Specificities of Cell Permeant Peptidyl Inhibitors for the Proteinase Activities of μ-Calpain and the 20 S Proteasome*

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Cell-permeant peptidyl aldehydes and diazomethylketones are frequently utilized as inhibitors of regulatory intracellular proteases. In the present study the specificities of several peptidyl inhibitors for purified human μ-calpain and 20 S proteasome were investigated. Acetyl-LLnL aldehyde, acetyl-LLM aldehyde, carbobenzyloxy-LLnV aldehyde (ZLLnVal), and carbobenzyloxy-LLY-diazomethyl ketone produced half-maximum inhibition of the caseinolytic activity of μ-calpain at concentrations of 1–5 × 10^{-7} M. In contrast, only ZLLnVal was a reasonably potent inhibitor of the caseinolytic activity of 20 S proteasome, producing 50% inhibition at 10^{-5} M. The other inhibitors were at least 10-fold less potent, producing substantial inhibition only at near saturating concentrations in the assay buffer. Further studies with ZLLnVal demonstrated that its inhibition of the proteasome was independent of casein concentration over a 25-fold range. Proteolysis of calpastatin or lysozyme by the proteasome was half-maximally inhibited by 4 and 22 μM ZLLnVal, respectively. Thus, while other studies have shown that ZLLnVal is a potent inhibitor of the hydrophobic peptidase activity of the proteasome, it appears to be a much weaker inhibitor of its proteinase activity. The ability of the cell permeant peptidyl inhibitors to inhibit growth of the yeast *Saccha ce ce e ae* was studied because this organism expresses proteasome but not calpains. Concentrations of ZLLnVal as high as 200 μM had no detectable effect on growth rates of overnight cultures. However, yeast cell lysates prepared from these cultures contained 2 μM ZLLnVal, an amount which should have been sufficient to fully inhibit hydrophobic peptidase activity of yeast proteasome. Degradation of ubiquitinylated proteins in yeast extracts by endogenous proteasome was likewise sensitive only to high concentrations of ZLLnVal. The higher sensitivity of the proteinase activity of calpains to inhibition by the cell permeant inhibitors suggests that calpain-like activities may be targets of these inhibitors in animal cells.

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diluted 10-fold to a final concentration of 20 nM in substrate solution ± inhibitor for further incubation at 37 °C. The duration of the assay was 30–60 min, and ^{14}C-methylated casein substrate was present at 0.5 mg/ml, except where noted. The generation of radiolabeled trichloroacetic acid-soluble casein peptides was monitored (20).

In some experiments, human erythrocyte calpastatin or chicken egg lysozyme was used as the proteasome substrate, utilizing assay conditions similar to those described above. Degradation of the native protein bands was assessed by densitometric scanning of Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis gels.

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Calpains and 20 S proteasome activity was measured in the same buffer system (50 mM HEPES, 0.5 mM EDTA, pH 7.8) at 37 °C. Both proteases were preincubated with lactacystin for 5 min before initiation of the assay (by addition of Ca^{2+} for calpain or casein for the proteasome).

strain RFY 231 was obtained from Dr. Robert Trumbly (Dept. of Biochemistry and Molecular Biology, Medical College of Ohio). Yeasts were cultured at ambient temperatures (23–26 °C) in YPD medium for a minimum of 4 h to overnight (16–18 h) in the presence of the various protease inhibitors or an equivalent volume of the solvent, dimethyl sulfoxide, for control cultures. Because the protease inhibitors were sometimes used at almost saturating concentrations, culture medium containing inhibitors was precleared of potential insoluble inhibitors by centrifugation for several minutes at top speed in a clinical centrifuge before inoculation with yeast. Cultures were seeded with 0.02–0.1 $_{600}$ units of yeast from a fresh culture, and the final $_{600}$

since it spontaneously hydrolyzes to form the active inhibitory component, lactacystin β-lactone, and the latter achieves peak concentrations well below the initial concentration of lactacystin in the assay buffer (28). Unlike the peptidyl aldehyde inhibitors used in this study, and in agreement with previous results (27), addition of lactacystin to the assay buffer did not significantly inhibit calpain activity (Fig. 3).

—To investigate the possibility that protein substrate concentration plays a role in the decreased sensitivity of proteasome to the inhibitors, as previously suggested (6), titrations with ZLLnVal were carried out over a 25-fold range of casein concentration. All assays were conducted under conditions which ensured initial rate measurements, and casein concentrations both above and below the (80 μg casein/ml, see Ref. 20) were used. There was no significant influence on the potency of this inhibitor at any of the casein concentrations tested (Fig. 4).

