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Binding of Hydrophobic Peptides to Several Non-catalytic Sites Promotes Peptide Hydrolysis by All Active Sites of 20 S Proteasomes

EVIDENCE FOR PEPTIDE-INDUCED CHANNEL OPENING IN THE α-RINGS*

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The eukaryotic 20 S proteasome contains the following 6 active sites: 2 chymotrypsin-like, 2 trypsin-like, and 2 caspase-like. We previously showed that hydrophobic peptide substrates of the chymotrypsin-like sites allosterically stimulate peptide hydrolysis by the caspase-like sites and their own cleavage. More thorough analysis revealed that these peptides also stimulate peptide hydrolysis by the trypsin-like site. This general activation by hydrophobic peptides occurred even if the chymotrypsin-like sites were occupied by a covalent inhibitor and was highly cooperative, with an average Hill coefficient of 7. Therefore, this stimulation of peptide hydrolysis at all active sites occurs upon binding of hydrophobic peptides to several non-catalytic sites. The stimulation by hydrophobic peptides was not observed in the yeast ΔNα3 mutant 20 S proteasomes, in 20 S-PA26 complexes, or SDS-activated proteasomes and was significantly lower in 26 S proteasomes, all of which appear to have the gated channel in the α-rings in an open conformation and hydrolyze peptides at much faster rates than 20 S proteasomes. Also the hydrophobic peptides altered K_m, V_{max} of active sites in a similar fashion as PA26 and the ΔNα3 mutation. The activation by hydrophobic peptides was decreased in K^+-containing buffer, which favors the closed state of the channels. Therefore, hydrophobic peptides stimulate peptide hydrolysis most likely by promoting the opening of the channels in the α-rings. During protein breakdown, this peptide-induced channel opening may function to facilitate the release of products from the proteasome.

The majority of proteins in mammalian cells is degraded by 26 S proteasomes (1). This 2.4-MDa proteolytic enzyme consists of the 20 S proteasome and one or two 19 S regulatory complexes (2, 3). The 20 S proteasome, which also exists in mammalian cells as a free 700-kDa particle, is a hollow cylinder composed of two outer α- and two inner β-rings (3). Each ring contains seven different subunits, and each β-ring contains three proteolytic sites, which differ in their substrate specificities. The "chymotrypsin-like" (β5) site cleaves peptide bonds preferentially after hydrophobic residues; the "trypsin-like" (β2) site cuts mainly after basic residues, and the third site (β1) cuts preferentially after acidic residues (4–7). This latter site has been traditionally termed "post-glutamyl peptide hydrolase" site. However, because it hydrolyzes standard fluorogenic substrates of caspases and cleaves after aspartate residues better than after glutamates, we prefer the more accurate and simpler term "caspase-like" site (8).

When isolated under gentle conditions (*e.g.* in the presence of glycerol), 20 S proteasomes are in a latent state (9) in which they are unable to degrade proteins and hydrolyze model peptide substrates only at low rates. This low peptidase activity is suppressed further by physiological concentrations of potassium ions (10), but the activity of such preparations increases dramatically upon a variety of treatments, such as heating, removal of glycerol, or addition of low concentrations of SDS (9). The explanation for the low basal activity (latency) of the 20 S proteasome is that all of its proteolytic sites are located within this cylindrical particle (11, 12), and access of substrates to these sites is restricted by two gated axial channels in the α-rings (11). These channels allow entry or exit of small peptides, but even in their most open state they can be traversed only by unfolded polypeptides (13). In the crystal structure of the yeast 20 S particle these channels were found to be completely sealed by the N-terminal portions of 7 α-subunits (12). However, when the nine N-terminal residues of the α3 subunit were deleted, an open channel was found (14). This ΔNα3 mutant also showed greatly increased rates of peptide hydrolysis, which were not further enhanced by SDS (14) nor suppressed by potassium (10). Thus, rates of peptide hydrolysis by 20 S proteasomes depend on whether or not these openings are in a closed or open position.

The association of 20 S proteasomes with the 19 S regulatory complexes to form 26 S proteasomes leads to much higher rates of peptide hydrolysis (15) and confers the ability to degrade ubiquitinated proteins as well as certain non-ubiquitinated polypeptides (16–18). Recent studies indicated that, in the yeast 26 S proteasome, the channel in the α-rings is primarily in an open conformation as the result of an interaction between the N terminus of the α3 subunit and the Rpt2 ATPase subunit of the adjacent 19 S particle (10). In addition to promoting gate opening, the ATPases of the 19 S ring appear to unfold protein substrates and translocate the unfolded polypeptide into the 20 S particle (19). A different protein complex, PA28 (also termed 11 S or REG), can attach to the α-rings and open the channel by an ATP-independent mechanism, as demonstrated by the x-ray diffraction of the complex of yeast 20 S proteasome with PA26, the PA28 homologue from *Trypanosoma brucei* (20). It is noteworthy that this hexameric ring-shaped activator stimulates peptide hydrolysis but not protein breakdown by 20 S proteasomes (21, 22).

We have reported recently (8) that hydrolysis of peptides by

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the caspase-like site of 20 S proteasomes is also stimulated by the peptide substrates of the chymotrypsin-like sites. Conversely, peptide substrates of the caspase-like sites allosterically inhibit the chymotrypsin-like activity and thereby reduce protein breakdown by the 26 S particle. These findings suggested that different proteolytic sites of proteasomes may function in an ordered, cyclical fashion in protein degradation (8). Because the concentration dependence of the stimulation of the caspase-like activity by hydrophobic peptides was similar to the concentration dependence of their cleavage at the chymotrypsin-like sites, we concluded that this activation of caspase-like activity is due to the binding of peptides to the chymotrypsin-like sites (8). However, Schmidtke *et al.* (23) demonstrated a similar activation even in the presence of inhibitors of the chymotrypsin-like sites and concluded that activation of the caspase-like site occurs upon peptide binding to a single unidentified "modifier" site.

The present study was undertaken to clarify the mechanisms of allosteric stimulation of the caspase-like activity by hydrophobic peptides and to determine whether these peptides act by binding to the active site or to distinct non-catalytic sites. We report here that multiple non-catalytic sites exist in the 20 S proteasome and that the binding of hydrophobic peptides to these sites stimulates peptide hydrolysis by all three of its active sites. Furthermore, we provide evidence that this stimulation occurs by peptide-induced opening of the channel in the α-rings of the 20 S proteasome. Specifically, we show that treatments that cause channel opening eliminate the stimulatory effects by hydrophobic peptides and cause similar changes in the kinetic properties of the proteasome as do the hydrophobic peptides.

EXPERIMENTAL PROCEDURES

Substrates and Inhibitors—Ac[1]-nLPnLD-amc and Ac-GPLD-amc were kindly provided by Dr. Mike Pennington (Bachem, King of Prussia, PA). The detailed characterization of these novel substrates of the caspase-like activity will be reported elsewhere.[2] All peptide substrates were from Bachem (Bubendorf, Switzerland) except Suc-LLVY-mna, which was from Enzyme System Products (Livermore, CA). NLVS was kindly provided by Dr. Matt Bogyo (University of California, San Francisco) and NP(4-hydroxy-3-nitrophenyl acetyl)-LLL-VS by Dr. Benedikt Kessler (Department of Pathology, Harvard Medical School). Radioiodination of NP-LLL-VS to generate [^{125}I]NLVS was performed as described (24), and [^{125}I]NLVS was subsequently separated from unreacted NP-LLL-VS by high pressure liquid chromatography. Recombinant *Trypanosoma* PA26 was kindly provided by Drs. Anthony Duff and Chris Hill (University of Utah).

Purification of Proteasomes—Purification of 20 S and 26 S proteasomes from rabbit muscles was performed using several modifications of the published protocol (16). Proteasomes from the 100,000 $\times$ *g* supernatant were batch-absorbed on DE52 DEAE-cellulose, and the resin (~50 ml) was washed on the filter with buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA) and packed in a column. The proteasomes were eluted with a 0–0.5 M NaCl gradient in 250 ml of buffer A. Suc-LLVY-amc cleaving fractions were pooled and loaded on a 6-ml Resource Q (Amersham Biosciences) column. 20 S and 26 S proteasomes were eluted as a single peak by a 0.10–0.35 M gradient of NaCl in 150 ml of buffer A and then separated on a 6-ml UnoQ column (Bio-Rad) as described (16). 26 S proteasomes were purified to homogeneity by a glycerol gradient as described (16). 20 S proteasomes were purified on a 5-ml hydroxylapatite (CHTII EconPack cartridge, Bio-Rad) column as described by Groll *et al.* (12), except that 10% glycerol was present in all buffers to maintain the particles in their native state. 20 S proteasomes were stored in aliquots at −80 °C in the buffer, containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 10% glycerol. Once thawed, the enzyme was kept at 0–4 °C and usually used within 1–3 days.

