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

Ronald L. Mellgren

From the Department of Pharmacology and Therapeutics, Medical College of Ohio, Toledo, Ohio 43614-5804

Cell-permeant peptidyl aldehydes and diazomethyl-ketones 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 $1–5 \times 10^{-7}$ 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 *Saccharomyces cerevisiae* 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.

Over the last several years, it has become increasingly apparent that regulated intracellular proteolysis plays a key role in diverse cell functions including signal transduction (1, 2), cell cycle progression (3), and apoptosis (4). Cell-permeant peptidyl protease inhibitors have been extensively employed in attempts to identify which intracellular proteases participate in these cell physiologic and pathologic functions. In some cases, the results have been readily interpretable. For example, the development of selective inhibitors of the interleukin-1β converting enzyme-like proteases have helped to established the central importance of these enzymes in apoptosis (5). On the other hand, interpretation of results obtained with other inhibitors is less clear cut. Most notably, CI-1,[1] which was developed as an inhibitor of the Ca^{2+}-dependent cysteine proteases calpains, has been shown to also inhibit the hydrophobic peptidyl hydrolase activity of the proteasome (6), the proteolytic component of the ATP-ubiquitin-dependent proteolytic system. CI-1 and related peptidyl inhibitors have recently been used by different laboratories as evidence for participation of calpains (7–10) or the proteasome (11–19) in a variety of intracellular proteolytic events. As a case in point, Mellgren and coworkers (9) recently reported that CI-1 and ZLLY-DMK inhibit progression of WI-38 fibroblasts through G_1 phase, and this appears to be the result of inhibition of p53 degradation in late G_1. The results of this investigation indicated that a calpain-like protease was involved in triggering p53 degradation. On the other hand, a concurrent, independent study utilizing FH109 fibroblasts concluded that p53 degradation in late G_1 was catalyzed by the proteasome, based largely on its inhibition by CI-1 (11).

The claimed specificity of hydrophobic peptidyl inhibitors like CI-1 for the proteasome is largely based on their potency in inhibiting hydrolysis of small hydrophobic fluorogenic peptides (6). The present investigation was conducted to determine the relative potencies of the peptidyl inhibitors for the proteinase activities of purified human μ-calpain and 20 S proteasome. Since the relevant intracellular substrates for these enzymes are proteins, this seemed like a reasonable basis for comparison.

EXPERIMENTAL PROCEDURES

Materials—CI-1 and CI-2 were obtained from Boehringer Mannheim. ZLLY-DMK was a kind gift from Dr. Elliott Shaw, Friedrich Miescher Institut, Basel. ZLLnVal and leupeptin were obtained from Sigma. Lactacystin was generously provided by Dr. Satoshi Omura, Kitasato Institute, Tokyo. The 20 S proteasome (20), μ-calpain (21), and calpastatin (22) were purified from human red blood cells to near homogeneity as described previously.

Calpain Inhibition Assay—Calpain activity was measured by release of trichloroacetic acid-soluble peptides from ^{14}C-methylated casein as described previously (23). Calpain, radiolabeled casein, and protease inhibitors were preincubated together at 25 °C for 1–5 min, and the reaction was started by addition of $CaCl_2$. Calpain was present at 10 nM; $CaCl_2$ was added to a final concentration of 2 mM, and radiolabeled casein concentration was 0.5 mg/ml. Duration of the assay was 10 min at which time trichloroacetic acid was added to the reaction mixture, and the soluble radioactive casein peptides were processed for counting as described (23).

Proteasome Inhibition Assay—Inhibition of the purified 20 S proteasome was assessed by including peptidyl inhibitors in the previously described caseinolytic assay (20). Proteasome was first activated by incubation with 0.2 mg protamine/ml for 2 min at 37 °C. It was then

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[1] The abbreviations used are: CI-1, acetyl-Leu-Leu-nLeu aldehyde; CI-2, acetyl-Leu-Leu-Met aldehyde; ZLLY-DMK, benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone; ZLLnVal, benzyloxycarbonyl-Leu-Leu-nVal aldehyde.

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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.

Inhibition of Calpain and the 20 S Proteasome by Lactacystin—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).

Yeast Culture—*Saccharomyces cerevisiae* 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 A_{600} units of yeast from a fresh culture, and the final A_{600} of the cultures varied from 0.4–10 depending upon the inoculum and time in culture.

