

Lack of Proteasome Active Site Allostery as Revealed by Subunit-Specific Inhibitors

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Summary

The chymotrypsin-like (CT-L) activity of the proteasome is downregulated by substrates of the peptidyl-glutamyl peptide hydrolyzing (PGPH) activity. To investigate the nature of such interactions, we synthesized selective α' , β' -epoxyketone inhibitors of the PGPH activity. In cellular proliferation and protein degradation assays, these inhibitors revealed that selective PGPH inhibition was insufficient to inhibit protein degradation, indicating that the CT-L and PGPH sites function independently. We also demonstrated that CT-L inhibition by a PGPH substrate does not require the occupancy of the PGPH site or hydrolysis of the PGPH substrate. Thus, these results support a model in which a substrate of one subunit regulates the activity of another via binding to a noncatalytic site(s) rather than through binding to an active site.

Introduction

Nonlysosomal protein degradation mediated by the proteasome determines the life span of most cellular proteins. This ATP- and ubiquitin-dependent process is responsible for intracellular activities as diverse as the removal of misfolded proteins, cell cycle regulation via cyclin degradation, and cellular immune responses by antigenic peptide processing (Goldberg et al., 1995; King et al., 1996; Bonifacino and Weissman, 1998; Hershko and Ciechanover, 1998). The proteasome consists of two regulatory complexes capping a 20S catalytic proteasome core, which contains six catalytic subunits responsible for at least three well-characterized proteolytic activities (Groll et al., 1997; Dick et al., 1998). These activities have been defined by their ability to cleave peptide bonds after hydrophobic, basic, and acidic amino acid residues (Wilk and Orłowski, 1983) and are referred to as the chymotrypsin-like (CT-L), the trypsin-like, and the peptidyl-glutamyl peptide hydrolyzing (PGPH) activities, respectively.

Although the biochemical basis of catalytic activity generated by each subunit has been extensively ana-

lyzed by classical genetics and the X-ray crystal structure determination of the 20S proteasome, it is not yet clear how each catalytic activity contributes to the overall biological processes described above. To further separate the role(s) of the proteasome catalytic subunits, inhibition of each proteolytic activity is required.

Among currently available proteasome inhibitors, many lack the subunit specificity required to perform a careful analysis of proteasome subunit function. Moreover, most of these lactone- (Ostrowska et al., 1997), aldehyde- (Rock et al., 1994; Lee and Goldberg, 1998), and vinyl sulfone-based (Palmer et al., 1995; Bogoy et al., 1997) proteasome inhibitors have been shown to inhibit other intracellular proteases as well. In contrast, we have recently shown that α' , β' -epoxyketone-based peptidyl proteasome inhibitors are highly specific for the proteasome (Meng et al., 1999b; Sin et al., 1999). As revealed by the crystal structure of the yeast 20S proteasome complexed with epoxomicin, this unique specificity of peptidyl α' , β' -epoxyketones is a result of a six-membered morpholino ring formation between amino-terminal catalytic Thr-1 of the proteasome and the α' , β' -epoxyketone pharmacophore of epoxomicin (Groll et al., 2000). We reason that other proteases, which are common targets of aldehyde- and vinyl sulfone-based proteasome inhibitors, cannot form the same morpholino adduct with epoxomicin, since they do not possess an amino-terminal catalytic Thr-1 residue. This proteasome specificity of α' , β' -epoxyketone-based inhibitors is proving useful in the development of reagents to understand the role of the proteasome in biological processes.

To address the role of individual proteolytic subunits in the proteasome catalytic cycle, we have developed α' , β' -epoxyketone-based subunit-specific proteasome inhibitors. We report here the development of proteasome inhibitors that displayed varying degrees of selectivity for the PGPH activity. Moreover, these specific, cell-permeable proteasome inhibitors were used in biochemical and biological assays to show that individual proteasome catalytic activities function independently of one another.

Results

Development of PGPH-Specific Peptidyl α' , β' -Epoxyketone-Based Inhibitors

As a step toward differentiating functions of the proteasome catalytic subunits, we attempted to develop inhibitors selective for the proteasome activity that cleaves peptide bonds after acidic residues (i.e., the peptidyl-glutamyl peptide hydrolyzing or PGPH activity). Initial efforts involved the synthesis of several peptidyl α' , β' -epoxyketones with glutamic acid at the P1 position. By analogy to peptide substrates, such as Z-Leu-Leu-Glu-AMC, we reasoned that the presence of Glu at the P1 position of peptide inhibitors alone would suffice to render higher selectivity toward the PGPH activity. Interestingly, compounds, such as isooctanoyl-Ser/Thr-Glu-, Ac-Ile-Ile-Thr-Glu-, Ac-Ala-Val-Glu-, Ac-Ile-Val-

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Table 1. Potency of α',β' -Epoxyketones for Inhibition of the 20S Proteasome Activities