Yeast *Saccharomyces cerevisiae* 20 S proteasomes were purified from SUB61 (wt) and SUB544 ($\Delta N\alpha 3$ mutant) strains (14), which were kindly provided by Dr. Daniel Finley (Harvard Medical School). Yeast cells grown to stationary phase were collected by centrifugation, washed several times with 0.1 M Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.25 M sucrose, 1 mM DTT. 250 g of cells were then mixed with 250 ml of the same buffer and lysed in the French press. The cell extract was centrifuged at 16,000 $\times$ *g* for 15 min and then at 150,000 $\times$ *g* for 1 h. The supernatant was filtered through glass wool to remove lipids, and mixed with 75 g (150 ml) of DEAE-cellulose DE52, equilibrated in the homogenization buffer. After stirring for 1 h at 4 °C, the mixture was poured onto a glass filter and washed with 350 ml of the homogenization buffer, followed by 700 ml of buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM DTT, 0.5 mM EDTA). Proteasomes were eluted by 300 ml of 0.25 M NaCl in buffer A and directly loaded on the 6-ml Resource Q column (same as for the preparation of rabbit muscle proteasomes). A gradient of 0.2–0.45 M NaCl was applied to the column, and fractions were assayed for their ability to cleave substrates specific for all three active sites (Suc-LLVY-amc for chymotrypsin-like, Boc-LRR-amc for trypsin-like, and Ac-nLPnLD-amc for caspase-like sites). Active fractions were pooled, dialyzed against 60 mM potassium phosphate, pH 7.5, containing 10% glycerol and 1 mM DTT. 20 S proteasomes were then purified on the 5 ml of CHTII hydroxylapatite (Bio-Rad) and Superose 6 (Amersham Biosciences) columns as described by Groll *et al.* (12) except that all buffers contained 10% glycerol.

Peptidase activities were assayed by continuously monitoring the production of 7-amino-4-methylcoumarin (amc) from fluorogenic peptides as described previously (8). The cleavage of amc substrates could be followed in the presence of Suc-LLVY-mna or Suc-FLF-mna because 4-methoxy-2-naphthylamine (mna), which is released upon cleavage of the latter peptides, does not fluoresce at the same wavelengths as amc and does not quench amc fluorescence. Rabbit muscle proteasomes were assayed at 37 °C, and yeast 20 S proteasomes were assayed at 30 °C. The buffer used in the assays contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT. For 26 S proteasomes, it also contained 5 mM $MgCl_2$, 1 mM ATP, 40 mM KCl, and 0.5 mg/ml bovine serum albumin. Concentrations of substrates and stimulators are indicated in the figures and table legends.

Inactivation of Chymotrypsin-like Sites by NLVS—20 S proteasomes (65–350 nM) were incubated at 37 °C for 30 min with 5 μM NLVS or in the absence of inhibitor. To increase the selectivity of the reaction, the inhibitor of the caspase-like sites (500 μM Ac-YVAD-aldehyde) was added in some experiments to prevent the reaction of NLVS with the caspase-like sites. The reaction with the inhibitor was stopped by a 10-fold dilution with the ice-cold storage buffer followed by dialysis.

RESULTS

Binding of a Peptide Inhibitor to the Chymotrypsin-like Site Does Not Stimulate the Caspase-like Activity—Initial experiments were undertaken to test whether simple occupancy of the chymotrypsin-like site by a peptide is sufficient to cause the stimulation of peptide hydrolysis by the caspase-like sites. If so, then binding of a peptide inhibitor to the chymotrypsin-like site should have a similar stimulatory effect as the binding of substrates to this site. Nearly all of the widely used proteasome inhibitors reduce protein breakdown primarily by blocking the chymotrypsin-like activity, but these agents can also block the other two activities, especially at high concentrations (25). Consequently, our prior studies of these allosteric effects used specific substrates (8) rather than such inhibitors to dissect the mechanism of allostericity. Careful analysis of the effects of various widely used inhibitors, including MG132, *clasto*-lactacystin-β-lactone, epoxomicin, and NLVS (see Ref. 25 for review), revealed that NLVS was the most selective for the chymotrypsin-like activity. When 20 S proteasomes were preincubated with NLVS for 30 min, the chymotrypsin-like activity was inhibited by 95–97% without significantly affecting the two other activities (data not shown). NLVS inhibits proteasomes by forming a covalent bond with the hydroxyl group of its catalytic threonine (24). In initial control experi-

[1] The abbreviations used are: Ac, acetyl; amc, 7-amido-4-methylcoumarin; Boc, *tert*-butyloxycarbonyl; DTT, dithiothreitol, IEF, isoelectrofocusing; mna, 4-methoxy-2-naphthylamide; na, 2-naphthylamide; nL, norleucyl; NLVS, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinyl sulfone; *p*na, 4-nitroanilide; Suc, succinyl; VS, vinylsulfone; Z, carbobenzoxy; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; wt, wild type.

[2] A. F. Kisselev *et al.*, manuscript in preparation.

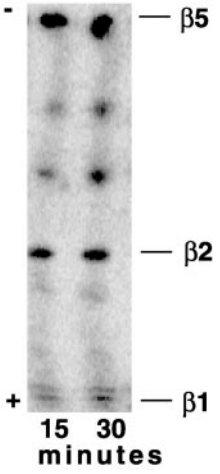

FIG. 1. **NLVS reacts with the β5 subunit of 20 S proteasomes much faster than with the two other catalytic subunits.** 20 S proteasomes (0.65 nM) from rabbit muscles were incubated with [^{125}I]NLVS (5 μM) at 37 °C. At times indicated, 10-μl aliquots were withdrawn and mixed with an equal volume of 8 M urea-containing IEF loading buffer. IEF focusing under denaturing conditions was performed as described (37) using pH 3.5–10 ampholines (Amersham Biosciences). The figure shows an autoradiogram of the IEF gel.

ments, a covalent adduct of the [^{125}I]NLVS with the β5 subunit, which contains the catalytic threonine of the chymotrypsin-like site, could be readily detected on the IEF gels (Fig. 1). (An IEF gel was used in these experiments because, unlike SDS-PAGE, it separates the β1 and β5 subunits.) The β2 subunit, which bears the catalytic residue of the trypsin-like site, reacted at a much lower rate (Fig. 1), and only very slight labeling of the β1 subunit, which contains the catalytic threonine of the caspase-like site, was detected after 30 min of incubation (Fig. 1). Thus, a 30-min incubation with NLVS allows complete inactivation of the chymotrypsin-like activity without any significant reaction of the inhibitor with the subunit responsible for the caspase-like activity. Because NLVS is an irreversible inhibitor, the chymotrypsin-like activity was still inhibited by 95–97% when the excess inhibitor was then removed by dilution or dialysis. Although the catalytic chymotrypsin-like site was completely occupied by the substrate analogue, NLVS, no stimulation of peptide hydrolysis by the caspase-like sites was observed (Table I). Thus, mimicking the transition state of the chymotrypsin-like active site does not allosterically activate peptide hydrolysis by the caspase-like sites.

Hydrophobic Peptides Stimulate the Caspase-like Activity by Binding to Non-catalytic Sites—In order to determine whether the stimulation of the caspase-like activity by hydrophobic peptide substrates of the chymotrypsin-like site is caused by their binding to the chymotrypsin-like site, we tested whether preventing substrate binding by modification with NLVS blocks this effect. For this purpose, the stimulatory effects of two hydrophobic substrates, Suc-LLVY-mna and Suc-FLF-mna, were studied in control and NLVS-treated proteasomes (after removal of the excess NLVS). Surprisingly, modification of the chymotrypsin-like site did not alter the ability of these peptides to enhance cleavages by the caspase-like sites. Upon addition of these hydrophobic compounds, the V_{max} of the AcnLPnLD-amc cleavage increased 18–25-fold, and the K_m decreased 59–68% (Table I), and similar dramatic changes in K_m and V_{max} were observed in the control and NLVS-treated proteasomes. Also NLVS treatment did not alter the ability of Suc-FLF-mna to stimulate hydrolysis of another substrate of the caspase-like site, Ac-GPLD-amc (data not shown). Furthermore, at all concentrations of Suc-FLF-mna (Fig. 2*a*) or Suc-LLVY-mna (data not shown) tested, the activation of caspase-like cleavages in the NLVS-modified proteasomes was similar to that in control particles, and their K_A values for this activation were indistinguishable (Table II).

TABLE I
Substrates of the chymotrypsin-like site stimulate the caspase-like activity even when the chymotrypsin-like sites are occupied by a covalent inhibitor

Values are means ± ranges of two independent experiments, except in the presence of Suc-LLVY-mna where only one experiment was performed to confirm findings with Suc-FLF-mna. Suc-FLF-mna was at 40 μM, and Suc-LLVY-mna was at 100 μM.

Peptide activator	Proteasomes			
	Control		NLVS-treated (chymotrypsin-like sites blocked)[a]	
	K_m	V_{max}	K_m	V_{max}
	μM	nmol/min·mg	μM	nmole/min·mg
None	310 ± 63	106 ± 11	160 ± 0	71 ± 17
Suc-FLF-mna	127 ± 39	2630 ± 100	84 ± 19	1903 ± 42
Suc-LLVY-mna	99	1856	58	1300

[a] In NLVS-treated proteasomes, Suc-LLVY-amc cleavage was inhibited by 97%.