Preparation of Yeast Lysates—Overnight cultures of yeast (5 ml) were centrifuged in a clinical centrifuge at top speed through a 3-ml 0.25 M sucrose cushion for 2 min at ambient temperature. Culture medium was aspirated along with approximately 0.5 ml of the sucrose layer. The walls of the centrifuge tube were then washed with 5 ml of 50 mM imidazole-HCl, pH 7.4, utilizing a Pasteur pipette and taking care not to disturb the sucrose layer. The wash was aspirated along with a small amount of sucrose layer. This wash procedure was repeated twice more. Finally the sucrose layer was removed, and the tube was inverted for drainage over Kimwipe® laboratory wipers. After several minutes, liquid remaining on the sides of the tubes was removed by inserting a Kimwipe®, and the yeast pellets were placed on ice. Pellets were suspended in approximately 4 pellet volumes (0.32 ml) of yeast lysis buffer (5 mM EGTA, 10 μM pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, pH 7.0), and homogenized in a chilled, frosted glass homogenizer for 20 ascents. The homogenates were centrifuged for 1 min at 10,000 × g, and the supernatants saved for inhibitor analysis.

Determination of Peptidyl Protease Inhibitor Concentrations in Culture Medium and Yeast Lysates—Concentrations of inhibitor in medium or lysates was usually assessed by assay as described above for μ-calpain. Generally 5 μl of lysate or 0.5 μl of medium was included in the routine calpain assay. Controls comprised lysates or medium from cultures carried out in the absence of inhibitors, but containing an equivalent volume of the solvent used for inhibitor stock solutions, dimethyl sulfoxide. The percent inhibition by inhibitor was taken as the difference between inhibitor-containing and control assays, the result being divided by the control assay value and multiplied by 100. The concentration of inhibitor was then estimated by reference to a standard curve generated with pure inhibitors (see Figs. 1 and 2). In other controls, inhibitors were incubated in medium without yeast, and subjected to the entire isolation and lysis protocol described above. At concentrations of inhibitor used in the experiments reported in this communication, these controls did not produce inhibition of calpain activity within the limits of detection of the standard assay (at least 10% inhibition).

In some experiments, ZLLnVal concentration in lysates was assayed by inhibition of caseinolytic activity of the proteasome. Since the latter is relatively insensitive to inhibition by ZLLnVal, it was necessary to add a larger volume of lysate. Yeast lysate was heated to 90 °C for 5 min to precipitate proteins and inactivate yeast proteases and then centrifuged to produce a supernatant. 20 μl of the supernatant was included in the standard proteasome assay mixture (total volume, 50 μl), and the volumes of the other assay component stock solutions were adjusted to ensure that their final concentrations in the assay would be the same as for the routine method.

Inhibition of Proteolysis of Polyubiquitinated Proteins in Yeast Lysates by ZLLnVal—Yeast lysates were prepared from overnight cultures as described above. A Tris-HCl buffer was added to 50 mM to adjust the pH to 8.0, and iodoacetamide was added to 10 mM to inhibit polyubiquitin isopeptidase T activity (24). Various concentrations of
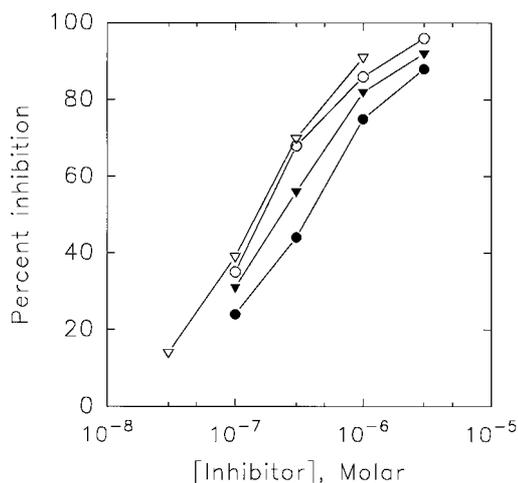

FIG. 1. **Inhibition of the proteinase activity of μ-calpain by hydrophobic peptidyl protease inhibitors.** Purified human erythrocyte calpain was assayed in the presence of various concentrations of inhibitors as described under "Experimental Procedures." ○, CI-1; ●, CI-2; ▽, ZLLY-DMK; ▼, ZLLnVal.