To ensure that the low potency of ZLLnVal was not a phenomenon specifically associated with utilization of casein as a protein substrate, its inhibitory effect was investigated using lysozyme or calpastatin as substrates for the 20 S proteasome (Fig. 5). Half-maximum inhibition of proteolysis was evident at 22 and 4 μM ZLLnVal using lysozyme and calpastatin, respectively, as substrates. Thus, ZLLnVal is a relatively weak inhibitor of proteasome-catalyzed cleavage of at least three different protein substrates.

The budding yeast do not contain calpains but express the proteasome and other components of the ATP and ubiquitin-dependent proteolytic system (29). They therefore represent a natural model system for investigating the roles of the inhibitors in blocking proteasome activity without potential influences on K

proteasome, the assay for ZLLnVal in yeast lysate was repeated using purified 20 S proteasome as described under “Experimental Procedures.” By proteasome assay, the content of this inhibitor in the yeast lysate was 1.92 ± 0.56 S.D.

of the inhibitors on the proteinase activities of purified μ-calpain and 20 S proteasome have been studied concurrently. The results demonstrated that μ-calpain is much more sensitive than the proteasome to inhibition by the hydrophobic peptidyl aldehydes and ZLLY-DMK. However, the selectivity of another proteasome inhibitor, lactacystin, was confirmed in the present investigation. It was capable of inhibiting the caseinolytic activity of the 20 S proteasome at micromolar concentrations while producing no apparent inhibition of calpain. Insensitivity of the proteasome to the peptidyl inhibitors was not uniquely associated with use of the usual assay substrate, casein, since half-maximum inhibition of the proteasome by ZLLnVal required greater than micromolar concentrations using lysozyme or calpastatin as substrates (Fig. 5). Moreover, high concentrations of ZLLnVal appeared to be required to inhibit degradation of ubiquitinylated proteins by endogenous proteasome in yeast lysates (Fig. 6).

To test the idea that the proteinase activity of the proteasome is relatively insensitive to cell-permeant peptidyl protease inhibitors, the influence of the latter on yeast cell growth was studied. While these experiments were in progress, it was reported that wild-type [illegible] are resistant to the effects of ZLLnVal and CI-1 on protein degradation and cell proliferation (30). This appeared to be related to an inability to penetrate into the yeast cells since a mutant yeast strain with altered permeability characteristics was affected by ZLLnVal. However, no attempt was made in these latter studies to estimate the uptake of inhibitors into yeast. Results from the present study indicate that greater than micromolar concentrations of ZLLnVal or ZLLY-DMK accumulate in yeast exposed to 200 μM inhibitor overnight. There are, of course, caveats regarding extrapolation of inhibitor concentrations in lysates relative to intracellular cytosolic concentrations. The potential problem of compartmentalization in cells cannot be addressed, and it is conceivable that a fraction of the inhibitor in lysates is derived from a noncytosolic pool. However, it should be noted that the potential for extraction from membranes was minimized in the present studies by use of a hypotonic lysis buffer lacking detergents. Furthermore, the cells were homogenized in a minimum volume of lysis buffer to minimize redistribution to the soluble phase by dilution. It seems unlikely that a large fraction of ZLLnVal or ZLLY-DMK in lysates was derived from extracellular bound inhibitor; exposure of yeast to 200 μM leupeptin, a structurally related inhibitor that does not permeate cell membranes well, did not result in its detectable accumulation in lysates. Although the precise intracellular distribution of the inhibitors is problematic, it seems likely that sufficient ZLLnVal would have accumulated to inhibit the hydrophobic peptidohydrolase activity of the yeast proteasome since this inhibitor has been reported to half-maximally inhibit 20 S proteasome at 21 nM and 26 S proteasome at 35 nM (6).

The observations reported herein have significant bearing on interpretation of the effects of the hydrophobic peptidyl protease inhibitors, especially in animal cells where calpains and proteasome coexist. Among the possibilities that must be considered is that calpain-like proteases are the direct targets for these inhibitors in animal cells. The influence of CI-1, ZLLnVal, and related inhibitors on proteasome function where apparent might be downstream from their direct inhibition of calpains, suggesting that members of these two protease families may share common signal-transduction pathways. A less likely but nonetheless intriguing possibility is that animal cells possess unique intracellular protein substrates for the proteasome containing regions that mimic the substrate properties of the small hydrophobic peptides. Proteolysis of these proteins should be specifically inhibited by low concentrations of the cell-permeant inhibitors, aiding in their identification and isolation. Lactacy3(therhe)-m

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