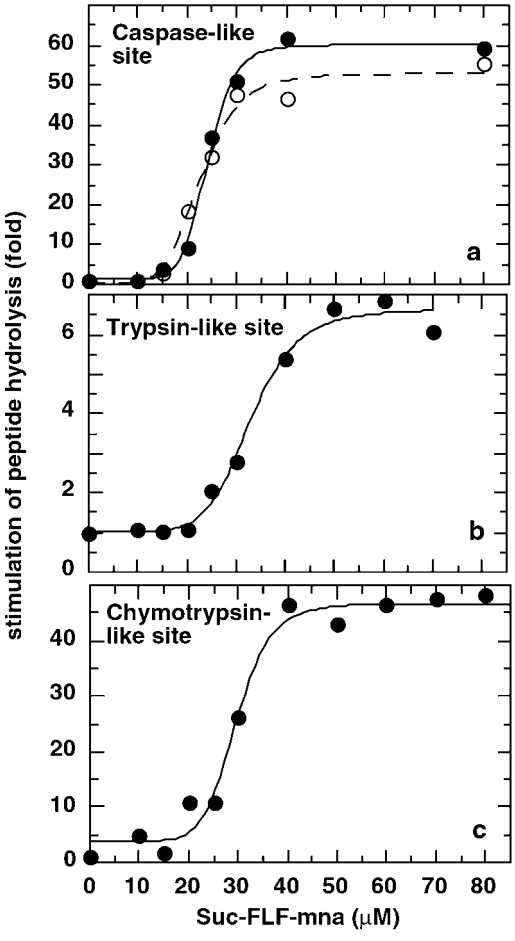

FIG. 2. **Stimulatory effect of different concentrations of Suc-FLF-mna on peptide hydrolysis by different active sites of 20 S proteasomes from rabbit muscle.** Substrates were 100 μM AcnLPnLD-amc (*a*), 20 μM Boc-LRR-amc (*b*), and 5 μM Suc-LLVY-amc (*c*). *Filled circles*, *solid lines*, control proteasomes; *open circles*, *dashed line*, NLVS-treated proteasomes (chymotrypsin-like sites occupied by NLVS). K_A, Hill coefficient, and V_{max} stimulation values obtained by fitting these curves to the Hill equation are presented in Table II.

Previously we found (8) that as the occupancy of the chymotrypsin-like sites by these hydrophobic peptides increased (measured as the concentration dependence of their cleavage

TABLE II

Hydrophobic peptides stimulate peptide hydrolysis by all active sites

Substrates were 100 μM Ac-nLPnLD-amc for the caspase-like activity, 5 μM Suc-LLVY-amc for the chymotrypsin-like activity, and 20 μM for Boc-LRR-amc for the trypsin-like activity. Cleavage rates of these peptides were determined at different concentrations of activators (Fig. 2) and fitted into the Hill equation to calculate Hill coefficients, K_A, and maximal stimulation using Kaleidagraph software package. Values are the mean ± S.E. obtained for the curve fits. Results of two experiments are shown. Stimulation was determined by dividing the specific activity in the presence of activator by the activity in its absence. $S_{0.5}$ (K_m) and Hill coefficients (n_H) for cleavage of hydrophobic peptides by the chymotrypsin-like sites were 28.8 ± 1.3 and 8 ± 3 μM for Suc-FLF-mna and 52 ± 4 and 3.7 ± 0.9 μM for Suc-LLVY-mna.

Hydrolysis by	Chymotrypsin-like sites		Hydrophobic peptide					
			Suc-FLF-mna			Suc-LLVY-mna		
			K_A	n_H	Maximal stimulation	K_A	n_H	Maximal stimulation
			μM		*-fold*	μM		*-fold*
Caspase-like site	Free	Exp.1	23.9 ± 0.4	8.6 ± 1	60 ± 2	52 ± 4	3.7 ± 0.9	34 ± 3
		Exp.2	27.2 ± 1.1	6.1 ± 1.5	92 ± 5			
	Blocked	Exp.1	22.6 ± 0.8	5.9 ± 1	53 ± 2	38 ± 16	12.5 ± 5.0	46 ± 24
		Exp.2	27.0 ± 1.1	5.7 ± 1.3	39 ± 2			
Trypsin-like site	Free	Exp.1	32.9 ± 1.2	7.0 ± 1.4	5.6 ± 0.3	38.2 ± 2.5	4.1 ± 1.0	12.5 ± 1.2
		Exp.2	31.5 ± 1.2	17 ± 9	6.7 ± 0.7	24 ± 4	5.8 ± 3.3	8.9 ± 1.3
Chymotrypsin-like site	Free	Exp.1	29.5 ± 0.9	8.5 ± 2.3	43 ± 2	31.2 ± 2.2	4.9 ± 1.4	36 ± 3
		Exp.2	23.4 ± 1.0	10 ± 3.6	6.2 ± 0.5	25.9 ± 2.4	4.4 ± 1.6	5.4 ± 0.6

rates), so did the stimulation of cleavages by the caspase-like sites. Thus, the K_A for this stimulation by substrates was similar to the $S_{0.5}$ for their hydrolysis at the chymotrypsin-like sites. This observation led us to conclude that the allosteric activation of the caspase-like activity was due to the binding of hydrophobic peptides to the chymotrypsin-like active site. We confirm these observations in this study (Fig. 2*a* and Table II), but based on the new findings with NLVS treatment, an alternative interpretation is necessary. Specifically, hydrophobic peptide substrates of the chymotrypsin-like site must stimulate the caspase-like activity by binding to one or more non-catalytic site(s), whose affinity for these peptides appears to be similar to that of the chymotrypsin-like sites (see "Discussion").

To obtain further evidence that the non-catalytic sites where Suc-LLVY-mna and Suc-FLF-mna act are distinct from the chymotrypsin-like active sites, we compared more thoroughly the ability of the different substrates of the chymotrypsin-like sites to stimulate the caspase-like and trypsin-like cleavages with their susceptibility to hydrolysis at the chymotrypsin-like sites. In fact, we found that Suc-AAF-*p*na and Z-GGL-na, although substrates of the chymotrypsin-like site, did not stimulate peptide hydrolysis by the caspase-like sites (data not shown). Thus, occupancy of the chymotrypsin-like sites by substrates does not activate the other two sites, and their stimulation must involve binding of certain hydrophobic peptides to non-catalytic sites, where specificity for ligands is distinct from that of the chymotrypsin-like site.

It is also noteworthy that the plot of stimulation of the caspase-like activity by different concentrations of Suc-FLF-mna (Fig. 2*a*) and Suc-LLVY-mna (not shown) was clearly sigmoid-shaped. This stimulatory effect showed strong positive cooperativity, which indicated that it is was due to the binding of the hydrophobic peptides not to one or two but to several non-catalytic sites. When data were fitted to the Hill equation, values of Hill coefficients ranging from 3.7 to 12.5 with an average of 7 ± 3 (Table II) were obtained. Because these coefficients indicate the minimal number of sites involved in cooperative interactions, substrates of the chymotrypsin-like site activate the caspase-like activity by cooperative binding to several non-catalytic sites.

Stimulation of the Trypsin-like Activity—In contrast to our prior observations (8), the cleavage of basic peptides was also found to be stimulated by these hydrophobic peptides in this more systematic study (Fig. 3). Unlike changes in the caspase-like activity, the stimulation of the trypsin-like site was entirely due to a very large decrease in the K_m for Boc-LRR-amc

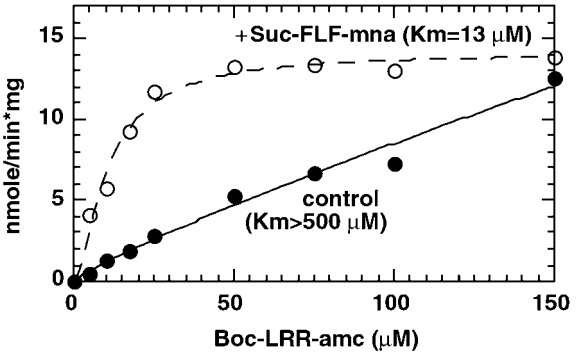

FIG. 3. **Effect of hydrophobic peptide on peptide hydrolysis by trypsin-like site of muscle 20 S proteasomes.** *Filled circles*, *solid line*, Boc-LRR-amc hydrolysis in the absence of an activator; *open circles*, *dashed line*, hydrolysis in the presence of 40 μM Suc-FLF-mna.

(Fig. 3) and Z-ARR-amc hydrolysis (data not shown). This marked activation, therefore, could be observed only at substrate concentrations less than 100 μM (Fig. 3). This finding explains why we (8) and others (23) failed to detect this activation in prior studies, where basic peptides were used only at high concentrations (100 μM in our studies).

We then tested whether this stimulation is also due to the interaction of hydrophobic peptides with multiple non-catalytic sites. The addition of hydrophobic peptides to the NLVS-treated proteasomes stimulated the trypsin-like activity to a similar extent (2.5–3-fold) as in control particles (data not shown). Furthermore, two substrates of chymotrypsin-like sites, Suc-AAF-*p*na and Z-GGL-na, did not stimulate peptide hydrolysis by the trypsin-like sites (data not shown). Thus, hydrophobic peptides stimulate the trypsin-like activity by binding to non-catalytic sites, presumably the same sites that regulate caspase-like activity. The concentrations of hydrophobic peptides that caused half-maximal activation of the trypsin-like and the caspase-like activity were quite similar (Table II), and the plots of concentration dependence for stimulation of both these activities had sigmoid shapes (Fig. 2, *a* and *b*) with similar Hill coefficients (3.7–12.5 for the caspase-like sites and 4.1–17 for the trypsin-like sites; see Table II). Thus, by binding to multiple non-catalytic sites, hydrophobic peptides allosterically activate not only the caspase-like but also the trypsin-like cleavages by the latent 20 S proteasomes.