ZLLnVal were added to aliquots of the lysate, and proteolysis was initiated by adding 15 mM $MgCl_2$ and 10 mM ATP and placing the samples in a 37 °C heat block. In one experiment, an ATP regenerating system comprised of ATP, phosphocreatine, and creatine phosphokinase (25) was added without qualitatively altering the outcome of the experiment. After 120 min of incubation, the samples were heated to 100 °C in SDS-polyacrylamide gel electrophoresis sample buffer, electrophoresed in a 12% polyacrylamide gel, transferred to nitrocellulose, and immunostained utilizing a polyclonal antibody against bovine ubiquitin (Sigma) at 0.25 mg/ml as first antibody. Alkaline phosphatase second antibody was used, and immunoreactive bands were visualized using a phosphoindolyl substrate and nitroblue tetrazolium (26). Relative loss of immunoreactive bands was determined by densitometry using a Bio-Rad model GS-670 densitometer and the accompanying Molecular Analyst software.

RESULTS

Inhibition of μ-Calpain and 20 S Proteasome by Peptidyl Protease Inhibitors—The caseinolytic activity of human μ-calpain, determined as described under "Experimental Procedures," was potently inhibited by all of the inhibitors tested (Fig. 1). ZLLY-DMK and CI-1 were nearly equipotent, producing half-maximum inhibition under standard assay conditions at 1 to 2 × 10^{-7} M. ZLLnVal was nearly as potent as the latter two inhibitors. As previously noted (6), CI-2 was somewhat less potent than CI-1 as an inhibitor of μ-calpain by approximately a factor of 3. In contrast, the caseinolytic activity of purified human 20 S proteasome was markedly resistant to inhibition by CI-1, CI-2, or ZLLY-DMK (Fig. 2). Full dose response curves could not be obtained with these inhibitors, because their solubility limits were reached in the assay buffer as evidenced by noticeable cloudiness in the solutions. Just as for inhibition of μ-calpain, there was a 2–3-fold difference in relative potencies of CI-1 and CI-2 as inhibitors of caseinolytic activity of the proteasome. ZLLnVal was much more potent than the other inhibitors; half-maximum inhibition being achieved at 10^{-5} M. In contrast, hydrolysis of succinyl-Leu-Leu-Val-Tyr aminomethylcoumarin by the 20 S proteasome assayed, as described previously (6), was fully inhibited by 2.5 × 10^{-7} M ZLLnVal (data not shown). This is consistent with the observation that ZLLnVal is a very potent inhibitor of hydrophobic peptidase activity associated with either the 20 S or 26 S proteasome (6).

The *Streptomyces* antibiotic lactacystin, a recently described proteasome-specific inhibitor (27), was found to be a potent inhibitor of the caseinolytic activity of the 20 S proteasome (Fig. 3). Its potential as a proteasome inhibitor is probably underestimated by experiments like the one depicted in Fig. 3,
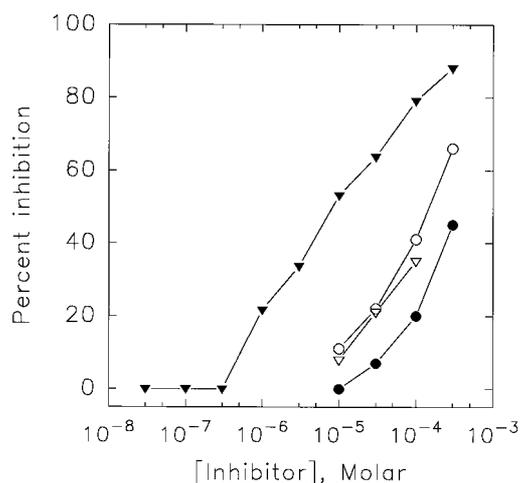
FIG. 2. **Inhibition of the proteinase activity of proteasome by hydrophobic peptidyl protease inhibitors.** Purified human erythrocyte 20 S proteasome was assayed in the presence of various inhibitors as described under "Experimental Procedures." Symbols are the same as in Fig. 1.
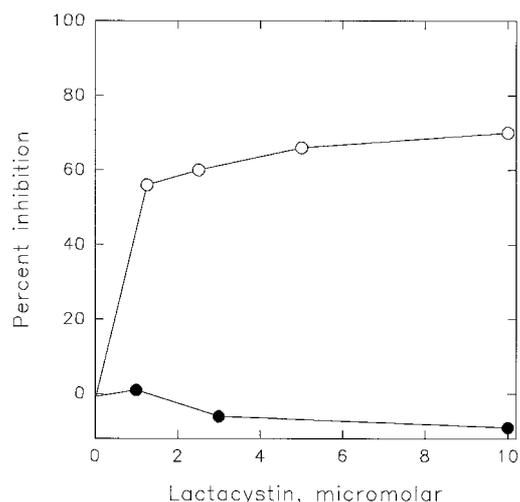

FIG. 3. **Inhibition of the proteinase activities of μ-calpain and the 20 S proteasome by lactacystin.** The proteasome ($\bigcirc$) and μ-calpain ($\bullet$) were assayed in the presence of various concentrations of lactacystin as described under "Experimental Procedures."

