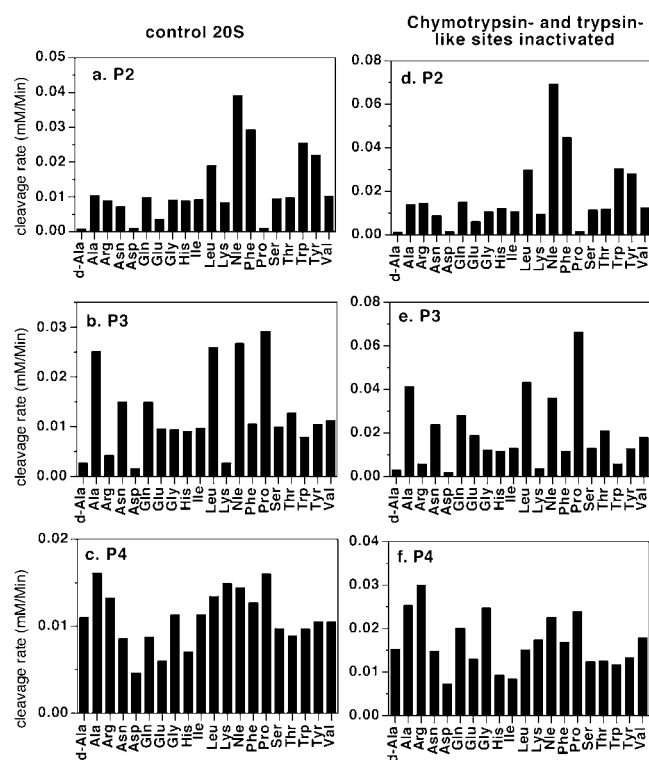


FIG. 1. **Substrate specificity of the caspase-like sites of the proteasome.** Cleavage of Ac-XXX-Asp-amc PS-SCL by 20 S proteasomes from rabbit muscle was assayed in the absence of inhibitors (*a-c*) or after a 30-min preincubation with inhibitors of the chymotrypsin-like ( $1 \mu\text{M}$  NLVS) and trypsin-like ( $0.5 \text{ mM}$  AEBSF) sites (*d-f*).

of three separate sublibraries (P2, P3, and P4). In each sublibrary, one position is defined with one of 20 amino acids, whereas the remaining two positions contain approximately equimolar mixtures of all amino acids (20). 20 S proteasomes from rabbit muscle were incubated simultaneously with all three sublibraries (20 samples each) in a 96-well plate, and the cleavage of the Asp-amc bond was followed by monitoring the fluorescence of released AMC.

This analysis revealed that in the P2 position, the caspase-like sites in proteasomes strongly prefer bulky hydrophobic residues (Nle, Phe, Trp, Tyr, and Leu) (Fig. 1*a*). This finding is surprising, because the x-ray diffraction of yeast proteasome did not reveal a pronounced substrate-binding pocket for the P2 residue in any active site of the yeast or archaeal proteasomes (4, 8). Cleavages after most other amino acid residues, except Pro and Asp, were also observed, but they occurred at lower rates. In the P3 position (Fig. 1*b*), nonaromatic hydrophobic (Leu, Nle, and Pro) and Ala residues were preferred, whereas Asp and Lys caused significantly lower cleavage rates. Proteasomes showed low selectivity for the P4 position of the substrate (Fig. 1*c*).

In order to confirm that these cleavages were performed by the caspase-like site, we repeated the screen after inactivation of the chymotrypsin-like site with the irreversible inhibitor NLVS (16). The trypsin-like sites were simultaneously inactivated with another irreversible inhibitor, AEBSF (36). When these sites were inactive (NLVS- and AEBSF-treated proteasomes cleaved standard substrates of chymotrypsin-like and trypsin-like sites at 2–5% of the control rates), the pattern of selectivity against this library (Fig. 1, *d-f*) was not different from that of the control proteasomes (Fig. 1, *a-c*). Therefore, the great majority of cleavages in this library are indeed performed by the caspase-like sites.

Interestingly, NLVS- and AEBSF-treated proteasomes

cleaved libraries at 2-fold higher rates than control particles (Fig. 1). This observation is surprising, because we have previously shown that treatment of proteasomes with NLVS and AEBSF does not enhance the caspase-like activity (16, 36). In the previous study, preincubation of proteasomes with these irreversible inhibitors was followed by the removal of the excess of the unreacted inhibitor by dilution or dialysis. In the present study, concentrations of inhibitors were not reduced prior to the addition of substrates. Hydrophobic peptides stimulate peptide hydrolysis by the caspase-like site and two other sites by binding to the noncatalytic sites (16). Because NLVS is hydrophobic (it contains 3 leucine residues), we tested whether NLVS can also enhance substrate hydrolysis by the caspase-like sites by reversible binding to the noncatalytic sites. When we removed excess NLVS by dilution or dialysis so that only NLVS irreversibly attached to the chymotrypsin-like sites remained, no activation of the caspase-like sites was observed (16). When  $1 \mu\text{M}$  NLVS (same concentration as used in the experiments on Fig. 1) was added back to the mixture of substrates and proteasomes with blocked chymotrypsin-like sites, it still stimulated cleavage of substrates by the caspase-like sites 2-fold (data not shown). Thus, enhanced rates of library cleavage in the presence of NLVS and AEBSF are due to binding of NLVS to the noncatalytic regulatory sites.

These studies were performed with latent 20 S proteasomes in which the gates in the  $\alpha$ -rings are largely in the closed conformation. Consequently, in these particles, substrate entry through this channel rather than their hydrolysis at the active sites is the rate-limiting step (16). To exclude the possibility that the results of the screen were affected by difference in substrate access to the proteolytic chamber, we repeated this analysis under several conditions where the channels are mainly in an open state. Screens were performed in the presence of 0.01% SDS or the hydrophobic peptides, Suc-FLF-mna and Suc-LLVY-mna, both of which trigger gate opening (16). As expected, the overall activity of the proteasomes increased considerably, but similar patterns of substrate preference were found (data not shown). Thus, the original screen with latent 20 S proteasomes (Fig. 1) indeed revealed subsite preferences of the caspase-like site and did not reflect the relative abilities of the substrates to enter the particles.