Compounds	$K_{\text{obs}}/[I] \text{ (M}^{-1}\text{s}^{-1})^{\text{a}}$		
	Chymotrypsin-like Activity	PGPH Activity	Trypsin-like Activity
(1) Ac-GPFL-EX YU102	5 ± 1 (50–150 μM) ^b	254 ± 57 (4–25 μM)	NI ^c (100–150 μM)
(2) Ac-NorLPFL-EX	1.9 ± 0.6 (100–150 μM)	115 ± 15 (10–40 μM)	NI (100–150 μM)
(3) Ac-PFL-EX	42 ± 2 (50–100 μM)	32 ± 6 (25–75 μM)	NI (100–150 μM)
(4) N-diethylaminobenzoyl-PFL-EX	220 ± 70 (1–2 μM)	150 ± 40 (10–40 μM)	NI (100–150 μM)
(5) Isooctanoic-PFL-EX	190 ± 60 (1–2 μM)	194 ± 4 (10–40 μM)	NI (100–150 μM)
(6) Benzoylbenzoic-PFL-EX	8.7 ± 0.2 (80–100 μM)	140 ± 30 (10–40 μM)	NI (100–150 μM)
(7) Pyrazinecarbonyl-PFL-EX	520 ± 40 (1–2 μM)	23 ± 3 (40–80 μM)	3.7 ± 1.1 (125–150 μM)
(8) Ac-GGPFL-EX	170 ± 60 (20–60 μM)	170 ± 30 (10–40 μM)	1.3 ± 0.3 (125–150 μM)
(9) Ac-PPFL-EX	6.4 ± 3.3 (20–60 μM)	10.6 ± 0.3 (40–80 μM)	NI (125–150 μM)
(10) N-acetyl-piperidinecarbonyl-PFL-EX	31.2 ± 0.6 (60–80 μM)	45.3 ± 2.2 (40–80 μM)	NI (125–150 μM)
(11) Ac-Ile-Val-Leu-EX	5 ± 0.1 (100–150 μM)	14.6 ± 4.2 (50–125 μM)	6.7 ± 0.1 (100–150 μM)
(12) Ac-Ala-Val-Leu-EX	10.8 ± 0.2 (100–150 μM)	12.7 ± 1.3 (50–100 μM)	1.8 ± 0.1 (125–150 μM)
(13) Eponemycin	114 ± 27 (8–10 μM)	217 ± 38 (8–50 μM)	17 ± 3 (100–150 μM)
(14) Z-GPFL-EX	34.7 ± 4.2 (20–50 μM)	236 ± 6 (1.5–15 μM)	NI (100–150 μM)
(15) Ac-hFLFL-EX YU101	150,000 ± 11,000 (5–15 nM)	3.9 ± 0.3 (80–150 μM)	17 ± 6 (60–100 μM)
(16) Epoxomicin	20,000 ± 3,000 (50–100 nM)	40 ± 10 (25–75 μM)	300 ± 100 (0.25–2.5 μM)

^a See Experimental Procedures for details.^b Values in parentheses indicate the range of inhibitor concentrations used.^c No inhibition was measured.

Glu-, Ac-Gly-Pro-Phe-Glu-, and Ac-Pro-Phe-Glu- α',β' -epoxyketones did not display any significant selectivity for the PGPH activity (data not shown).

Given these results, we next tested several isooctanoyl-Ser-X- α',β' -epoxyketones with various natural and unnatural amino acid residues (i.e., Leu, Ala, Val, Phe, Norleucine, cyclohexyl, and t-butyl) in place of Glu at the P1 position. Analysis of this series of compounds indicated that Leu provided the best PGPH selectivity (data not shown). This was not anticipated, since the catalytic subunits $\beta 1/\text{Pre-3}$ in yeast and Y in mammalian proteasome, known to be responsible for the PGPH activity, have also been found to be responsible for the activity that cleaves after hydrophobic branched-chain amino acids (Dick et al., 1998; McCormack et al., 1998; Cardozo et al., 1999).

Incorporation of leucine at the P1 position in an amino-protected peptidyl aldehyde proteasome inhibitor (i.e., Z-GPFL-CHO) has also been shown to inhibit competitively the PGPH activity with a ~ 13 -fold selectivity over the chymotrypsin-like activity (Vinitsky et al., 1994). Expanding on this finding, we synthesized several new α',β' -epoxyketone-based inhibitors in order to explore the importance of length, steric bulk, and amino-terminal protection on specific proteasomal subunit inhibitory activity.

Table 1 shows that α',β' -epoxyketone inhibitors with

Pro-Phe-Leu at the P3-P1 positions (1–10) displayed no significant, if any, inhibition of the trypsin-like activity, regardless of the nature of residues at the P4 position. These results suggest that the Pro-Phe-Leu tripeptide prevents these inhibitors from binding to the subunit responsible for the trypsin-like activity.

Secondly, the presence of a bulky aromatic protecting group (4 and 7) in place of an acetyl group (3) at the amino terminus provided a stronger inhibition (5- to 12-fold) toward the chymotrypsin-like activity, thus making them less PGPH selective. Similarly, compounds with larger groups at the P4 position (5 vs. 3 and 8 vs. 3) displayed a modest increase in inhibition of the chymotrypsin-like activity. These results are consistent with previous studies using aldehyde- (Iqbal et al., 1995), vinyl sulfone- (Bogyo et al., 1998), glyoxal- (Lynas et al., 1998), and α',β' -epoxyketone-based (Elofsson et al., 1999) proteasome peptidyl inhibitors. In those studies, inhibitors with longer peptide backbone displayed stronger inhibition toward the chymotrypsin-like activity compared to their truncated tripeptide counterparts. It should be noted, however, this trend is not strictly followed, as shown in 3 vs. 2 and 3 vs. 6, reflecting the difficulty of designing a subunit-specific inhibitor.

As anticipated, synthesis of Z-GPFL-EX (14) afforded an irreversible proteasome inhibitor displaying moderate selectivity (7-fold) for the PGPH activity over the

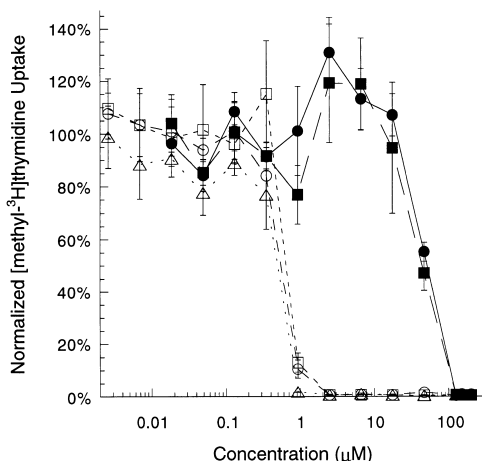


Figure 1. Inhibition of BAE Cell Proliferation by Peptidyl α',β' -Epoxyketones

Sparingly plated BAE cells were treated with increasing concentration of compounds 1 (filled circles), 2 (filled squares), 4 (open circles), 5 (open squares), and 7 (open triangles), respectively, and [methyl- ^3H]thymidine uptake was measured, as described in Experimental Procedures. The amount of [methyl- ^3H]thymidine uptake at each concentration of inhibitors is normalized to cells treated with DMSO. The lines are drawn to connect the data points. Data represent a mean of triplicate samples. Error bars indicate the standard deviation.

chymotrypsin-like activity (Table 1). However, based on our previous results indicating that large bulky protecting groups contribute to inhibition of the chymotrypsin-like activity, we synthesized several α',β' -epoxyketone peptides possessing a smaller amino-terminal group (i.e., acetyl) instead of a Z protecting group. The two best compounds from this series, 1 (YU102) and 2, showed the highest selectivity toward the PGPH activity with ~ 50 - and ~ 60 -fold higher values of $k_{\text{obs}}/[I]$ for inhibition of the PGPH activity than the chymotrypsin-like activity, respectively (Table 1).