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).

Effect of Casein Concentration on Inhibition of the Proteasome by ZLLnVal—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 K_m (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).

Inhibition of Proteasome-catalyzed Proteolysis of Lysozyme and Calpastatin by ZLLnVal—To ensure that the low potency of ZLLnVal was not a phenomenon specifically associated with
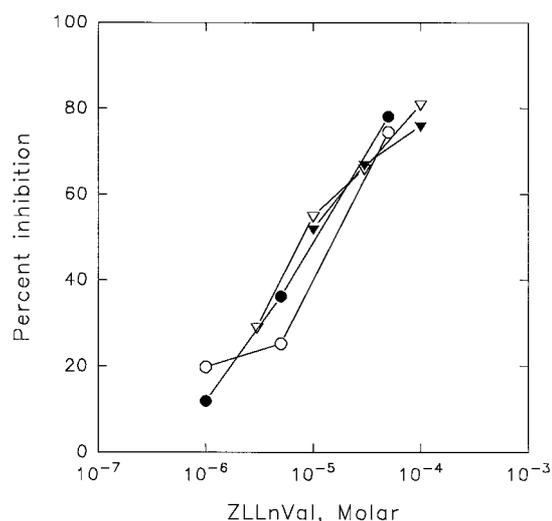

FIG. 4. **The influence of casein concentration on inhibition of the proteasome by ZLLnVal.** $\bigcirc$, 40 μg ^{14}C-methyl casein/ml; $\bullet$, 100 μg ^{14}C-methyl casein/ml; $\triangledown$, 200 μg ^{14}C-methyl casein/ml; $\blacktriangledown$, 1000 μg ^{14}C-methyl casein/ml.

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.

Uptake of Peptidyl Protease Inhibitors by Yeast Cells—The budding yeast *S. cerevisiae* 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 calpain activity. Since the ATP-ubiquitin system is an established component of the cell cycle regulatory machinery (3), proteasome inhibitors should effectively arrest growth of yeast cells. Including as much as 200 μM, CI-1, CI-2, ZLLY-DMK, or ZLLnVal in medium of overnight yeast cultures produced no detectable inhibition of yeast cell growth as assessed by A_{600} of the cultures (data not shown). Similar results have previously been reported, and at least part of this lack of effect on proliferation has been attributed to instability of the inhibitors in yeast culture medium (30). In the present studies, 100 μM ZLLnVal was found to be relatively stable after overnight exposure to yeast in culture; 72 ± 1.7 μM ($n = 3$ culture tubes) remained in the medium after overnight incubation with an initial yeast inoculum of approximately 0.1 A_{600} unit. Other inhibitors used in the present studies were also relatively stable except for CI-1, which was depleted by approximately two-thirds.