**New Substrates of the Caspase-like Sites**—The caspase-like sites of the proteasome are currently the most difficult to assay. The most commonly used substrate Z-LLE-na is cleaved not only at the Glu-na bond but also at Leu-Glu bond (5). Cleavage of this substrate results in the release of potentially carcinogenic  $\beta$ -naphthylamine, which is less fluorescent than AMC used in substrates of the other active sites. Furthermore,  $\beta$ -naphthylamine fluoresces at different wavelengths and thus requires different filters than those used for AMC, which may cause inconvenience to investigators using filter-based fluorimeters. To eliminate some of these problems,  $\beta$ -naphthylamine was replaced by AMC. However, the resulting Z-LLE-amc was cleaved at a 600-fold lower rate than Z-LLE-na (data not shown). Previously, we used the standard caspase substrate, Ac-YVAD-amc, to assay the caspase-like site in proteasomes (5). It was cleaved more than 50-fold faster than Z-LLE-amc but significantly slower than standard substrates of the chymotrypsin- and trypsin-like sites (5). Another disadvantage of Ac-YVAD-amc was that it was also cleaved by the proteasome (probably by the chymotrypsin-like site) N-terminally of the Asp-amc bond (data not shown). Because of this clear need for more sensitive and more specific substrates of the caspase-like site, we used the results of the screens with PS-SCL to design new fluorogenic substrates.

Our goal was to generate substrates cleaved rapidly at the

TABLE I  
Cleavage of new substrates by the proteasomes

All substrates were at 100  $\mu$ M. The specific activity of 26 S proteasomes is mean  $\pm$  range of two different enzyme preparations.

Substrate	Specific activity		
	26 S (muscle) proteasome	20 S (yeast) proteasome	
		Wild type	$\beta$ 1T1A
		<i>nmol/min*mg</i>	
Ac-YVAD-amc	3.5	0.11	0
Ac-nLPnLD-amc	113 $\pm$ 2	6.6	0
Ac-GPLD-amc	42 $\pm$ 3	2.4	0
Ac-GPLE-amc	19	1.1	0
Ac-GPLL-amc	28 $\pm$ 3	3.1	3.1
Ac-APnLL-amc	62 $\pm$ 8	5.0	4.1

Asp-amc amide bond, but not at any of the peptide bonds. Since analysis of the specificity by PS-SCL revealed that the caspase-like site prefers hydrophobic residues in the P2 and P3 positions, it was possible that any newly designed substrates might also be cleaved after these residues by the chymotrypsin- or caspase-like sites. In order to prevent such cleavage, we took advantage of the fact that a Pro residue, when present in the P3 position, allowed rapid cleavages at the Asp-amc bond (Fig. 1b), although it almost completely prevented such cleavages when present in the P2 position (Fig. 1a). In addition, we had observed that several fluorogenic peptides with a Pro in the P2 position and a hydrophobic residue in P1 are not cleaved by the chymotrypsin-like sites.<sup>2</sup> Therefore, a Pro in P3 should not only allow rapid cleavage after aspartate; it also should prevent cleavage after hydrophobic residues in P2. Consequently, all new substrates were designed with a Pro in the P3 position. For the P2 position, we chose Nle, which gave the fastest rates of hydrolysis in the screen, and Nle was arbitrarily chosen for the P4 position to yield Ac-Nle-Pro-Nle-Asp-amc. We further synthesized a second fluorogenic peptide, Ac-Gly-Pro-Leu-Asp-amc.

The results of the PS-SCL screen predict that Ac-GPLD-amc would be cleaved at 2–3-fold slower rates than Ac-nLPnLD-amc, largely because Nle in the P2 position allowed at least 2-fold faster cleavages than Leu. Indeed, Ac-GPLD-amc was hydrolyzed by 26 S proteasomes from rabbit muscle 2.5–3-fold slower than Ac-nLPnLD-amc (Table I). Remarkably, Ac-nLPnLD-amc was cleaved 30-fold faster than Ac-YVAD-amc and Ac-GPLD-amc 12-fold faster than Ac-YVAD-amc (Table I). Unfortunately, these substrates all showed high  $K_m$  values ( $\sim$ 500  $\mu$ M or higher) (16), which did not allow accurate measurements of  $V_{max}$  values. To show that the cleavage of these substrates is indeed carried out by the caspase-like sites, we used a mutant of the yeast proteasome in which the catalytic threonine of the  $\beta_1$  subunit responsible for caspase-like activity was changed to alanine. This mutation ( $\beta$ 1T1A) completely abolished the cleavage of Z-LLE-na (37, 38) and blocked hydrolysis of Ac-nLPnLD-amc and Ac-GPLD-amc (Table I) as well. Therefore, these substrates are specific for the caspase-like sites. The HPLC analysis of the cleaved substrates revealed the only fluorescent product for each reaction was free AMC (Fig. 2). Thus, in contrast to the commonly used substrates of the caspase-like sites, Z-LLE-na and Ac-YVAD-amc, our new substrates are cleaved exclusively at the Asp-amc bond.