The Chymotrypsin-like Activity Degrades Protein In Vivo Independently of the PGPH Activity

By exploiting the observation that proteasome inhibition results in apoptosis at elevated proteasome inhibitor concentrations (Imajoh-Ohmi et al., 1995; Shinohara et al., 1996; Orłowski et al., 1998; Adams et al., 1999), we assessed the importance of different catalytic activities of the proteasome in the degradation of proteins required for cell growth and survival. For this, we measured inhibition of bovine aortic endothelial (BAE) cell proliferation with α',β' -epoxyketone-based inhibitors possessing varying degrees of specificity for the PGPH and chymotrypsin-like activities. We reasoned that if inhibition of the PGPH activity inhibits the protein degradation, then inhibitors with comparable PGPH inhibitory activities would yield similar biological effects irrespective of their chymotrypsin-like inhibitory activities. Thus, four compounds (YU102, 2, 4, and 5) possessing similar values of $k_{\text{obs}}/[I]$ for the inhibition of the PGPH activity but different chymotrypsin-like inhibitory activities were tested for their ability to arrest DNA synthesis. One com-

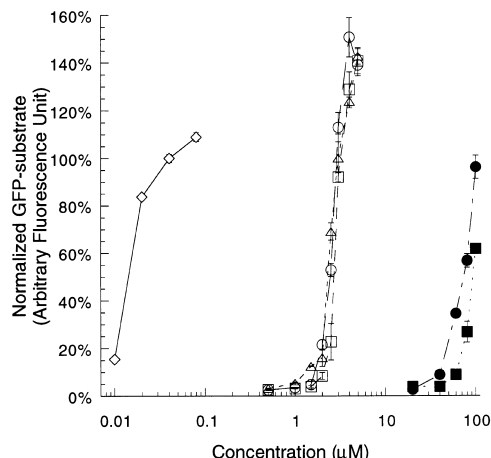


Figure 2. Inhibition of Degradation of Destabilized GFP in Living Cells

Subconfluent HeLa cells stably transfected with Ub^{G76V}-GFP were treated with increasing concentration of compounds 1 (filled circles), 2 (filled squares), 4 (open circles), 5 (open squares), 7 (open triangles), and epoxomicin (open diamonds), respectively. Accumulation of the proteasome substrate Ub^{G76V}-GFP was quantified by measuring the mean fluorescence intensity per cell by flow cytometry. The lines are drawn to connect the data points. Cells treated with 40 nM epoxomicin were included in each experiment and used for normalization of the accumulation of GFP-substrate. Data represent a mean of triplicate samples. Error bars indicate the standard deviation.

ound (7), with lower PGPH and higher chymotrypsin-like inhibitory activities was included as a control.

All compounds proved to be cytotoxic when incubated with BAE cells (Figure 1). However, two classes emerged from these studies: the more potent class (compounds 4, 5, and 7) inhibited DNA synthesis with IC_{50} values of ~ 0.5 μM , whereas the other class (YU102 and 2) required a 100-fold higher concentration to inhibit 50% of proliferation with IC_{50} values of ~ 50 μM . These results show that selective inhibition of the PGPH activity alone was not sufficient to inhibit intracellular proteasome-mediated protein degradation.

We exploited another cell-based assay that directly monitors proteasome function in living cells to confirm these results. It has recently been shown that the fluorescence of cells expressing the inherently unstable proteasome substrate ubiquitin^{G76V}-green fluorescent protein (Ub^{G76V}-GFP) significantly increases upon incubation with cell-permeable proteasome inhibitors (Dantuma et al., 2000). Using this convenient reporter system of intracellular proteasomal activity, different peptide epoxyketone inhibitors were assayed for their ability to inhibit proteasome activity in vivo. The six compounds tested for antiproliferative activity (YU102, 2, 4, 5, 7, and epoxomicin) were assayed using the short-lived GFP assay (Figure 2). Incubation of reporter cells with each compound resulted in the accumulation of intracellular fluorescence indicating that in vivo proteasome activity was inhibited. The most potent inhibitor was the natural product epoxomicin, which inhibited intracellular GFP degradation at low nanomolar concentrations. The other compounds, again, fell into two classes. Incubation of reporter cells with compounds possessing higher values

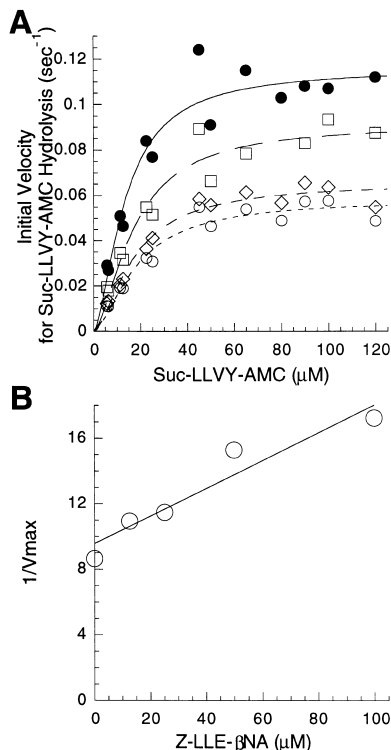


Figure 3. Allosteric Inhibition of the Chymotrypsin-like Activity by a PGPH Substrate

(A) Michaelis-Menten plot for Suc-LLVY-AMC hydrolysis. The initial velocity for Suc-LLVY-AMC hydrolysis was measured in the absence (filled circles) and in the presence of 12.5 μM (open squares), 50 μM (open diamonds), or 100 μM (open circles) Z-LLE- βNA . The lines through the data were drawn with $K_{1/2} = 13.4 \mu\text{M}$ and $V_{\text{max}} = 0.115 \text{ s}^{-1}$ (filled circles), $K_{1/2} = 17.1 \mu\text{M}$ and $V_{\text{max}} = 0.091 \text{ s}^{-1}$ (open squares), $K_{1/2} = 17.2 \mu\text{M}$ and $V_{\text{max}} = 0.065 \text{ s}^{-1}$ (open diamonds), and $K_{1/2} = 18.3 \mu\text{M}$ and $V_{\text{max}} = 0.058 \text{ s}^{-1}$ (open circles), respectively.