Hydrophobic Peptides Activate Their Own Hydrolysis by Binding to the Non-catalytic Sites—Sigmoid concentration de-

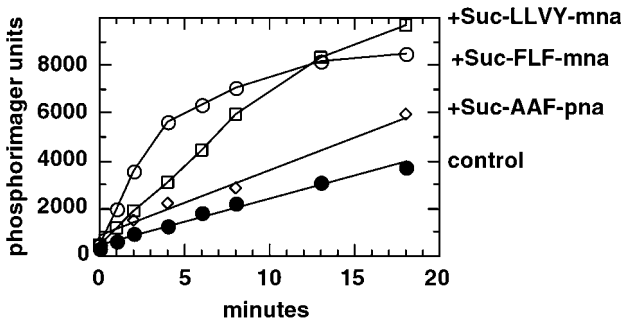

FIG. 4. **Ability of different substrates of the chymotrypsin-like site to stimulate the reaction of this site with [^{125}I]NLVS.** 65 nM 20 S proteasomes from rabbit muscle were incubated at 37 °C with 0.1 μM [^{125}I]NLVS (50–80 Ci/mmol). Under these conditions, only the β5 subunit reacted with NLVS. At specific time points, aliquots were withdrawn, and the reaction was stopped by the addition of the SDS-PAGE loading buffer. The samples were run on NuPAGE® 4–12% gradients Bistris gels (Invitrogen). The amount of the radioactivity in the β5 bands was quantified by the FX Molecular Imager (Bio-Rad). *Closed circles*, no activator; *open circles*, 60 μM Suc-FLF-mna; *open rectangles*, 90 μM Suc-LLVY-mna; *open diamonds*, 1 mM Suc-AAF-*p*na.

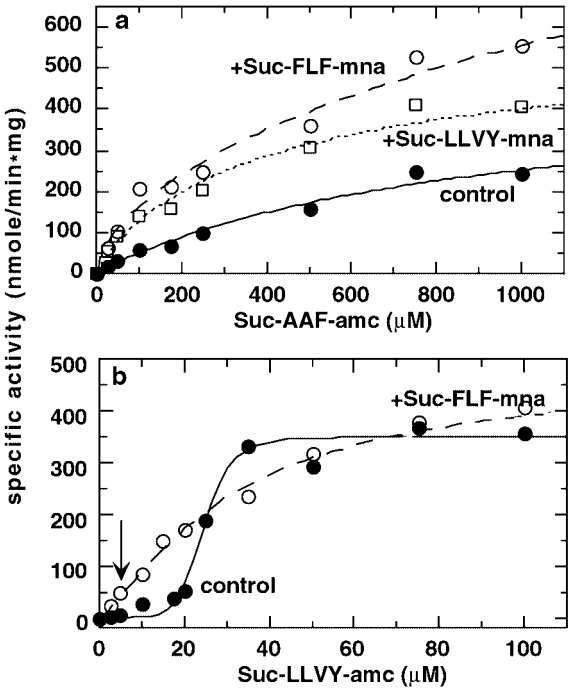

FIG. 5. **Effect of hydrophobic peptides on peptide hydrolysis by the chymotrypsin-like site of muscle 20 S proteasomes.** *Closed circles*, *solid line*, no activator; *open circles*, *dashed lines*, 40 μM Suc-FLF-mna; *open rectangles*, *dotted line*, 90 μM Suc-LLVY-mna. *Arrow* in (*b*) indicates the concentration of Suc-LLVY-amc, where the maximal stimulation was observed (5 μM).

pendence of the cleavage of the hydrophobic peptides by the chymotrypsin-like sites was observed previously (8, 21), and therefore the existence of positive cooperativity between these two catalytic sites had been suggested. The present finding that substrates of the chymotrypsin-like sites bind also to several distinct non-catalytic sites raises the possibility that the sigmoid kinetics of their hydrolysis may also be due to their binding to the non-catalytic sites. The first indication that Suc-LLVY-mna and Suc-FLF-mna might promote their own cleavage at the chymotrypsin-like sites by binding to the same non-catalytic sites was that concentration dependence of their hydrolysis by 20 S proteasomes exhibited strong positive cooperativity with Hill coefficients always ranging from 4 to 8 (Table II). Such high values, which resemble those for the stimulation of the caspase-like and the trypsin-like sites, could not result from positive cooperativity between just two chymotrypsin-like sites. Also the half-maximal stimulation of caspase- and trypsin-like activities was reached with Suc-LLVY-mna and Suc-FLF-mna at the same concentrations that gave half-maximal rates of the cleavage of these peptides (8). These finding suggest that the binding of hydrophobic peptides to the same non-catalytic sites must also enhance peptide cleavage by the chymotrypsin-like sites.

In order to demonstrate further that binding of these peptides to the non-catalytic sites indeed stimulates their hydrolysis by the chymotrypsin-like sites, the rate of reaction of these sites with the [^{125}I]NLVS was measured. The catalytic residues of the sites modified by the peptide vinylsulfone are located within the particle on the β5 subunits, and the rates of this modification were measured by quantifying the amount of radioactivity incorporated into this subunit on SDS-PAGE. Although the β1 subunit responsible for the caspase-like activity is not separated from β5 by SDS-PAGE, the concentration of NLVS used in this experiment (0.1 μM) was much lower than that required for modification of the β1 subunit (5 μM, Fig. 1). Furthermore, at this low concentration of NLVS, there was no labeling of the β2 subunit (which migrates more slowly than β5 on SDS-PAGE (26, 27), not shown). Because the β2 subunit reacts with NLVS faster than β1 (Fig. 1), this lack of any detectable reaction of the β2 subunit with 0.1 μM NLVS excludes the possibility that some of the radioactivity in the β5 band resulted from a reaction with co-migrating β1 subunit. Therefore, we were able to follow specifically the reaction of NLVS with the β5 subunit by SDS-PAGE. This reaction occurred at a slow linear rate (Fig. 4), but the addition of Suc-FLF-mna caused a 6-fold increase in the rate of labeling (Fig. 4), whereas the addition of Suc-LLVY-mna stimulated this process 4- to (Fig. 4) 13–fold (data not shown). However, the actual degree of stimulation is probably much greater because these substrates, in the absence of the allosteric activation, would be expected to reduce labeling by NLVS by simple competition for the active sites. By contrast, the substrates of the chymotrypsin-like site that appear not to bind to the non-catalytic sites, Suc-AAF-*p*na (Fig. 4) and Suc-AAF-amc (not shown), caused only a very small increase in labeling by NLVS, even when used at almost saturating concentrations. Thus, hydrophobic peptides that bind to the non-catalytic sites enhance the reaction of the chymotrypsin-like sites with the covalent inhibitor.

A variety of experiments with substrates of the chymotryptic sites demonstrated that this activity was also enhanced by hydrophobic peptide binding to the non-catalytic sites. As shown above, Suc-AAF-amc, although a substrate of the chymotrypsin-like site, appears not to bind to the non-catalytic sites, as evidenced by its inability to stimulate hydrolysis of acidic and basic peptides and the non-sigmoidal kinetics of its own cleavage (Fig. 5*a*). However, cleavage of Suc-AAF-amc was stimulated 2–3-fold by other hydrophobic peptides (Suc-FLF-mna and Suc-LLVY-mna) that bind to the non-catalytic site, and this effect was largely through an increase in V_{max} (Fig. 5*a*). In addition, the highly sigmoid concentration dependence of Suc-LLVY-amc hydrolysis (Fig. 5*b*) indicates that this widely used substrate of the chymotrypsin-like site also binds to the non-catalytic sites. Therefore, at the high concentrations typically used (above 40 μM), this peptide occupies all the catalytic and non-catalytic sites, and the addition of other activators should not enhance its rates of hydrolysis as indeed was observed (Fig. 5*b*). However, at lower concentrations of Suc-LLVY-amc (below 20 μM), the majority of the non-catalytic sites must be unoccupied, and the addition of other activators at saturating concentrations would be expected to occupy these

TABLE III
Hydrophobic peptides stimulate peptide hydrolysis by the wild-type yeast 20 S proteasome but not by its complex with PA26 activator and by the ΔNα3 mutant

The concentrations of peptides used are as follows: 20 μM Ac-nLPnLD-amc, 50 μM Suc-LLVY-amc, 20 μM Boc-LRR-amc, 50 μM Suc-FLF-mna, and 80 μM Suc-LLVY-mna. 20 S proteasomes were at 1 μg/ml (1.4 nM). PA26 (1 μg/ml) was at 4-fold molar excess over 20 S proteasomes, and higher PA26 concentrations did not enhance the stimulation of peptidase activities. Values are means ± ranges of two independent experiments.

Active site (substrate)	Activator	wt	ΔNα3-mut	Wt + PA26	wt	ΔNα3-mut[a]	Wt + PA26
		specific activity (nmol/min·mg)			*-fold stimulation*		
Caspase-like	None	0.7 ± 0.4	48.2 ± 1.7	84.2 ± 11.6	1.0	1.0	1.0
(Ac-nLPnLD-amc)	Suc-FLF-mna	50.7 ± 1.7	43 ± 0	68.1 ± 10.6	72	0.9	0.8
	Suc-LLVY-mna	40.8 ± 6.2	49.8 ± 2.8	74.0 ± 9.6	58	1.0	0.9
Chymotrypsin-like	None	0.9 ± 0.7	109 ± 1	190 ± 29	1.0	1.0	1.0
(Suc-LLVY-amc)	Suc-FLF-mna	107 ± 6	103 ± 7	151 ± 19	118	0.9	0.8
	Suc-LLVY-mna	90.0 ± 6.4	63.2 ± 2.8	104 ± 11	100	0.6	0.5
Trypsin-like	None	1.5 ± 1.2	10.7 ± 2.0	22.2 ± 3.8	1.0	1.0	1.0
(Boc-LRR-amc)	Suc-FLF-mna	6.0 ± 0.9	11.3 ± 3.2	23.3 ± 4.8	4.0	1.1	1.0
	Suc-LLVY-mna	25.7 ± 3.0	29.2 ± 7.4	63.9 ± 13.4	17	2.7	2.9

[a] Stimulation was calculated by dividing the specific activity in the presence of hydrophobic peptide by the specific activity of the same particle or complex in the absence of peptide stimulator.

non-catalytic sites and enhance rates of Suc-LLVY-amc cleavage, as was indeed observed (Fig. 5*b*).