The caspase-like sites can cleave not only after aspartic acid but also after glutamic acid. In fact, this activity was defined originally as postglutamyl peptide hydrolase or PGPH sites (6). They also cleave after leucines and are largely responsible for the “branched chain amino acid-preferring” (BrAAP) activity

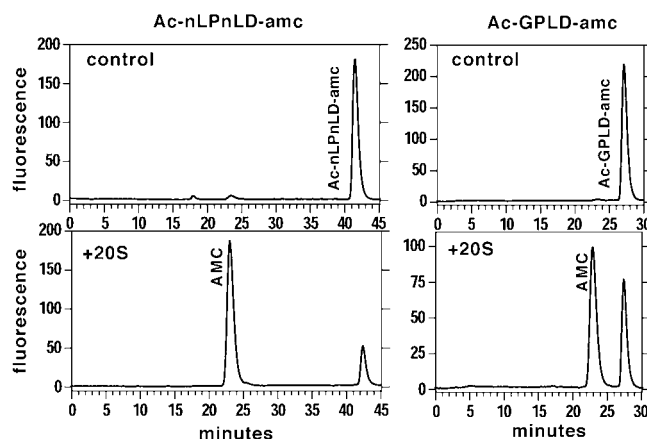


FIG. 2. **Ac-nLPnLD-amc and Ac-GPLD-amc are cleaved only at Asp-amc bonds.** Ac-nLPnLD-amc and Ac-GPLD-amc (each at 100  $\mu$ M) were incubated with 20 S proteasomes from rabbit muscles (1.2  $\mu$ g/ml) for 6.5 h at room temperature (in order to facilitate the detection of even minor reaction products). Substrates incubated without proteasomes served as control. Reaction mixtures were separated by HPLC on a Vydac C18 (2.1  $\times$  250 mm) column using fluorescent detection. Excitation (380 nm) and emission (420 nm) wavelength were chosen to allow detection of free AMC and amc-containing peptides. Buffer A was 10 mM sodium phosphate, pH 6.8, and buffer B was a mixture of 70% acetonitrile and 30% A. The gradient was 10–50% B in 40 min, followed by 50–70% B in 10 min. The flow rate was 0.2 ml/min. Peaks of substrates and reaction product are indicated. Note that only a single fluorescent peak of the product is observed, the retention time of which corresponds to the retention time of free AMC (not shown).

(5, 38–41). There have been no studies of the relative preferences for aspartate, glutamate, and branched chain residues in substrates with identical P4-P2 residues and the same fluorogenic reporter group. To make such a comparison and further characterize the specificity of these sites, peptides Ac-GPLE-amc, Ac-GPLL-amc, and Ac-APnLL-amc were synthesized. Ac-GPLE-amc was cleaved by mammalian 26 S proteasomes and yeast 20 S proteasomes at half the rate of Ac-GPLD-amc cleavage (Table I). This reaction was completely blocked by the  $\beta$ 1T1A mutation, confirming that hydrolysis is carried out by the caspase-like sites. Thus, these sites are indeed more “caspase-like” (*i.e.* aspartate-preferring) than “postglutamyl peptide hydrolase” in their specificity.

The two substrates with leucines in P1 were hydrolyzed by mammalian 26 S proteasomes at half the rate of similar substrates with Asp in P1, and Ac-GPLL-amc was cleaved even faster than Ac-GPLE-amc (Table I). Cleavage of these BrAAP substrates by the yeast particles was not blocked by the inactivating mutation in the caspase-like sites, indicating that another site(s) (most likely the chymotrypsin-like) is responsible for this activity (Table I).

To test which active sites in mammalian proteasomes cleave these substrates, we used selective inhibitors of different active sites (Table II). Covalent inhibitors of the chymotrypsin- and trypsin-like sites, NLVS and AEBSF, had very little effect on hydrolysis by 26 S proteasomes of substrates containing Asp in P1 (Table II), and even this inhibition could be due to partial modification of the caspase-like sites by these inhibitors. In contrast, cleavages after aspartates were inhibited by more than 90% by the inhibitors of the caspase-like sites, peptide epoxyketone YU102 (19) and the specific peptide aldehydes developed in this study (Table III; see below). Cleavage of the substrate with glutamic acid in P1 by the 20 S proteasomes was sensitive to the same inhibitors as was cleavage of Asp-containing peptides (data not shown). These data are in agreement with findings with yeast mutants (Table I) that postaspartic and postglutamic cleavages are performed by the same sites in

<sup>2</sup> A. Kisselev, unpublished observations.

TABLE II  
Effect of inhibitors of individual active sites on hydrolysis of substrates by 26 S proteasomes

26 S proteasomes (0.15 mg/ml in the storage buffer) were preincubated with inhibitors (1  $\mu$ M NLVS, 1 mM AEBSF, 15  $\mu$ M YU102, 25  $\mu$ M Ac-APnLD-al, 50  $\mu$ M Z-PnLD-al) for 30 min at 37°C and then diluted 440-fold into 100  $\mu$ M solutions of substrates. In case of reversible aldehyde inhibitors (Ac-APnLD-al and Z-PnLD-al), substrate solutions also contained the same concentrations of these inhibitors as those used for preincubations with the proteasome. Inhibition was calculated by relating the activity of inhibitor-treated proteasomes to the control proteasomes treated in a similar fashion but in the absence of inhibitor.

Substrates	Inhibition				
	NLVS	AEBSF	YU102	Ac-APnLD-al	Z-PnLD-al
	%	%	%	%	%
New					
Ac-nLPnLD-amc	4	25	89	99	99
Ac-GPLD-amc	5	22	89	100	100
Ac-GPLL-amc	7	19	85	95	95
Ac-APnLL-amc	12	21	84	89	91
Standard					
Suc-LLVY-amc <sup>a</sup>	81	26	29	24	12
Boc-LRR-amc <sup>b</sup>	7	98	0	0 <sup>c</sup>	0 <sup>c</sup>

<sup>a</sup> A standard substrate for the chymotrypsin-like activity.

<sup>b</sup> A standard substrate for the trypsin-like activity.