(B) Dixon plot for Z-LLE- βNA at saturating concentrations of Suc-LLVY-AMC. The values of V_{max} for Suc-LLVY-AMC hydrolysis were obtained in the absence and in the presence of 12.5, 25, 50, and 100 μM Z-LLE- βNA , respectively. The solid line is a nonlinear least-squares best fit to the data. If Suc-LLVY-AMC competed with Z-LLE- βNA for the same sites, the values of V_{max} at saturating concentration of Suc-LLVY-AMC would have remained constant.

of $k_{\text{obs}}/[I]$ for chymotrypsin-like inhibition (4, 5, and 7) accumulated intracellular Ub^{G76V}-GFP at concentrations ~ 50 -fold lower than those PGPH-specific compounds lacking significant chymotrypsin-like inhibitory activity (YU102 and 2).

A PGPH Substrate Allosterically Inhibits the Chymotrypsin-like Activity

Since the results of our *in vivo* studies deviate from the recently proposed model of proteasome allostery (Kisselev et al., 1999), we next explored possible allosteric regulation between different proteasome subunits in *in vitro* proteolytic assays. First, we reproduced the reported allosteric PGPH substrate-mediated downregulation of the chymotrypsin-like activity. The chymotrypsin-like activity of the 20S proteasome, measured by the Suc-LLVY-AMC hydrolysis, was compared in the presence of PGPH substrate, Z-LLE- βNA . As shown in Figure 3A, increasing concentration of Z-LLE- βNA de-

creased the initial rate of Suc-LLVY-AMC hydrolysis. This result shows that the presence of Z-LLE- βNA inhibits the chymotrypsin-like activity.

Several lines of evidence indicate, however, that this inhibition was not due to competitive binding of Z-LLE- βNA to the active sites that are responsible for the chymotrypsin-like activity. First, if competitive binding had occurred, the concentration of Suc-LLVY-AMC required to give half-maximal velocity ($K_{1/2}$) would have increased dramatically as the concentration of a competitive inhibitor, Z-LLE- βNA , increased. Values of $K_{1/2}$ for Suc-LLVY-AMC hydrolysis, however, showed no significant increase as the concentration of Z-LLE- βNA increased (Figure 3A). Second, this inhibition was not overcome by increasing substrate concentration, which strongly suggests that Z-LLE- βNA does not compete with Suc-LLVY-AMC for the same active sites (Figures 3A and 3B). This observation is consistent with previous results described by Goldberg and colleagues (Kisselev et al., 1999).

Selective Occupancy of the PGPH Subunits Is Not Sufficient for an Allosteric Inhibition of the Chymotrypsin-like Activity

Next, to test whether occupancy of the PGPH sites could be responsible for the allosteric downregulation of the CT-L activity, the most potent and selective PGPH inhibitor, YU102, was used to saturate the PGPH sites irreversibly. After testing various concentrations, 8 μM YU102 (open squares) were found to inhibit only the PGPH activity but not the chymotrypsin-like activity (Figures 4A and 4B). By comparison, the natural product dihydroeponemycin (open triangles) inhibited both the PGPH and the chymotrypsin-like activities at the same concentration (Figures 4A and 4B). Moreover, YU102 is a very poor inhibitor against the trypsin-like activity; even at concentrations of 100–150 μM , it displayed no significant inhibition of the trypsin-like activity (Table 1).

To specifically occupy the PGPH sites, the 20S proteasome was preincubated with 8 μM YU102 for ~ 2 min prior to addition of either the chymotrypsin-like substrate Suc-LLVY-AMC or the PGPH substrate Z-LLE-AMC. Greater than 55% of the PGPH activity was found to be inhibited during the initial preincubation period, followed by increasing inhibition up to above 90% under the assay conditions (Figure 5A, open squares). The possibility that YU102 could bind to sites other than the PGPH catalytic sites and yet still inhibit the PGPH activity is highly unlikely, since X-ray analysis of crystal structure of the 20S proteasome complexed with various peptide-based proteasome inhibitors, including epoxomicin possessing the same pharmacophore as YU102, has revealed that the peptide moiety of such inhibitors occupy the substrate binding sites and their pharmacophores modify the catalytic Thr-1 (Groll et al., 1997, 2000). Moreover, YU102 as well as epoxomicin showed a time-dependent inhibition, indicating the irreversible modification of the catalytic Thr-1 of the proteasome. Therefore, a greater than 90% inhibition of the PGPH activity under these conditions suggests that the PGPH subunits were at least 90% saturated irreversibly with YU102.