The strongest stimulation of Suc-LLVY-amc hydrolysis was observed when it was present at 5 μM (indicated by an *arrow* in Fig. 5*b*), and we subsequently used this concentration to study how ligand binding to the non-catalytic sites stimulate peptide cleavage by the chymotrypsin-like sites. At this concentration, the cleavage of Suc-LLVY-amc was stimulated up to 43-fold (Fig. 2*c* and Table II) by other hydrophobic peptides (Suc-FLF-mna and Suc-LLVY-mna), and the plot of this activation as a function of the concentration of these stimulatory peptides clearly had a sigmoid shape (Fig. 2*c*), as was observed for the stimulations of the hydrolysis of basic and acidic substrates (Fig. 2, *a* and *b*). Values for the K_A and Hill coefficients were similar for all these processes and ranged from 4 to 17 with a mean of 7 (Table II), which is therefore our best estimate of the minimal number of non-catalytic sites. Thus, binding of hydrophobic peptides to several non-catalytic sites enhances peptide hydrolysis by all active sites of 20 S proteasomes from rabbit muscle.

Activation of Peptide Hydrolysis Is Not Observed in Mutant Proteasomes with an Open Entrance Channel—A simple mechanism by which hydrophobic peptides might enhance their own hydrolysis as well as peptide hydrolysis by the trypsin-like and caspase-like sites would be by promoting entry of peptides into the proteasome, *i.e.* if the occupancy of the non-catalytic sites favors an open state of the channel in the α-ring. A specific prediction of this model is that these hydrophobic peptides should have much less or no effect when the gate in the α-ring is already in an open state. In order to test this possibility, we compared the effects of these activators on peptide hydrolysis by the wild type yeast 20 S proteasomes and its ΔNα3 mutant. In this mutant, the channels are open because of a deletion of the nine N-terminal residues of the α3 subunit (14). As a consequence of this open channel, rates of peptide entry and hydrolysis by all active sites are increased (14). Because such mutants have been isolated only in *S. cerevisiae*, we first tested whether hydrophobic peptides can activate hydrolysis by all three active sites of yeast 20 S proteasomes as they do in the mammalian particles. Indeed, a similar large activation does occur in the wild type yeast proteasomes (Table III).

In the ΔNα3 ("open channel") mutant, the caspase-like activity was up to 65–fold higher than in the wild type 20 S proteasome; the chymotrypsin-like activity was up to 120-fold higher, and the trypsin-like activity was 7-fold higher (Table III). This difference is larger than reported previously (14), probably because our wild type proteasomes purified in the presence of glycerol have lower activity (*i.e.* are more native) than when isolated in its absence, as was done by Groll *et al.* (14). The specific activity of the ΔNα3 mutant was comparable with the activity of the wild type stimulated by hydrophobic peptides. In these particles, unlike the wild type, the hydrophobic peptides Suc-LLVY-mna and Suc-FLF-mna did not cause any further enhancement of cleavage of Ac-nLPnLD-amc (Table III) and Ac-GPLD-amc (not shown). Furthermore, cleavage of Suc-LLVY-amc (Table III) and Suc-AAF-amc (data not shown) was even inhibited by the homologous mna peptides, presumably due to competition between these activators and substrates for cleavages at the chymotrypsin-like site. Finally, Suc-FLF-mna also had no effect on cleavage of Boc-LRR-amc (Table III) and Z-ARR-amc (not shown) by the trypsin-like site, and Suc-LLVY-mna caused only 3-fold stimulation of cleavages of these basic peptides, which was much lower than the 17-fold stimulation seen with the wild type. This stimulation of trypsin-like cleavages by Suc-LLVY-mna in the ΔNα3 mutant proteasomes was not due to the binding of Suc-LLVY-mna to the chymotrypsin-like site, because it was still observed when binding was blocked by NLVS (data not shown). Thus, if the gate into the 20 S proteasome is in the open form, activation of peptide hydrolysis by hydrophobic peptides does not occur or is significantly reduced. This observation strongly suggests that hydrophobic peptides stimulate hydrolysis of peptides generally by opening the entrance channel into the particle and thus facilitating substrate cleavage by all three active sites.

Hydrophobic Peptides Do Not Activate Peptide Hydrolysis by 20 S-PA26 Complexes—Opening of the entrance channel also occurs upon association of 20 S proteasomes with the heptameric PA26 proteasome activator complex from *T. brucei*, as was demonstrated by x-ray diffraction (20). Therefore, we tested whether cleavages by different active sites are stimulated by hydrophobic peptides in the 20 S-PA26 complexes. As shown in Table III, PA26 increased peptide hydrolysis of yeast wt-20 S proteasomes 15–210-fold, in a similar fashion as the mammalian PA28 stimulates this process by mammalian 20 S particles (21, 22). Interestingly, PA26 caused even a higher increase in the rates of peptide hydrolysis than the deletion of the N-terminal tail of the α3 subunit (Table III). These differences may indicate that the effective diameter of the channel is wider in PA26-wt complexes than in the mutant.

Importantly, when the hydrophobic peptide activators were added to PA26-stimulated proteasomes, they did not cause a further activation of hydrolysis of Ac-nLPnLD-amc (Table III) and Ac-GPLD-amc (not shown) by the caspase-like site. Cleavages of Suc-LLVY-amc (Table III) and Suc-AAF-amc (not

shown) by the chymotrypsin-like site were even inhibited, presumably because of the competition between mna- and amc-containing peptides. Finally, Suc-FLF-mna had no effect on Boc-LRR-amc (Table III) and Z-ARR-amc (not shown) cleavage by the trypsin-like site, and Suc-LLVY-mna stimulated cleavage of these substrates by only 3-fold, much less than the 17-fold stimulation seen in the absence of PA26 (Table III). These findings and the lack of stimulation in the ΔNα3 mutant (Table III) indicate that if the entrance channel is maintained in an open conformation, hydrophobic peptides cannot further stimulate peptide hydrolysis by the caspase- and chymotrypsin-like sites, and their capacity to stimulate the trypsin-like activity is greatly reduced. These effects are consistent with peptides inducing gate opening rather than altering the catalytic efficiency of the three active sites.

Hydrophobic Peptides Alter the K_m and V_{max} for All Substrates in a Similar Fashion as the ΔNα3 Mutation and PA26—Further strong evidence that these stimulatory peptides promoted gate opening came from systematic comparisons of their effects on the kinetic parameters (K_m and V_{max}) with those treatments known to cause channel opening. As demonstrated above, hydrophobic peptides stimulate hydrolysis by the caspase-like site of mammalian 20 S proteasomes through both a large increase in V_{max} and a decrease in K_m values (Table I), but the stimulation of cleavages by the trypsin-like sites (uncovered in these studies) was strictly the result of a decrease in K_m values (Fig. 3). In addition, although hydrophobic peptides did not change the K_m and V_{max} values for the cleavage of Suc-LLVY-amc by the chymotrypsin-like site, they altered markedly the kinetics from highly cooperative to standard Michaelis-Menten, and they stimulated the hydrolysis of this substrate at a concentration below K_m (Fig. 5*b*). In order to determine whether opening of the channels in the α-rings alters the kinetic properties in a similar manner as the hydrophobic peptides, we determined K_m and V_{max} values and the Hill coefficient for all three active sites of the wild type yeast 20 S proteasomes, ΔNα3 mutant, and the wild type 20 S after addition of PA26 complexes both in the presence and absence of peptide activators.

For all three types of substrates the concentration dependence of their cleavage by wild type proteasomes after stimulation with Suc-FLF-mna (Fig. 6*a*) or Suc-LLVY-mna (Table IV) resembled more closely the data obtained with the ΔNα3 mutant proteasome and with 20 S-PA26 complexes than with control wild type particles. For example, the K_m for Ac-nLPnLD-amc cleavage by the caspase-like site was decreased 5-fold by the peptide activators, 6-fold by PA26, and 2–3-fold by the ΔNα3 mutations (Fig. 6*a* and Table IV). Also, the hydrophobic activators caused a 7–8-fold increase in V_{max}, which approached the 12-fold increase with PA26 and the 11-fold increase in the mutant. In addition, the hydrophobic peptides, PA26, and the ΔNα3 mutation all caused a large decrease in the K_m for cleavage by the trypsin-like site (even larger than the decrease of the K_m for the caspase-like activity). In fact, although the untreated wild type particles were not saturated even by 1 mM Boc-LRR-amc, in the other cases saturation was clearly evident (Fig. 6*b*) with the K_m values all in the 45–300 μM range (Table IV).