<sup>c</sup> 19% activation of the trypsin-like activity by acidic peptide aldehydes was observed (see also Fig. 3).

yeast and mammalian proteasomes. In sharp contrast with data obtained with yeast particles, the cleavage of substrates with leucines in P1 by mammalian 26 S (Table I) and 20 S (data not shown) proteasomes showed similar sensitivity to the inhibitors as substrates with acidic residue in P1, indicating that most of the postleucine cleavages are performed by the caspase-like sites in mammalian proteasomes. Thus, the ability of the caspase-like sites in mammalian proteasomes to cleave after a hydrophobic residue (at least when a Pro residue is present in the P3 position) is comparable with their PGPH activity. These data also demonstrate an interesting difference in substrate specificity of the caspase-like sites between yeast and mammalian proteasomes.

**New Inhibitors of the Caspase-like Sites**—The information about the specificity of the caspase-like sites obtained with PSL was then used to design specific inhibitors of the caspase-like sites. The majority of proteasome inhibitors are short peptides with a threonine-reactive pharmacophore at their C terminus (42). We chose an aldehyde as the pharmacophore because of the simplicity of synthesis (compared with epoxyketones and peptide boronates) and because aldehyde derivatives of the aspartic acid are stable, unlike vinyl sulfones. We used the sequence of the best substrate Ac-nLPnLD-amc as the basis for peptide portions of the inhibitors. However, the bulky norleucine in P4 was replaced by a smaller alanine residue to give Ac-APnLD-al in order to decrease the chances that this compound would bind to the chymotrypsin-like sites at high concentrations. Large hydrophobic residues in the P4 position of the inhibitors tend to increase the potency of inhibitors toward chymotrypsin-like sites, even when the P1 residue is not hydrophobic.<sup>3</sup> Replacement of the Nle by Ala, according to the results with PS-SCL, should not affect the affinity of the inhibitor for the caspase-like sites (Fig. 1c). In addition, because substrates with the lowest  $K_m$  and presumably the best affinity for caspase-like site, Z-LLE-na and Z-LLE-amc, have a Z group in the P4 position, we also synthesized another inhibitor, Z-PnLD-al.

The two peptide aldehydes synthesized inhibited the caspase-like activity of 26 S proteasomes from rabbit muscle

with  $K_i$  values of 0.4–1  $\mu$ M (Table III). As expected for peptide aldehydes, these compounds are reversible inhibitors. Preincubation of the proteasomes with these inhibitors followed by dilution into substrate solution resulted in the immediate loss of the inhibition by Z-PnLD-al. A much slower loss of inhibition was seen after the removal of Ac-APnLD-al, with complete recovery after 10–20 min (data not shown). With Z-PnLD-al, the inhibition was also evident immediately after the addition to the proteasome, but Ac-APnLD-al required several minutes incubation to reach steady state inhibition (data not shown). The chymotrypsin-like and trypsin-like sites were not inhibited even at concentrations greater than 50  $K_i$  (Fig. 3) or even 100  $K_i$  (data not shown). In contrast, epoxyketone YU102 (Ac-GPFL-ex), which was reported previously to be a selective inhibitor of the caspase-like activity (19), showed 50% inhibition of the chymotrypsin-like activity at concentrations exceeding its  $IC_{50}$  for the caspase-like activity only by 10-fold (Fig. 3). Thus, both peptide aldehydes are more selective inhibitors of the caspase-like sites than any agent previously described.

The inhibition of latent rabbit muscle 20 S proteasome by these new inhibitors was ~10-fold weaker than that of 26 S proteasomes (Table III). It is believed that the 19 S regulatory complex stimulates peptide hydrolysis in the 20 S core largely by opening the channel in the 20 S  $\alpha$ -rings (14), although additional allosteric effects have not been ruled out. To test whether the greater potency of the inhibitor in 26 S proteasomes is a consequence of the open channel, we measured the  $K_i$  of the inhibitor under a variety of conditions that lead to gate opening in the  $\alpha$ -rings. When channel opening in the 20 S proteasomes was stimulated by the addition of the hydrophobic peptide Suc-FLF-mna (16), the  $K_i$  value of both new inhibitors of the caspase-like sites decreased almost to the level found with the 26 S proteasomes (Table III). Similarly, in the yeast 20 S proteasomes, opening of the channels in the  $\alpha$ -ring by mutation or by addition of the protein activator PA26 (15) significantly enhanced the sensitivity of the caspase-like site to these new peptide aldehydes (Table III). These treatments also increased the degree of inhibition of the chymotrypsin-like site by MG132 (Z-LLL-al) by 5–25-fold (data not shown). It is noteworthy that opening of the channel generally results in the lower  $K_m$  values of the substrates (16). These lower  $K_i$  values of the open gate forms may simply reflect the fact that effective concentrations of inhibitors in these forms are higher than when the gates are closed. Alternatively, opening of the channel in the  $\alpha$ -ring may result in stronger binding of inhibitors and substrates to the active sites.

PS-SCL results indicated that the P4-P2 preference of proteasome (Fig. 1) is significantly different from that of caspases (20). As expected from those observations, the peptide aldehydes synthesized in this work were weak inhibitors of caspases, with  $K_i$  for different caspases ranging from 30 to 130  $\mu$ M (Table III). Also, the nanomolar inhibitor of caspase-1 Ac-YVAD-al (33) inhibited the 26 S proteasome with a  $K_i$  of 40  $\mu$ M (not shown).

**Inhibitors of Caspase-like Sites Stimulate Peptide Cleavage by the Trypsin-like Sites**—While analyzing effects of these new inhibitors of the caspase-like sites on two other peptidase activities of the 26 S proteasome, we noticed that these compounds increased the trypsin-like activity by 15–20% (Fig. 3). This stimulation of cleavage of Boc-LRR-amc was reproducibly observed in three different preparations of 26 S proteasomes. In yeast 20 S proteasomes, Ac-APnLD-al caused a 50% increase in the trypsin-like activity (Table IV), and a 2-fold activation by epoxyketone YU102 was also observed (data not shown). In the presence of the PA26 proteasome activator, the aldehydes stimulated cleavage of Boc-LRR-amc (Table IV) and Z-ARR-amc

<sup>3</sup> T. N. Akopian and A. L. Goldberg, unpublished observations.