If occupancy of the PGPH sites were sufficient for the

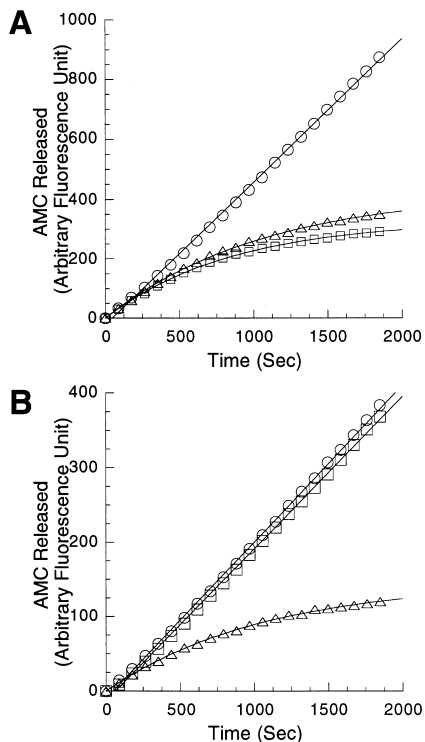


Figure 4. Kinetics for the Proteasome Activity in the Presence of DMSO, YU102, or Dihydropeponemycin

(A) Kinetics for the PGPH activity. DMSO (open circles), 8 μM dihydropeponemycin (open triangles), or 8 μM YU102 (open squares) was added to the assay buffer containing purified 20S proteasome and 10 μM Z-LLE-AMC after the steady state of hydrolysis was established. Subsequently, fluorescent AMC production was continuously monitored. The lines drawn through the data are nonlinear least-squares best fits to an equation, Fluorescence = $V_o t + \text{constant}$ with $V_o = 0.48 \text{ s}^{-1}$ (open circles) or to an equation, Fluorescence = $V_s t + [(V_o - V_s)/k_{\text{obs}}][1 - \exp(-k_{\text{obs}}t)]$ with $V_o = 0.4 \text{ s}^{-1}$, $V_s = 0.0001 \text{ s}^{-1}$, and $k_{\text{obs}} = 0.00094 \text{ s}^{-1}$ (open triangles) and $V_o = 0.39 \text{ s}^{-1}$, $V_s = 0.0001 \text{ s}^{-1}$, and $k_{\text{obs}} = 0.00118 \text{ s}^{-1}$ (open squares), respectively, where V_o and V_s are the initial and final velocities, respectively, and k_{obs} is the apparent rate constant for a time-dependent inhibition.

(B) Kinetics for the chymotrypsin-like activity. The rate of the Suc-LLVY-AMC hydrolysis was measured essentially identical as described above, except 10 μM Suc-LLVY-AMC was used. The lines drawn through the data are nonlinear least-squares best fits to an equation, Fluorescence = $V_o t + \text{constant}$ with $V_o = 0.209 \text{ s}^{-1}$ (open circles) or 0.204 s^{-1} (open squares), respectively, and to an equation, Fluorescence = $V_s t + [(V_o - V_s)/k_{\text{obs}}][1 - \exp(-k_{\text{obs}}t)]$ with $V_o = 0.14 \text{ s}^{-1}$, $V_s = 0.0001 \text{ s}^{-1}$, and $k_{\text{obs}} = 0.00095 \text{ s}^{-1}$ (open triangles).

allosteric inhibition of the chymotrypsin-like activity, a noticeable decrease in the rate of Suc-LLVY-AMC hydrolysis is expected under the same conditions as above. The chymotrypsin-like activity of the 20S proteasome, however, was not affected (Figure 5B, open squares). This result shows that near quantitative occupancy of the PGPH sites with YU102 did not trigger inhibition of the chymotrypsin-like activity.

PGPH Substrate Inhibits the Chymotrypsin-like Activity without Its Hydrolysis

We next tested whether the PGPH substrate has any effect on the chymotrypsin-like activity while its hydroly-

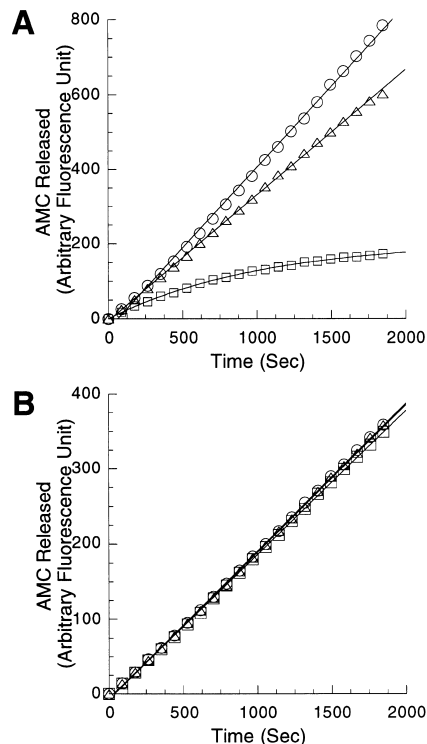


Figure 5. Selective Occupancy of the PGPH Sites with YU102

(A) Kinetics for the PGPH activity of the 20S proteasome pretreated with YU102. The 20S proteasome was preincubated with DMSO (open circles), 1 μM (open triangles), or 8 μM (open squares) YU102 for ~ 2 min prior to the addition of Z-LLE-AMC. Subsequently, the fluorescent AMC production was continuously monitored. The lines drawn through the data are nonlinear least-squares best fits to an equation, Fluorescence = $V_o t + \text{constant}$ with $V_o = 0.43 \text{ s}^{-1}$ (open circles) or 0.34 s^{-1} (open triangles), respectively, and an equation, Fluorescence = $V_s t + [(V_o - V_s)/k_{\text{obs}}][1 - \exp(-k_{\text{obs}}t)]$ with $V_o = 0.2 \text{ s}^{-1}$, $V_s = 0.0001 \text{ s}^{-1}$, and $k_{\text{obs}} = 0.00098 \text{ s}^{-1}$ (open squares).