Finally, the hydrophobic peptides Suc-FLF-mna (Fig. 6*c*) and Suc-LLVY-mna (data not shown) changed the kinetics of Suc-LLVY-amc cleavage by yeast 20 S proteasomes from clearly sigmoid-shaped to classical Michaelis-Menten (Fig. 6*c*), as was found with the latent mammalian 20 S proteasome (Fig. 5*b*). (Interestingly, $S_{0.5}$ of the yeast 20 S was much higher (450 *versus* 25 μM) perhaps because of a lower affinity of the non-catalytic sites for this peptide.) In addition, these hydrophobic

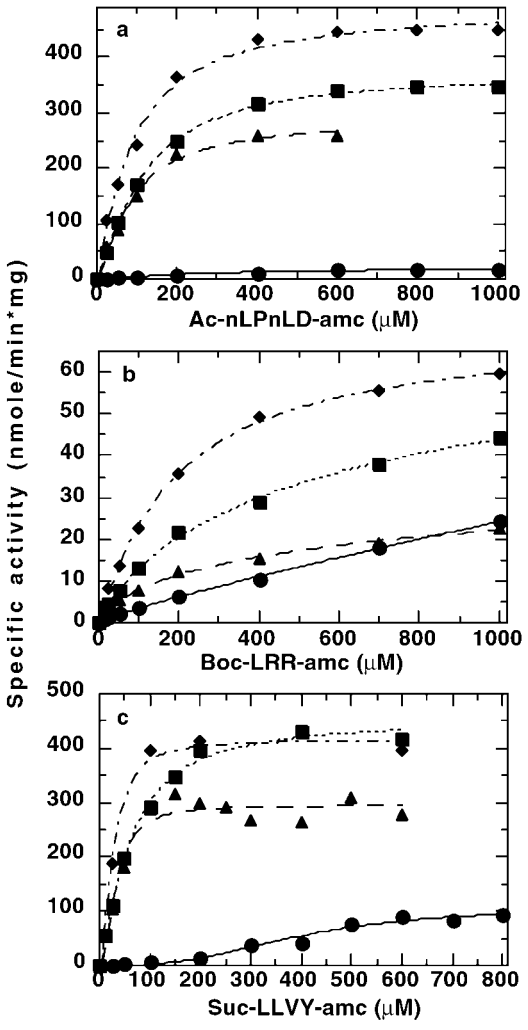

FIG. 6. **Hydrophobic peptides mimic the effect of PA26 and of the ΔNα3 mutation on peptide hydrolysis by all active sites of yeast 20 S proteasomes.** Concentration dependence of cleavages by caspase-like (*a*), trypsin-like (*b*), and chymotrypsin-like (*c*) sites. *Filled circles*, *solid lines*, wild type 20 S proteasomes; *filled triangles*, *dashed line*, wild type in the presence of 50 μM Suc-FLF-mna; *filled diamonds*, *dashed-dotted line*, wild type 20 S-PA26 complexes; *filled squares*, *dotted line*, ΔNα3 mutant.

peptides caused a 5–8-fold decrease in K_m and 2-fold increase in V_{max} values. A similar loss of cooperativity and similar changes in the K_m and V_{max} values were seen in the ΔNα3 mutant and in PA26-stimulated wild type particles (Fig. 6*c* and Table IV). Thus, careful kinetic analysis demonstrates that hydrophobic peptides change the kinetic parameters of all three active sites of the wild type 20 S proteasome in a similar fashion as the ΔNα3 mutation and PA26 activator. These data strongly support the conclusion that hydrophobic peptides stimulate peptide hydrolysis generally by opening the channels in the α-rings. It is also noteworthy that treatments that open the channel not only increase the V_{max} but also reduce the K_m values of 20 S proteasomes; thus, both K_m and V_{max} values should not be viewed simply as properties of the active sites of the proteasome (especially in its native state).

Significantly, the addition of hydrophobic peptides to the ΔNα3 mutant (Table IV) and wt-PA26 complexes (data not shown) did not decrease the K_m or increase the V_{max} of all active sites, except for a small decrease in the K_m of the trypsin-like site caused by Suc-LLVY-mna. Thus, hydrophobic peptides do not cause general stimulation of peptide hydrolysis when the entrance channel of the 20 S proteasomes is open. These

TABLE IV

Hydrophobic peptides change K_m and V_{max} of yeast 20 S proteasomes in a similar way as PA26 and the ΔNα3 mutation

Values are means ± ranges of two independent experiments, except for the cleavage of Ac-nLPnLD-amc by the ΔNα3 mutant where means ± S.D. of four experiments are shown. Concentrations of reagents were as in Table III.

Proteasomes	Peptide activator	Caspase-like site (Ac-nLPnLD-amc)		Chymotrypsin-like site (Suc-LLVY-amc)		Trypsin-like site (Boc-LRR-amc)	
		K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
		μM	*nmole/min·mg*	μM	*nmole/min·mg*	μM	*nmol/min·mg*
wt	None	484 ± 6	37 ± 13	447 ± 78[a]	144 ± 36	>1000	ND[b]
	Suc-FLF-mna	104 ± 28	311 ± 15	56 ± 18	350 ± 13	183 ± 3	30 ± 6
	Suc-LLVY-mna	96 ± 29	250 ± 23	82 ± 30	330 ± 102	45 ± 8	38 ± 6
wt + PA26	None	79 ± 15	455 ± 45	56 ± 22	531 ± 91	178 ± 20	96 ± 16
ΔNα3 mut	None	191 ± 40	435 ± 57	75 ± 21	424 ± 127	294 ± 50	58 ± 18
	Suc-FLF-mna	217 ± 63	504 ± 52	65 ± 21	338 ± 68	225 ± 30	124 ± 3
	Suc-LLVY-mna	185 ± 5	375 ± 65	90 ± 65	300 ± 176	57 ± 4	78 ± 1

[a] Cleavage of Suc-LLVY-amc by the control wild-type proteasomes showed positive cooperativity (Fig. 6*c*) with a Hill coefficient value of 2.5 ± 0.2. In all other cases, it obeyed Michaelis-Menten kinetics.

[b] ND, not determined because saturation could not be reached under conditions used.

data further support the model that binding of hydrophobic peptides to the non-catalytic sites favors an open conformation of the channel in the α-rings.

Although hydrophobic peptides caused a very large stimulation of the active sites of the proteasome, the V_{max} of peptide hydrolysis by the ΔNα3 mutant and by the wt-PA26 complexes were still higher than for the wild type 20 S proteasomes activated by hydrophobic peptides. This difference may be due to the effective diameter of the channel being larger in the wt-PA26 complex and in the ΔNα3 mutant than in the peptide stimulated wild type 20 S proteasome.

The Stimulation of Peptidase Activities Is Smaller in 26 S than 20 S Proteasomes—If hydrophobic peptides enhance cleavages by 20 S proteasome by promoting the opening of the entry channels, then these peptides should cause a smaller stimulation in the 26 S proteasomes, where the channels in one or both α-rings are primarily in the open state due to the presence of the 19 S regulatory complex at one or both ends of the core particle (10). Accordingly, Suc-FLF-mna did not stimulate peptide hydrolysis by the 26 S proteasome from rabbit muscle, whereas Suc-LLVY-mna caused a modest 2–3-fold activation (Fig. 7), which was much lower than the 12–35-fold activation seen with latent 20 S proteasomes (Table II). Because these data were obtained with 26 S proteasomes from rabbit muscle, the activation of peptide hydrolysis in mammalian 20 S proteasomes also seems to occur by facilitating peptide entry (as in yeast 20 S particles).

Hydrophobic Peptides Do Not Stimulate Peptide Hydrolysis in SDS-treated 20 S Proteasomes—Low concentrations of SDS (0.01–0.02%) stimulate peptide cleavages by latent 20 S proteasome from yeast and mammals (9), but this detergent does not stimulate these cleavages in the ΔNα3 mutant of yeast 20 S proteasomes (14). Therefore, this activation most likely also involves opening of the gated channels in the α-rings. We tested the effects of hydrophobic peptides on peptide hydrolysis by SDS-activated 20 S proteasomes, except for cleavages by the trypsin-like sites, which cannot be assayed in the presence of SDS, because SDS causes a precipitation of a guanido group containing substrates. As expected, the caspase-like activity of the 20 S proteasomes from rabbit muscle was stimulated 25–45-fold by SDS but was not further stimulated by hydrophobic peptides (Fig. 8). In fact, the chymotrypsin-like activity of the SDS-activated proteasomes was even slightly inhibited by activator peptides, probably due to a competition of these substrates with the amc substrates used to assay activity. These data are consistent with the model that hydrophobic peptides and SDS stimulate peptide hydrolysis in mammalian 20 S proteasomes by opening the entrance channels in the α-rings. In

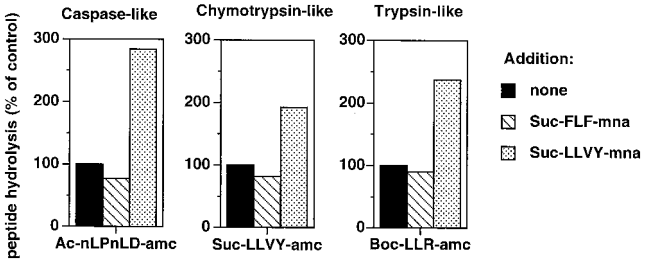

FIG. 7. **Stimulation by Suc-FLF-mna and Suc-LLVY-mna of hydrolysis of different peptide substrates by 26 S proteasomes from rabbit muscle.** *Solid bars*, control (no activator present); *hatched bars*, 40 μM Suc-FLF-mna; *stippled bars*, 100 μM Suc-LLVY-mna. Concentrations of substrates are as follows: 5 μM for Suc-LLVY-amc and 20 μM for the others. Similar effects were observed with 50 μM Ac-GPLD-amc (caspase-like site), 50 μM Suc-AAF-amc (chymotrypsin-like site), and 20 μM Z-AAR-amc (trypsin-like site). Note that the 2–3-fold stimulation of 26 S proteasomes by Suc-LLVY-mna is much lower than the stimulation of different peptidase activities in 20 S proteasomes by this peptide.