TABLE III  
 New peptide aldehyde inhibitors of the caspase-like sites

Enzyme	Source	Addition	$K_i$	
			Ac-APnLD-al	Z-PnLD-al
$\mu\text{M}$				
Proteasomes <sup>a</sup>				
26 S	Rabbit muscle		0.40 ± 0.07	1.0 ± 0.1
20 S	Rabbit muscle		16.3 ± 4.7	12.1 ± 4.7
20 S	Rabbit muscle	Suc-FLF-mna	1.34 ± 0.12	2.7 ± 1.1
20 S	Yeast		20 ± 3	21 ± 7
20 S	Yeast	PA26	1.6 ± 0.6	3.9 ± 1.5
$\Delta\text{N}\alpha\text{3-20 S}$	Yeast		2.7 ± 0.8	7.0 ± 2.2
Caspase-1			83	30
Caspase-3			45	22
Caspase-7			133	126

<sup>a</sup> Inhibition of the proteasomes' caspase-like activity was measured using Ac-nLPnLD-amc as substrate. Values are means ± S.D. of three experiments for 26 S proteasomes and mean ± range of two experiments for 20 S proteasomes.

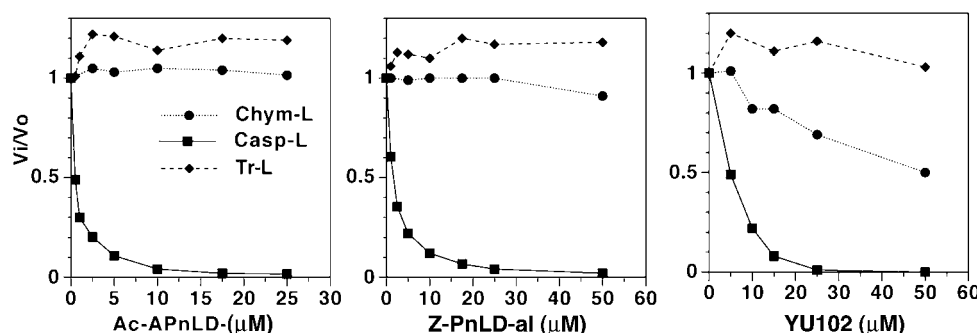


FIG. 3. Effect of peptide aldehydes and epoxyketone on different active sites of 26 S proteasomes from rabbit muscles. The chymotrypsin-like, caspase-like, and trypsin-like activities were assayed with Suc-LLVY-amc, Ac-nLPnLD-amc, and Boc-LRR-amc (each at 100  $\mu\text{M}$ ), respectively. To measure the effect of peptide aldehydes, 26 S proteasomes were added to a mixture of aldehydes and substrates, and reaction velocities in the presence and absence of inhibitor were determined from the steady state slopes of the reaction progress curves. 15–20% stimulation of the trypsin-like activity by 50  $\mu\text{M}$  Z-PnLD-al and Ac-APnLD-al was reproducibly observed with three different preparations of 26 S proteasomes. To measure effects of irreversible epoxyketone YU102 (Ac-GPFL-ex), 26 S proteasomes (0.2 mg/ml) were preincubated with YU102 for 15 min and then diluted 930-fold in substrate solutions containing no inhibitor.

TABLE IV  
 Occupancy of the caspase-like sites in yeast proteasomes stimulates the trypsin-like activity

The trypsin-like activity was assayed using 100  $\mu\text{M}$  Boc-LRR-amc as the substrate. Ac-APnLD-al was at 50  $\mu\text{M}$ .

Proteasome	Specific activity		Increase by inhibitor	Increase by propeptide
	Control	+Ac-APnLD-al		
	<i>nmol/min*mg</i>		<i>-fold</i>	<i>-fold</i>
20 S (WT)	2.2	3.4	1.5	1.3
20 S ( $\beta\text{1T1A}$ )	2.9	3.1	1.1	
20 S (WT) + PA26	20.1	64.3	3.2	2.2
20 S ( $\beta\text{1T1A}$ ) + PA26	43.8	41.7	1	

(not shown) by 3-fold. This stimulation in the yeast (Fig. 4) and mammalian (data not shown) proteasomes was entirely due to an increase in the  $V_{\text{max}}$  (Fig. 4).

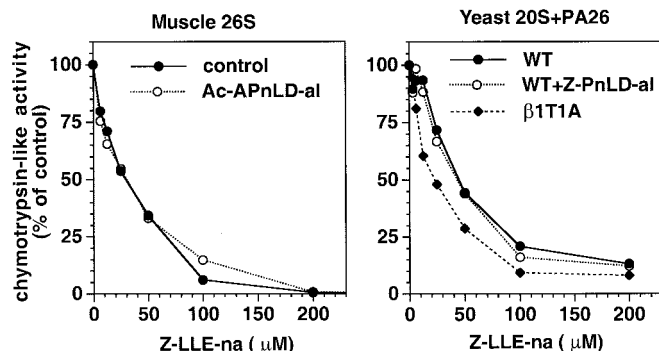
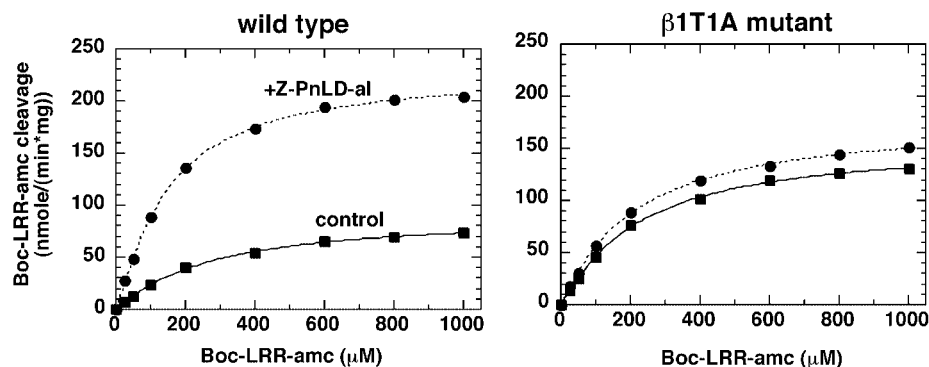
To test whether this activation of the trypsin-like activity is indeed due to the binding of inhibitors to the caspase-like site and not to a distinct regulatory site (16), we studied whether blocking inhibitor binding to the caspase-like site would prevent this allosteric stimulation. As mentioned above, mutation of catalytic threonine of the  $\beta_1$  subunit in yeast *S. cerevisiae* proteasomes into an alanine completely eliminated the caspase-like activity (37, 38). This  $\beta\text{1T1A}$  mutant fails to cleave off the propeptide, which was shown by x-ray diffraction to occupy the substrate-binding site (12). We therefore compared the effects of inhibitors of the caspase-like sites on the trypsin-like activity in the wild type yeast 20 S proteasomes and in the  $\beta\text{1T1A}$  mutant, where they could not bind to the caspase-like sites.