(B) Occupancy of the PGPH sites has no effect on the chymotrypsin-like activity. The 20S proteasome was preincubated with DMSO (open circles), 1 μM (open triangles), or 8 μM (open squares) YU102 for ~ 2 min prior to the addition of Suc-LLVY-AMC. Subsequently, the fluorescent AMC production was continuously monitored. The lines drawn through the data are nonlinear least-squares best fits to an equation, Fluorescence = $V_o t + \text{constant}$ with $V_o = 0.197 \text{ s}^{-1}$ (open circles), 0.196 s^{-1} (open triangles), or 0.192 s^{-1} (open squares), respectively.

sis is inhibited by a chemical modification of the catalytic Thr-1 of the PGPH sites by YU102. First, 20S proteasomes were preincubated with the 8 μM YU102 for ~ 20 min prior to addition of both the CT-L substrate Suc-LLVY-AMC and the PGPH substrate Z-LLE- β NA together. During the initial 20 min preincubation period, more than 95% of the PGPH activity was found to be inhibited, indicating a quantitative chemical knockout of the PGPH sites with YU102. Surprisingly, Z-LLE- β NA inhibits the CT-L activity, although hydrolysis of this PGPH substrate was prevented (Figure 6A, open squares). Moreover, the extent of this inhibition was indistinguishable between the YU102-treated and unmodified proteasomes (Figure 6A, open circles).

To confirm the previous result and exclude the possibility that the PGPH substrate could have been hy-

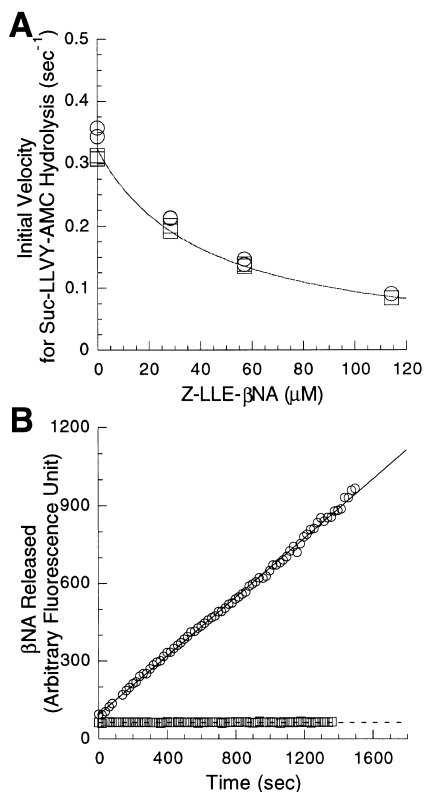


Figure 6. A PGPH Substrate Still Downregulates the CT-L Activity Even without Its Hydrolysis

(A) Allosteric Inhibition of the CT-L activity by a PGPH substrate with or without pretreatments with YU102. The 20S proteasome was preincubated with DMSO (open circles) or 8 μ M YU102 (open squares) for \sim 20 min prior to the addition of 25 μ M Suc-LLVY-AMC and increasing concentrations of Z-LLE- β NA. Subsequently, inhibition of the CT-L activity was measured by continuous monitoring of the fluorescent AMC production. The line drawn through the data is a nonlinear least-squares best fit to an equation describing a typical reversible competitive inhibition.

(B) Z-LLE- β NA is not hydrolyzed when the PGPH site is blocked with YU102. The 20S proteasome was preincubated with DMSO (open circles) or 8 μ M YU102 (open squares) for \sim 20 min prior to the addition of Z-LLE- β NA. Subsequently, the fluorescent β NA production was continuously monitored. The lines drawn through the data are nonlinear least-squares best fits to an equation, Fluorescence = $V_o t + \text{constant}$ with $V_o = 0.567 \text{ s}^{-1}$ (open circles) or 0.001 s^{-1} (open squares), respectively.

drolyzed by an unknown catalytic site(s) that cannot be chemically knocked out by YU102, the hydrolysis of Z-LLE- β NA was measured after the 20S proteasome was preincubated with the 8 μ M YU102 for \sim 20 min. As expected, the modified proteasome does not hydrolyze Z-LLE- β NA (Figure 6B, open squares). Taken together, our results show that the hydrolysis of the PGPH substrate is not required for the downregulation of the CT-L activity.

Discussion

Although it is well established that the proteasome possesses six catalytic subunits having three different substrate cleavage specificities (Dick et al., 1991; Ma et al., 1992; Mykles, 1996), it is not clear how each proteolytic

activity contributes to intracellular protein degradation. Adding a new dimension to any simple mechanistic model of proteasome-mediated proteolysis are reports of allosteric interactions between individual proteasome catalytic subunits. In addition to evidence suggesting a positive cooperativity between subunits responsible for the hydrolysis of a given substrate, for example, between the two chymotrypsin-like sites (Chu-Ping et al., 1992; Kuckelkorn et al., 1995) or between the two PGPH sites (Arribas and Castano, 1990; Orłowski et al., 1991; Djaballah and Rivett, 1992), mutual allosteric regulation between catalytic subunits possessing different substrate specificities has also been recently recognized (Kisselev et al., 1999). Ingenious use of two proteasome substrates with different fluorogenic leaving groups allowed the measurement of the CT-L (chymotrypsin-like) and PGPH activities in the presence of both substrates. As a result, this approach revealed provocative mutual allosteric regulations between these two sites.

Based on these findings, Goldberg and colleagues proposed an ordered mechanism for protein degradation in the proteasome (Kisselev et al., 1999): the chymotrypsin-like sites first cleave (or “bite”) an incoming protein substrate, followed by a gross conformational change of the proteasome resulting in activation of the PGPH activity. The PGPH sites then hydrolyze (or “chew”) the substrate previously cleaved (or “bitten”) by the chymotrypsin-like sites, while simultaneously inhibiting the chymotrypsin-like activity (or additional “biting”). Protein degradation continues until the protein substrate is hydrolyzed to small peptides, which eventually diffuse from the catalytic chamber. While this model elegantly explains the observed allosteric regulations between the CT-L and the PGPH sites observed *in vitro*, it is not clear whether the proposed interplay or interdependence of both sites is essential for proteasome-mediated protein degradation. Furthermore, the structural and the biochemical bases for these allosteric interactions also remain to be determined. Hence, we investigated the biochemical nature of the allosteric interactions between different subunits and their possible role in intracellular protein degradation, using newly developed proteasome subunit-specific inhibitors.