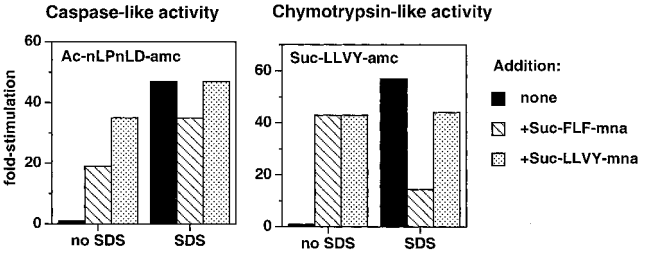

FIG. 8. **Hydrophobic peptides do not stimulate peptide hydrolysis by SDS-activated muscle 20 S proteasomes.** Rates of peptide hydrolysis by 20 S proteasomes from rabbit muscles were normalized to the rate of hydrolysis in the absence of 0.01% SDS and hydrophobic peptides. *Solid bars*, control (no activator present); *hatched bars*, 40 μM Suc-FLF-mna; *stippled bars*, 100 μM Suc-LLVY-mna. Concentrations of substrates were as on Fig. 7. The figure shows the average stimulation in 4 different preparations. Similar effects were observed with 50 μM Ac-GPLD-amc (caspase-like site) and 50 μM Suc-AAF-amc (chymotrypsin-like site).

fact, these findings raise the possibility that the activation by low concentrations of SDS may be because this detergent binds to the same non-catalytic sites as the hydrophobic peptides and mimics their activities.

Activation of 20 S Proteasomes Is Decreased by Potassium Ions—We then tested the effects of hydrophobic peptides under a condition which suppresses spontaneous opening of the channel. Hydrolysis of peptides by 20 S proteasomes is significantly reduced in the presence of physiological concentrations of sodium and potassium (10, 28). Although KCl retards spontane-

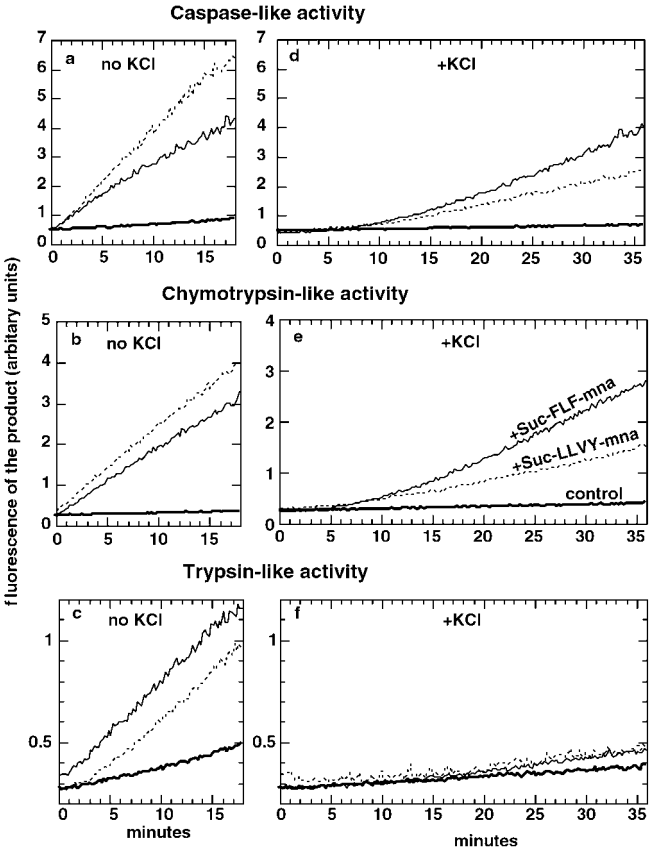

FIG. 9. **Inhibitory effect of KCl on the stimulation of peptide hydrolysis by hydrophobic peptides.** Reaction progress curves for the hydrolysis of 20 μM Ac-nLPnLD-amc (*a* and *d*), 5 μM Suc-LLVY-amc (*b* and *e*), and 20 μM Boc-LRR-amc (*c* and *f*) by 20 S proteasomes from rabbit muscles in the presence (*d–f*) or absence (*a–c*) of 100 mM KCl. *Thick solid line*, control (no activators added); *thin solid line*, 40 μM Suc-FLF-mna added; *dotted line*, 90 μM Suc-LLVY-mna added. The concentrations of the proteasome were 1.5 nM (*a* and *d*), 3 nM (*c* and *f*), or 6 nM (*b* and *e*). One fluorescence unit corresponds to 0.1 μM of the reaction product.

ous activation of yeast 20 S proteasomes, it does not have any effect on the $\Delta N\alpha 3$ mutant (10), indicating that potassium suppresses peptide hydrolysis by maintaining the channel in a closed conformation. Therefore, potassium would be expected to suppress the stimulation caused by hydrophobic peptides. In accord with prior observations, the basal activity of 20 S proteasomes from rabbit muscle was decreased in the presence of potassium (Fig. 9). Although hydrophobic peptides were able to overcome this inhibition by potassium, and to stimulate all three peptidase activities, this stimulation occurred only after a significant delay of 10–15 min (Fig. 9, *d–f*). In contrast, there was no delay in the activation of peptide hydrolysis in the absence of KCl (Fig. 9, *a–c*). Furthermore, the magnitude of activation was always less in the presence of potassium. Thus, potassium ions, which help maintain the entrance channel into the 20 S proteasome in the closed conformation, partially suppress the activation by hydrophobic peptides.

DISCUSSION

Hydrophobic Peptides Appear to Stimulate Peptide Hydrolysis by Opening the Channel in the α-Rings—This study has uncovered the following two new regulatory properties of the 20 S proteasome: 1) that the binding of hydrophobic peptides to multiple non-catalytic sites stimulates the hydrolysis of peptides by all of its active sites, and 2) that this stimulation occurs most probably by peptide-induced opening of the channel in the α-rings through which substrates enter the 20 S particle. This latter conclusion is based on our finding that the hydrophobic peptides did not stimulate peptide hydrolysis under various conditions where the entrance channels are primarily in an open state, including the $\Delta N\alpha 3$ mutant of yeast proteasomes, 20 S-PA26 complexes, 26 S proteasomes, and SDS-activated 20 S proteasomes. Furthermore, the changes induced by these peptides in K_m and V_{max} of the yeast 20 S proteasome, as well as the loss of cooperativity for Suc-LLVY-amc cleavage, were similar to the changes seen after association of the proteasome with PA26 and in the $\Delta N\alpha 3$ mutant. Moreover, the activation by hydrophobic peptides was decreased by potassium ions, which stabilize the closed conformation of the channels (10).

Recent studies of 20 S proteasomes by atomic force microscopy (29) also suggested that in solution there is a dynamic equilibrium between the closed and open forms of the channels, and that the addition of Suc-LLVY-amc dramatically increases the proportion of proteasomes with an open channel. It is also noteworthy that the peptide alcohol, Z-IE(OtBu)AL-ol, like the hydrophobic peptides studied here, mimics the activation of peptide hydrolysis at the chymotrypsin- and caspase-like sites seen with mammalian PA28 but has no further effect on activity of PA28–20 S complexes (30). These observations were interpreted as evidence that this hydrophobic peptide alcohol interacts with the PA28-binding site. Another explanation is that, like hydrophobic peptide substrates, the peptide alcohol binds to non-catalytic sites and triggers channel opening.

Opening of the Channel Is Caused by Binding of Peptides to Multiple Non-catalytic Sites—In our original report that substrates of the chymotrypsin-like site allosterically activate the caspase-like sites, we concluded that these peptides do so by binding to the chymotrypsin-like sites (8), because the concentration dependence of cleavage of these peptides was very similar to that of their stimulatory effects. However, the present experiments, while confirming and extending those observations, indicate that substrates of the chymotrypsin-like sites stimulate peptide hydrolysis not only by the caspase-like but also by the trypsin-like and the chymotrypsin-like sites, and they do so by binding to multiple non-catalytic sites. This conclusion is based on the following observations. 1) Selective covalent modification of the two chymotrypsin-like sites by a substrate analogue, the peptide vinylsulfone NLVS, causes little or no stimulation of peptide cleavage by the other sites (Tables I and III). 2) Even with the chymotrypsin-like sites fully occupied by NLVS (Fig. 2 and Tables I and II), and thus unable to bind substrates, the hydrophobic peptides still stimulate cleavages by the other active sites as they do in control proteasomes. 3) The specificities of the chymotrypsin-like and non-catalytic sites, although overlapping, are different because some substrates (*e.g.* Suc-AAF-*p*na and Z-GGL-na) cannot allosterically stimulate peptide hydrolysis by the caspase-like sites. Our conclusion that hydrophobic peptides stimulate the caspase-like activity by binding to the non-catalytic sites is supported by the observation of Schmidtke *et al.* (23) that another inhibitor of the chymotrypsin-like activity, β-lactone, does not prevent stimulation of the caspase-like activity by Suc-FLF-mna. However, these authors, like our prior study (8), did not detect the stimulation of the trypsin-like activity by this peptide apparently because both studies used high concentrations of basic peptides.