Although the peptide aldehyde Ac-APnLD-al stimulated the trypsin-like activity of the wild-type proteasomes 3-fold at all concentrations of two different substrates tested, it failed to cause a significant increase in the  $\beta\text{1T1A}$  mutant (Fig. 4). Interestingly, the basal activity of the mutant was 2-fold higher than that of the wild type, probably because of the presence of the propeptide in the active site. Thus, occupancy of the caspase-like sites by peptides allosterically stimulates peptide hydrolysis by the trypsin-like sites.

*Occupancy of the Caspase-like Sites Does Not Prevent Allosteric Inhibition of Chymotrypsin-like Sites by Acidic Peptides*—A substrate of the caspase-like site, Z-LLE-na, allosterically inhibits the chymotrypsin-like activity in the 26 and 20 S particles (5). Because the  $K_i$  for this allosteric inhibition resembled the  $K_m$  of the caspase-like site for this substrate, we concluded that this inhibition was due to the binding of this acidic peptide to the caspase-like sites. Myung *et al.* (19) and Schmidtke *et al.* (18) confirmed these observations but demonstrated that inhibitors of the caspase-like site do not prevent these allosteric effects of its substrate. Therefore, they concluded that this acidic peptide acts by binding to a noncatalytic site.

To resolve this controversy, we tested whether occupancy of the caspase-like site by more selective inhibitors developed in this study or by the  $\beta_1$  propeptide could prevent allosteric inhibition of the chymotrypsin-like activity by substrates of the caspase-like site. The two inhibitors developed in this study, which by themselves did not affect the chymotrypsin-like activity of mammalian 26 S proteasomes (Fig. 3), did not prevent the allosteric inhibition of the chymotrypsin-like activity by Z-LLE-na (Fig. 5). Similarly, this substrate of the caspase-like

**FIG. 4. Occupancy of the caspase-like sites stimulates the trypsin-like activity.** Hydrolysis of Boc-LRR-amc by wild-type yeast 20 S proteasomes and its  $\beta$ 1T1A mutant (1  $\mu$ g/ml each) was measured in the presence of PA26 (1  $\mu$ g/ml) and in the presence or absence of 100  $\mu$ M Z-PnLD-al. Similar results were obtained with another substrate of the trypsin-like site, Z-ARR-amc.



**FIG. 5. Substrate of the caspase-like sites inhibits the chymotrypsin-like activity even when their binding to the caspase-like sites is blocked by inhibitor or propeptide.** Inhibition of the chymotrypsin-like activity of mammalian 26 S proteasomes and yeast 20 S-PA26 complexes by Z-LLE-na is not affected by the occupancy of the caspase-like site by inhibitors Ac-APnLD-al (50  $\mu$ M) and Z-PnLD-al (100  $\mu$ M) or uncleaved propeptide ( $\beta$ 1T1A mutant). The chymotrypsin-like activity was assayed with 100  $\mu$ M Suc-LLVY-amc.

site was able to inhibit the chymotrypsin-like activity in the  $\beta$ 1T1A mutant of yeast 20 S proteasomes where the caspase-like site was occupied by the uncleaved propeptide. Thus, occupancy of the caspase-like site does not prevent its substrate from inhibiting the chymotrypsin-like site. Therefore, acidic peptides must be exerting their allosteric effects by two distinct mechanisms. They inhibit the chymotrypsin-like activity by binding to a noncatalytic, regulatory site and activate trypsin-like sites by binding to the caspase-like sites.

#### DISCUSSION

**Specificity of the Caspase-like Sites**—Positional scanning combinatorial libraries have been used to characterize the substrate specificity of a variety of proteases (20–23), including that of the 20 S proteasome and its modulation by PA28 activators (24). By using selective inhibitors of the chymotrypsin- and trypsin-like site of the proteasome, we were able to show that the vast majority of cleavages in the libraries with Asp in P1 position are carried out by the caspase-like sites (an issue not addressed in the previous studies) (24). The preference for hydrophobic residues in P2 position was unexpected, because no obvious binding pocket for the P2 residue could be identified from x-ray diffraction analysis of complexes of proteasomes with inhibitors (4, 8).