Development of PGPH-Specific Inhibitors Allows a “Chemical Knockout” of the PGPH Sites

To investigate the contribution of the PGPH sites to proteasome-mediated proteolysis, we first synthesized a series of proteasome-specific α',β' -epoxyketone-based inhibitors, with varying degrees of PGPH selectivity (Table 1). From these series, two compounds, 1 (YU102) and 2, were shown to inhibit selectively the PGPH activity with \sim 50- and \sim 60-fold higher values of $k_{obs}/[I]$ for inhibition of the PGPH activity than the chymotrypsin-like activity, respectively. These two compounds are the first series of PGPH-specific inhibitors that selectively target only the proteasome but not other cellular proteases due to the presence of the proteasome-specific α',β' -epoxyketone pharmacophore. Moreover, both PGPH-specific inhibitors displayed a time-dependent inhibition, indicative of covalent and irreversible modification of the PGPH sites, which is equivalent to the genetic knockout mutant of the PGPH sites. Given its selectivity, YU102 was used to investi-

gate the role of the PGPH site of the proteasome in mediating *in vitro* and *in vivo* proteolysis.

PGPH-Specific Inhibitors Reveal Proteasome Catalytic Site Independence

One could anticipate that if the catalytic sites responsible for the hydrolysis of different substrates are interdependent and such allosteric interactions are vital to the overall protein degradation, then proteasome-mediated protein degradation could be inhibited by blocking only one proteolytic activity (e.g., the PGPH activity). However, if two sites function independently (or their interactions contribute to the overall protein degradation in only a minor manner), one would not expect to observe an inhibition of the protein degradation by just inhibiting one proteolytic activity.

To investigate whether both catalytic sites could act independently from each other in mediating protein degradation in living cells, we used two cell-based assays to probe proteasome-mediated protein degradation. In the first series of experiments (Figure 1), two classes of compounds emerged, with significantly different antiproliferative IC_{50} values, irrespective of their comparable PGPH inhibitory activities. We anticipated that if selective inhibition of the PGPH activity inhibits the turnover of intracellular proteasome activity, all compounds with similar PGPH inhibitory activity (YU102, 2, 4, and 5) would have yielded similar IC_{50} values for antiproliferation. The greater than 100-fold difference in IC_{50} values of these compounds, therefore, suggests that inhibition of the PGPH activity is not sufficient to inhibit the turnover of intracellular proteasomal activity, as reflected by the lack of proliferation inhibition in BAE cells. These results show that the PGPH activity is not required for *in vivo* protein degradation. This is consistent with previous work showing that cytotoxicity associated with proteasome inhibition correlates with inhibition of the CT-L activity (Elofsson et al., 1999). Moreover, using the short-lived GFP reporter system (Figure 2) to monitor directly intracellular proteasome activity, an essentially identical trend was obtained as in the antiproliferative assays. Taken together, these results support the conclusion that the CT-L activity degrades proteins independently of the PGPH activity and the PGPH activity is dispensable in proteasome-mediated protein degradation.

The conclusion drawn here using the mammalian proteasome is consistent with several elegant yeast genetic studies that investigated potential proteasome catalytic subunit interplay. Here, we demonstrated that the PGPH and chymotrypsin-like activities of the mammalian proteasome function independently. Similarly, in several yeast studies, mutation of the catalytic amino-terminal Thr-1 in the yeast proteasome $\beta 2$ /Pup1 and $\beta 1$ /Pre3 subunits resulted in the loss of the trypsin-like and the PGPH activities with no significant effect on cell growth and viability (Arendt and Hochstrasser, 1997, 1999; Heinemeyer et al., 1997).

Neither Active Site Occupancy nor Continuous Hydrolysis of the PGPH Substrate Is Required for *In Vitro* Chymotrypsin-like Activity Inhibition

While we were able to demonstrate that the PGPH and the chymotrypsin-like active sites function indepen-

dently in mediating protein degradation *in vivo*, the question remains whether these results are inconsistent with a model of proteasome allosteric interactions. To investigate the nature of possible allostery between the CT-L and the PGPH proteasome subunits, we first confirmed the original observation (Kisselev et al., 1999) that a model PGPH substrate inhibits the *in vitro* chymotrypsin-like activity of the proteasome (Figure 3A). Although the trivial possibility that the chymotrypsin-like and the PGPH substrates compete for the same sites has been excluded (Figure 3B and Kisselev et al., 1999), the mechanism of this allosteric interaction remained unknown. Therefore, we asked the next obvious questions: Does this allosteric inhibition require the occupancy of the PGPH active site or require the hydrolysis of the PGPH substrate?

Several lines of evidence suggest that PGPH-specific inhibitor, YU102, binds to the PGPH active sites in the same manner as the PGPH substrates. First, our previous structural studies with a similar peptide epoxyketone inhibitor complexed with the 20S proteasome (Groll et al., 1997, 2000) showed that the pharmacophore α',β' -epoxyketone covalently modifies the catalytic Thr-1 that is normally used in peptide hydrolysis by the proteasome. Moreover, the crystal structure revealed that the peptide moiety snugly occupies the substrate binding sites located at the active site. In addition, we show here that YU102 specifically inhibits the PGPH activity in *in vitro* assays, indicating that it binds preferentially to the PGPH catalytic sites. Therefore, as a suicide or an active site-directed irreversible inhibitor, YU102 was used to determine whether peptide binding (occupancy) of the PGPH catalytic subunit would be responsible for the inhibition of the chymotrypsin-like activity that is observed in the presence of the PGPH substrate.

As shown in Figure 5, near quantitative occupancy at the PGPH sites by YU102 did not result in the allosteric inhibition of the CT-L activity. This lack of any significant inhibition of the CT-L activity upon saturation of the PGPH catalytic subunits indicates that the allosteric inhibition of the CT-L sites requires more than peptide binding at the PGPH active sites alone.