Although these findings may indicate that hydrophobic peptides have the same affinity for the non-catalytic sites as to the chymotrypsin-like sites (Table II), the presence of two types of binding sites with such similar properties within the same particle seems quite unlikely. A more attractive explanation for the almost identical binding curves is that the concentration dependence for hydrolysis of these hydrophobic substrates ac-

tually reflects the concentration dependence of their binding to the non-catalytic sites, which by controlling channel opening determines the rates of peptide hydrolysis at the chymotrypsin-like sites. These observations argue that it is in fact impossible to determine the true kinetic constants of the active sites of the latent 20 S proteasomes by standard approaches, because binding to the non-catalytic sites is the rate-limiting event. For example, the V_{max} of latent proteasomes is probably a measure of the rate of substrate entry and not of their hydrolysis by the active sites. Similarly, a decrease in K_m upon channel opening observed in this study may simply reflect the different affinities of peptides for the catalytic and non-catalytic sites. Thus, the actual kinetic parameters of the active sites can be determined only under conditions where the entry channel is in an open conformation.

Possible Number and Location of the Gate-regulating Sites—Although Scmidtke *et al.* (23) also demonstrated a distinct "non-catalytic modifier site" in the proteasome and proposed a kinetic model to account for its behavior, this model could not predict the number of such sites. Our findings clearly indicate that there are several gate-regulating sites, because the stimulation of all three activities by hydrophobic peptides shows a very similar concentration dependence and a high degree of cooperativity. In fact, values for the Hill coefficients, which indicate the minimal number of non-catalytic sites, ranged between 4 and 17 in 14 different experiments (Table II). When these numbers were averaged on the assumption that they all reflected the same non-catalytic sites (as suggested by similar K_A values for stimulation of different activities), they gave a mean of 7 with an S.D. of 3, which is our best estimate of the minimal number of the gate-regulating sites.

The exact location of these non-catalytic sites remains unclear, but the average value of 7 ± 3 for the Hill coefficient and the 7-fold symmetry of the particle raise the possibility that there is a similar gate-regulating site on each α-subunit. If these sites are located inside the particle, then the spontaneous opening of the channel (10, 29) presumably enables the peptides to bind to these sites in our experiments, and thereby prevents the return of the channel to the closed conformation. The observation that potassium ions, which decrease spontaneous opening of the channel (10), delay (but do not prevent) the onset of the activation by the hydrophobic peptides (Fig. 9) favors this model of an interaction with internal gate-regulating sites. Conversely, if these sites are on the outside of the 20 S particle or on the channel itself, binding of hydrophobic peptides to them could directly favor channel opening.

It had been suggested that the hydrophobic peptide alcohol Z-IE(OtBu)AL-ol, which has similar effects as the hydrophobic peptides, stimulates by interacting with the PA28-binding site (30). However, we believe it unlikely that these compounds bind to this site, because they are structurally distinct from the C-terminal α-helixes of PA28/PA26, which mediate binding of these activators to 20 S proteasomes (20). These stimulatory tri- and tetrapeptides are too short to form an α-helix, are significantly more hydrophobic than C termini of PA26/PA28, and lack free C-terminal carboxyl group which appears to be important for PA26–20 S interactions (20). The mechanism of gate opening by these peptides must also differ from the channel opening induced by the 19 S (PA700) complex in the 26 S proteasome, which involves binding of ATP to certain of the ATPases of the 19 S complex (10). On the other hand, our data that hydrophobic peptides cannot stimulate peptide hydrolysis in SDS-activated proteasomes raises the possibility that this detergent which appear to stimulate channel opening at low concentrations (14) may bind to the same regulatory sites as hydrophobic peptides.

Thus, binding of hydrophobic peptides to several non-catalytic sites distinct from the chymotrypsin-like site appears to trigger opening of the channel in the α-rings or to inhibit the closing of the spontaneously opened channel. However, we cannot exclude the possibility that additional effects are required for maintaining the channel in the open conformation. Osmulski and Gaczynska (29) reported that blocking Suc-LLVY-amc cleavage at the chymotrypsin-like site by the inhibitor abolishes the effect of these peptides on the channel in the α-ring and suggested that a catalytic event is required to promote channel opening. Although the present results demonstrate that hydrophobic peptides need to bind to the non-catalytic sites to stimulate peptide hydrolysis, we cannot exclude the possibility that peptides may also have to bind to any of the catalytic sites to trigger channel opening, because in all of our experiments peptide substrates had to be present for us to assay the activities of the catalytic sites. In the future, by using selective ligands of the gate-regulating sites, it should be possible to test this possibility directly and to localize these unknown sites.

Although the present results indicate that the opening of the channels in the α-rings can account for most of the stimulation by hydrophobic peptides, some of it may be due to an additional effect beyond channel opening. Specifically, Suc-LLVY-mna can stimulate peptide hydrolysis by the trypsin-like site of the $\Delta N\alpha 3$ mutant and of 20 S-PA26 (Table III) complexes. This 3-fold stimulation is much lower than the 17-fold stimulation in the latent wild type and is a K_m effect (Table IV); it may thus be due to some structural changes in the particle or the active site. In fact, recent findings suggest a capacity of PA28 regulators to alter active sites in addition to (or perhaps as a consequence of) causing the gated channel in the α-rings to open (31, 32).

Possible Roles of Peptide-induced Gating in Protein Degradation—The channels in the α-rings control not only the entrance of substrates into the 20 S proteasome but also the exit from the particle of the products of protein breakdown (10). Although the present experiments used tetrapeptide substrates as activators, presumably a similar activation would occur with hydrophobic peptides produced during degradation of a protein. Thus, hydrophobic products, by triggering the opening of the channel, may facilitate the release of the products of proteolysis. These peptides range in length from 3 to 24 residues (16), and therefore their exit from the particle must be even more dependent on the opening of the channel than entrance of tetrapeptides studied here. Peptide-induced product release may be particularly important in the asymmetric 26 S proteasomes that contain a single 19 S regulatory particle (33, 34). In these complexes, protein substrates must first bind to the 19 S component, which catalyzes an ATP-dependent translocation through the adjacent α-ring into the 20 S core particle, whereas peptide products probably exit from the opposite, free end of the 20 S particle. Presumably, the latter channel remains primarily in the closed position until hydrophobic peptides generated by the active sites bind to the non-catalytic site and promote channel opening. Such an exit function for peptide-induced gating would seem most likely if the non-catalytic sites were located inside the 20 S cylinder but would also be possible if located on the outer surface of the α-rings in the vicinity of the channel.

It seems much less likely that peptide-induced gating *in vivo* functions as a mechanism by which hydrophobic peptides in the cytosol activate their own breakdown by 20 S proteasomes. Oligopeptides are not found in significant amounts within cells (35), and once peptide products exit the 26 S proteasome, they are rapidly degraded by the many exo- and endopeptidases

present in the cytosol (32), which are much more efficient than the proteasome in digesting small substrates.

It is presently unclear if the gate-regulating sites can bind much longer peptides than the tetrapeptides studied here or if they can even be activated by hydrophobic domains in proteins. This issue is an important topic for future study with major functional implications. If the gate-regulating sites can bind hydrophobic stretches in unfolded proteins and are located within the proteasome, then the binding of such domains (or large fragments) could trigger product exit or could even help maintain the gate in an open position facilitating polypeptide entry into the 20 S core. If these allosteric sites are on the exterior of the particles and bind hydrophobic sequences in proteins, it is conceivable that such stretches in denatured or oxidatively damaged polypeptides could also trigger opening of the channel in free 20 S particles and thus induce their own degradation. Such a theoretical possibility, however, is unlikely to play a major role in protein breakdown *in vivo*, because the great majority of intracellular proteolysis requires ATP for the function of 26 S particles and for substrate ubiquitination. Also even if the channels in free 20 S proteasomes become open within cells, these particles may still be unable to degrade proteins. Finally, it is still unclear if the channel opening demonstrated here allows the transit of only small peptides or also of proteins. In fact, gate opening by PA28, unlike that by $\Delta N\alpha 3$ and SDS, stimulates only cleavage of short peptides and not protein breakdown by 20 S proteasomes (21), and *in vivo* channel opening by PA28 seems to serve primarily to promote release of degradation products (20).

The new features of the 20 S core particle uncovered in this and related studies necessitate a revision of the cyclical ("bite-chew") model that we proposed to account for the role of the allosteric effects in protein degradation (8). A key observation leading to that model was that acidic peptides cause allosteric inhibition of the chymotrypsin-like activity. Two recent reports (23, 36) and our observations[3] demonstrate that this inhibition (8) also occurs upon binding of acidic peptides to a non-catalytic site, which appears distinct from the sites that stimulate channel opening.[3] These findings suggest that during protein breakdown the cleavages by the rate-limiting chymotrypsin-like site are slowed when acidic peptide fragments bind to the inhibitory non-catalytic site(s), whereas build-up of hydrophobic peptides and interaction with the non-catalytic sites described here probably facilitates the release of degradation products by promoting channel opening. Although this peptide-induced gating appears to be an important new feature of proteasomes, actual proof of such a role in protein breakdown will be a major challenge for future experiments.

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[3] A. F. Kisselev and A. L. Goldberg, unpublished observations.

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