Interestingly, the positional preference of rabbit muscle 20 S proteasomes (Fig. 1) differed from that of human erythrocyte 20 S proteasomes found with the similar PS-SCL library (24). These differences cannot be due to the presence of interferon- $\gamma$  inducible subunits (43) in any of the preparations, because proteasomes from muscle and erythrocytes lack these subunits (44). Furthermore, the presence of these subunits does not affect the preference for P2–P4 positions (data not shown). A

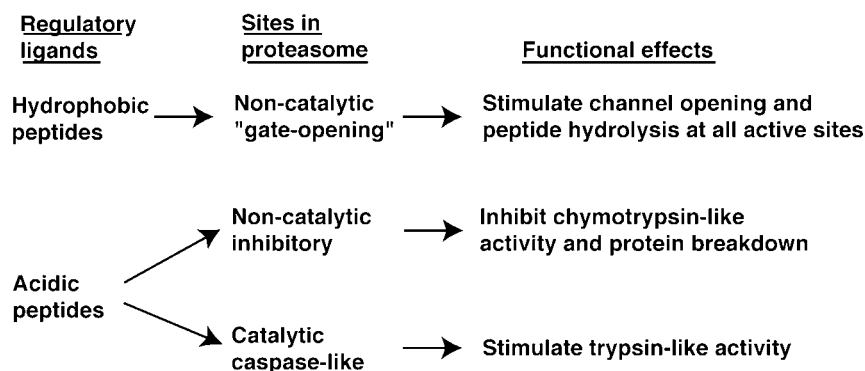
possible reason for differences could be post-translational modifications due to aging of proteasomes from erythrocytes. The half-life of proteasomes in red blood cells is significantly longer than in any other cell types or tissues.

In the presence of PA28 $\alpha\beta$ , the preferences of erythrocyte proteasomes for the P4–P2 positions resembled more closely the preferences observed in our study (24). Since the best documented effect of PA28 on 20 S proteasomes is the opening of the channel in the  $\alpha$ -rings (15), these changes could be caused by peptides' different abilities to traverse this channel in the absence of PA28. To test whether our results (Fig. 1) obtained with the latent muscle 20 S proteasomes could also be affected by different abilities of the peptides to enter the particle, we repeated the screen with PS-SCL in the presence of SDS and hydrophobic peptides, which stimulate opening of the channel (16). Because no differences were found (data not shown), we conclude that we determined the true specificity of the caspase-like sites and that the state of the channel does not affect the cleavage preferences established with this library. This result suggests also that PA28 changes the pattern of preferences of proteasomes (24) not because of the opening of the gate but rather by causing yet to be defined alterations to the active sites as suggested by Rechsteiner and colleagues (24, 45).

Although the term “caspase-like” seems most appropriate for the proteasome active sites cleaving after acidic residues, the subsite preferences of these sites in the P2–P4 positions are different from those of the majority of caspases (20). Most caspases prefer acidic residues or their amides in the P3 position (20), whereas the proteasome shows a preference for proline, alanine, and large aliphatic residues (Fig. 1b). Finally, caspases prefer histidine, threonine, or valine in the P2 position (depending on the class), whereas proteasomes show a preference for bulky hydrophobic residues.

The most striking differences between the subsite preferences of caspases and caspase-like sites in the proteasome are in the P1 position. Caspases cleave exclusively after aspartic acid residues (35), whereas caspase-like sites in proteasomes can also cleave after glutamic acid and even leucine residues (38, 40, 41). Indeed, this ability of proteasomes to cleave after glutamic acid residues was the activity initially discovered by Wilk and Orłowski (reviewed in Ref. 6) and was the basis for it being termed PGPH until we found that it cleaves after aspartates as well (5). The present study compared the ability of proteasomes to cleave after glutamic and aspartic acid residues following identical P4–P2 residues and preceded by the same fluorogenic group. Both 26 S proteasomes from rabbit muscles and yeast 20 S proteasomes cleave after aspartates 2-fold faster than after glutamates (Table I) and are indeed more “caspase-like” than PGPH. Furthermore, the PGPH activity of these sites in rabbit 26 S proteasomes is actually lower than their ability to cleave after leucines (Table I), sometimes referred to as BrAAP activity (39). This activity has actually been consid-

FIG. 6. Different regulatory effects of peptides on proteasome active sites.



ered to be catalyzed by a distinct active site based on kinetic studies (39), but structural studies and site-directed mutagenesis failed to reveal the presence of such a site (4, 38). Thus, the "caspase-like" site is more accurate term than PGPH.

We found some differences in the specificity of caspase-like sites between mammalian and yeast proteasomes. Two substrates with Leu in P1 and Pro in P3 were cleaved by caspase-like sites in mammalian proteasomes (Table II). In contrast, the rates of cleavage of these substrates by yeast proteasomes were not affected by the inactivation of the caspase-like sites by mutation, indicating that they are cleaved by some other site, perhaps the chymotrypsin-like sites (Table I). We also noticed that the peptide epoxyketone YU102 (Ac-GPFL-ek), which also has Leu in P1 and Pro in P3, is a much less potent inhibitor of the caspase-like sites in yeast than in mammalian proteasomes (data not shown). In contrast, peptide aldehydes with acidic residues in P1 showed a similar inhibition of the yeast and mammalian proteasomes (Table III). Thus, the yeast proteasome is not always an adequate model for mammalian particles.

*New Inhibitors of the Caspase-like Sites*—PS-SCL are excellent tools for the design of enzyme inhibitors (35). In this study, analysis of the specificity of the caspase-like sites by this approach allowed us to generate potent and selective inhibitors of the caspase-like sites without the laborious synthesis and purification of numerous compounds. In fact, it was possible by synthesis of just three compounds (the third compound, Z-PnLE-al, had similar potency as Z-PnLD-al), to create more potent and specific inhibitors than peptide epoxyketone YU102 (Ac-GPFL-ex) (Fig. 3), whose development required synthesis of about 20 compounds (19).