It should be noted that X-ray structural analysis of the yeast 20S proteasome (Groll et al., 1997) and studies of the human 20S proteasome by means of immunoelectron microscopy and chemical cross-linking (Kopp et al., 1997) have revealed that subunits responsible for the chymotrypsin-like and the PGPH activities are separated by ≥ 18 Å and are not in contact with each other. This suggests that long-range interactions required for any cooperativity between these sites would need to be "propagated" through other catalytic as well as structural subunits, as previously suggested (Kisselev et al., 1999). Although the nature of this type of long-range interaction is not clear, it does not seem to be feasible to "lock" the inhibitory state of the chymotrypsin-like subunits by occupying the PGPH subunits with the PGPH site-specific inhibitors. In addition, comparison of the 20S proteasome and of inhibitor-bound proteasome crystal structures revealed no significant structural change that could influence the activity of other sites (Groll et al., 1997).

We next investigated whether the PGPH substrate would continue to downregulate the chymotrypsin-like activity when its access or binding to the corresponding

catalytic substrate binding sites is prevented. Upon preincubation of 20S proteasome with YU102, we surprisingly found that the PGPH substrate still inhibited the chymotrypsin-like activity (Figure 6A). Further investigation revealed that under the same conditions, the PGPH substrate is not hydrolyzed, while inhibiting the chymotrypsin-like activity (Figure 6B). Therefore, given that YU102 did not alter the ability of the PGPH substrate to inhibit allosterically the chymotrypsin-like activity, one can conclude that the PGPH substrate does not require binding to or hydrolysis by the PGPH active site in order to produce this inhibition.

These results suggest that the PGPH substrate binds to an as of yet uncharacterized noncatalytic site(s) that regulates the CT-L catalytic sites. This is consistent with the recently proposed kinetic "two-site modifier" model that assumes that a substrate (or effector) may bind to an active site as well as to a second noncatalytic modifier site (Schmidtke et al., 2000). Definitive proof for this noncatalytic allosteric site(s), however, awaits its identification and mapping within the proteasome. We are currently developing a series of inhibitors that can bind to this putative regulatory site(s). These reagents will hopefully help determine whether these allosteric regulations contribute to protein degradation in vivo.

Refinement of the Proteasome Allosteric Model

Taken together, our new findings, therefore, support a refinement of the proposed "bite and chew" allosteric model (Kisselev et al., 1999). Whereas the original model proposed interdependent allosteric regulation of proteasome active sites responsible for the different proteolytic activities, we demonstrate in in vitro and in vivo assays that at least two active sites function independently of one another. Moreover, using a PGPH-specific suicide inhibitor, we were able to show that the originally observed allosteric interaction between the PGPH and the chymotrypsin-like sites can be explained by the presence of a noncatalytic site(s). However, it remains to be seen whether this newly discovered allosteric regulation between the catalytic subunits and noncatalytic site(s) plays an important role in in vivo protein degradation catalyzed by the proteasome.

Experimental Procedures

Materials

20S proteasome was purified from bovine reticulocyte lysates by batch DE-52 binding, DEAE Sephacel chromatography, gel filtration on Sephacryl S-300, and chromatography on hydroxyapatite. This procedure is as previously described (McGuire and DeMartino, 1986; McGuire et al., 1989) with the exception that bovine reticulocytes were used as a starting source.

Synthesis of Peptidyl α' , β' -Epoxyketones

All inhibitors were synthesized as previously described (Kim et al., 1999; Sin et al., 1998, 1999) and characterized by 500 MHz ^1H NMR spectroscopy and FAB-MS.

Kinetic Analysis of Proteasome Activity

Values of k_{obs} for inactivation of the proteasome were determined as previously described (Meng et al., 1999a): peptide-AMC (10 μM Suc-LLVY-AMC, 10 μM Z-LLE-AMC, or 20 μM Boc-LRR-AMC) and 20S proteasome were added to assay buffer (20 mM Tris/HCl [pH 8], and 0.5 mM EDTA). For Suc-LLVY-AMC and Z-LLE-AMC assays, 0.035% (w/v) SDS was added to the assay buffer. After the steady

state of hydrolysis for each substrate was established, an inhibitor was added to the assay buffer containing substrate and enzyme in a Dynex™ 96-well plate at room temperature. Release of fluorescent 7-amino-4-methylcoumarin (AMC) was measured using a Cytofluor spectrofluorometer with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Values of k_{obs} were obtained using Kaleidograph by a nonlinear least-squares fit of the data to the following equation: Fluorescence = $V_s t + [(V_o - V_s)/k_{\text{obs}}][1 - \exp(-k_{\text{obs}}t)]$, where V_o and V_s are the initial and final velocities, respectively, and k_{obs} is the apparent rate constant for a time-dependent inhibition (Morrison and Walsh, 1988). To measure Z-LLE- β NA cleavage, release of fluorescent β -naphthylamide (β NA) was measured with an excitation wavelength of 342 nm and an emission wavelength of 425 nm.

Cell Proliferation Assays

Bovine aortic endothelial (BAE) cells were cultured in Dulbecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in 7% CO_2 . On day 1, ~1000 cells were plated into each well of a Becton-Dickinson Microtest 96-well plate. After an overnight incubation, BAE cells were treated with different concentrations of proteasome inhibitors for 48 hr. Subsequently, 74 kBq of [methyl- ^3H]thymidine was added to each well, followed by an additional 4 hr incubation. Cells were harvested using a Skatron Cell Harvester, and radioactivity was quantified using liquid scintillation.

In Vivo Inhibition Assays for Degradation of Green Fluorescent Protein-Based Protein Substrates

Subconfluent HeLa cells stably expressing Ub^{G76V}-GFP reporter were incubated in a 24-well plate for 15 hr in the presence of inhibitor as described elsewhere (Dantuma et al., 2000). Cells were harvested by trypsinization and analyzed with a flow cytometer. The fluorescence intensity was quantified as mean fluorescence intensity per cell.

Acknowledgments

This work was supported by the National Institute of Health (GM62120) (C. M. C.) and by an Anderson Postdoctoral Fellowship (J. M.). K. L. is supported by grants awarded by the Swedish Cancer Society, the Swedish Foundation of Strategy Research, and the Hedlund Foundation (Stockholm, Sweden). N. P. D. is supported by a postdoctoral fellowship awarded by the European Commission Training and Mobility Program (ERBFMRXCT960026).

Received June 5, 2000; revised December 12, 2000.

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