Proteasomes play an essential role in the generation of MHC class I antigenic peptides and their N-terminally extended precursors (see Ref. 43 for a review). Organs of the immune system contain a different set of proteasomes, called immunoproteasomes. In these particles, catalytic subunits LMP2, LMP7, and MECL replace subunits  $\beta_1$  (Y),  $\beta_5$  (X), and  $\beta_2$  (Z), catalytic subunits of constitutive proteasomes (44, 46). Expression of immunoproteasome subunits is also induced by interferon- $\gamma$ , a cytokine that stimulates antigen presentation (43). Immunoproteasomes have higher chymotrypsin-like, trypsin-like activity and lower caspase-like activity. These observations suggest an important role of the caspase-like sites in the generation or, more likely, destruction of antigenic peptides. This hypothesis could not be tested due to lack of sufficiently selective cell-permeable inhibitors of caspase-like sites and appropriate genetic tools in mammalian cells. Compounds developed in this study are sufficiently selective to address this question, but their ability to inhibit caspase-like sites of the proteasomes in cells could not be tested for several reasons. Substrates developed in this study are not cell-permeable (data not shown), and because caspase-like sites are not essential for

overall protein breakdown (37, 47), there is no easy readout for the inhibition of these sites in cells by compounds that do not form a stable covalent bond with an active site threonine.

*Stimulation of the Trypsin-like Activity by Inhibitors of the Caspase-like Sites*—Surprisingly, peptide aldehyde inhibitors of the caspase-like sites enhanced the trypsin-like activity of proteasomes (Fig. 3), especially in yeast, where this effect was much stronger than in mammals (Table IV). When binding of an inhibitor to the caspase-like site was prevented by the uncleaved propeptide, no stimulation of the trypsin-like activity was observed. Furthermore, the presence of a propeptide by itself stimulated the trypsin-like activity 2-fold (see Table IV, Fig. 4, and Ref. 38). Thus, occupancy of the caspase-like sites allosterically stimulates the trypsin-like activity.

In the presence of the PA26 complex, the allosteric stimulation of the trypsin-like activity in yeast 20 S proteasomes increased from 1.5- to 3-fold (Table IV). The simplest explanation for this result would be that, because these aldehydes are weaker inhibitors of free 20 S proteasomes than of 20S-PA26 complexes (Table III), the caspase-like sites are not fully occupied by inhibitors in 20 S proteasomes at the concentrations used in the experiments (50  $\mu$ M). However, in the  $\beta$ 1T1A mutant, where all active sites are occupied by the propeptide, PA26 enhances the allosteric effect of the propeptide in a similar fashion as it does with the allosteric effects of the inhibitor (Table IV).

A more likely explanation of the stronger stimulatory effect of the inhibitors in the PA26–20 S complexes is the open state of the channel in the  $\alpha$ -rings. PA26 was shown by x-ray diffraction to open this channel in the latent 20 S proteasomes (15). Opening of the channel by PA26, as well as by mutation, hydrophobic peptides, low concentrations of SDS, or the 19 S regulatory complexes significantly increases the rates of peptide hydrolysis by all three active sites. Therefore, substrate entrance into the proteolytic chamber is a rate-limiting step in their hydrolysis by the latent 20 S proteasomes (13, 16). We previously demonstrated that stimulation by hydrophobic peptides of peptide cleavages by all three active sites was completely abolished in the presence of PA26 (16). In contrast, the allosteric activation of the trypsin-like sites by occupancy of the caspase-like sites observed in this study behaved in the opposite fashion (*i.e.* it was stronger when the channel was open). In other words, the effect of the occupancy of caspase-like sites on trypsin-like activity in yeast 20 S proteasomes is stronger when cleavages at the active sites are rate-limiting and weaker when the rates of cleavages depend on the ability of substrates to traverse the channels. This observation provides further evidence that acidic peptides exert allosteric effects on the trypsin-like sites rather than stimulate them indirectly as hydrophobic peptides do (16).

*Substrates of the Caspase-like Sites Inhibit the Chymotrypsin-like Site by Binding to a Noncatalytic Site*—In contrast to



their effects on the trypsin-like sites, inhibitors of the caspase-like sites had little effect on the chymotrypsin-like activity. Furthermore, the results of this (Fig. 5) and two previous studies (18, 19) clearly demonstrate that such inhibitors do not prevent allosteric inhibition of the chymotrypsin-like sites by substrates of the caspase-like sites. In addition, these acidic peptides are able to inhibit the chymotrypsin-like activity when their binding to the caspase-like site is blocked by the uncleaved propeptide (Fig. 5). Therefore, substrates of the caspase-like site decrease the chymotrypsin-like activity by binding to a distinct noncatalytic site.

Cooperative binding of hydrophobic peptides to several noncatalytic regulatory sites on the latent 20 S proteasome stimulates peptide hydrolysis by all three active sites (16). Several observations indicate significant differences between these two effects of peptides and suggest that sites to which hydrophobic peptides bind are functionally quite different from the sites interacting with acidic peptides. First, hydrophobic peptides stimulate all three peptidase activities, whereas acidic peptides inhibit only the chymotrypsin-like activity. Second, hydrophobic peptides act only on latent 20 S proteasomes and not on 26 S proteasomes or 20 S-PA28 complexes. In contrast, acidic peptides inhibited chymotrypsin-like activity in all forms of proteasome tested. Third, acidic peptides inhibit protein breakdown by the proteasomes, whereas hydrophobic peptides have little effect. These functional differences of two effects also suggest that regulatory sites responsible for them are topologically distinct, but this suggestion can be confirmed only after both sites are localized in the particle. The mechanism by which acidic peptides inhibit chymotrypsin-like activity or protein breakdown by proteasomes is unclear.

In summary, we have distinguished three different regulatory effects of peptides in proteasomes (Fig. 6). First, binding of hydrophobic peptides to multiple noncatalytic sites stimulates opening of the channels in the  $\alpha$ -rings and thus enhances peptide hydrolysis by all active sites in the latent 20 S proteasome (16). Second, binding of acidic peptides to a distinct noncatalytic site inhibits the chymotrypsin-like activity and protein breakdown by proteasomes. Finally, occupancy of the caspase-like sites stimulates the trypsin-like activity of the proteasome.

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**The Caspase-like Sites of Proteasomes, Their Substrate Specificity, New Inhibitors and Substrates, and Allosteric Interactions with the Trypsin-like Sites**

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