S. cerevisiae are believed to be relatively impermeant to the peptidyl inhibitors, and this might account for their tolerance toward high concentrations of the latter in culture medium (30). To estimate uptake of inhibitors by yeast cells, the washing procedure described under "Experimental Procedures" was employed. After 16 h of exposure to 200 μM ZLLnVal or ZLLY-DMK in the medium of triplicate yeast cultures, the inhibitors were detected in lysates from washed yeast cells at concentrations of 1.96 ± 0.36 and 1.34 ± 0.56 S.D. μM, respectively, using the calpain inhibitory assay. Taking into consideration the dilution of the inhibitor by the homogenization buffer, this would represent approximately 10 μM ZLLnVal and 7 μM ZLLY-DMK in yeast cytosolic fraction. To ensure that ZLLnVal was not metabolically converted by yeast to a form that inhibits calpains but not the
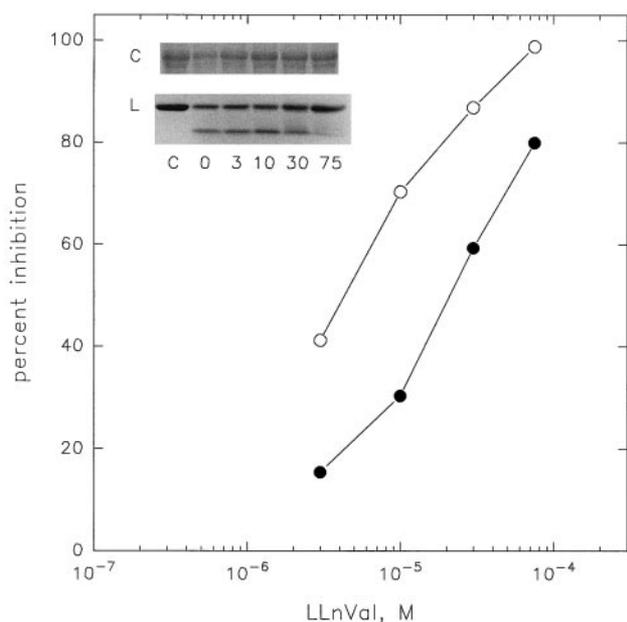
FIG. 5. **Inhibition of the proteasome-catalyzed proteolysis of calpastatin and lysozyme by ZLLnVal.** Purified human erythrocyte calpastatin or chicken egg lysozyme were incubated in the standard proteasome assay at a concentration of 200 μg/ml in the presence of 30 nM proteasome and 0, 3, 10, 30, or 75 μM ZLLnVal. After 20 min for calpastatin and 90 min for lysozyme, SDS-sample buffer was added, and the samples were heated to 100 °C. 3 μg of the substrate proteins were loaded per lane on a 15% SDS-polyacrylamide gel electrophoresis slab gel, electrophoresed, and stained with Coomassie Blue (*inset*; *C*, calpastatin; *L*, lysozyme). The gel was scanned by densitometry, and loss of the native protein band was estimated as the fractional decrease in staining intensity of the 0 inhibitor sample compared with untreated substrate (*C lanes* in *inset*). Protection against proteolysis by ZLLnVal was then determined as percent prevention of this decrease.

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. μM. In similar experiments, the positively charged and hence impermeant calpain inhibitor leupeptin was not detectable in yeast lysates. Within the limits of the calpain assay for this inhibitor, this means that less than approximately 0.5 μM leupeptin was present in lysates derived from yeast cells exposed overnight to 200 μM leupeptin in the medium.

Effect of ZLLnVal on Proteolysis of Ubiquitinylated Proteins in Yeast Lysates—Incubation of yeast lysates in the presence of ATP as described under "Experimental Procedures" resulted in the disappearance of high molecular mass ubiquitin immunoreactivity (Fig. 6). This probably reflects digestion of polyubiquitinylated proteins and not disassembly of polyubiquitin to ubiquitin monomers by isopeptidases, since no free ubiquitin was detected after incubation (Fig. 6). Including ZLLnVal in the incubation mixture resulted in protection against loss of ubiquitin immunoreactivity but only at high concentrations of the inhibitor (Fig. 6).

DISCUSSION

ZLLnVal, CI-1, and structurally related hydrophobic peptide aldehydes have been shown to be very potent inhibitors of the hydrophobic peptidohydrolase activity of both the 20 S and 26 S proteasomes (6). Based on this observation, it is tempting to speculate that their intracellular target is the proteasome. Indeed, much evidence has been provided supporting their ability to allow accumulation of polyubiquitinylated forms of proteins in mammalian cells and to suppress proteolysis of known proteasome substrates (11, 12, 31, 32). However, in a
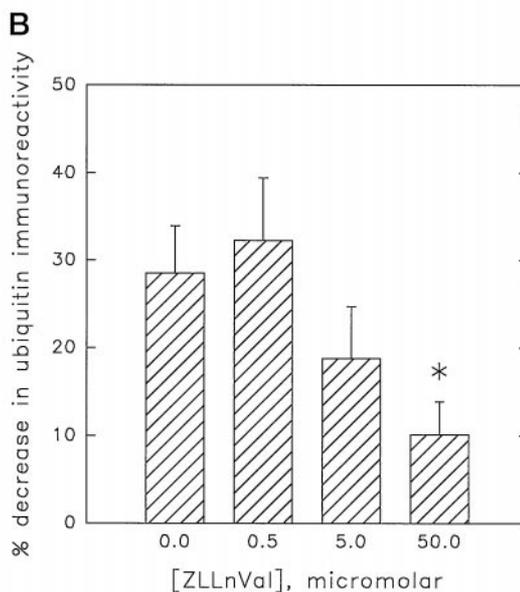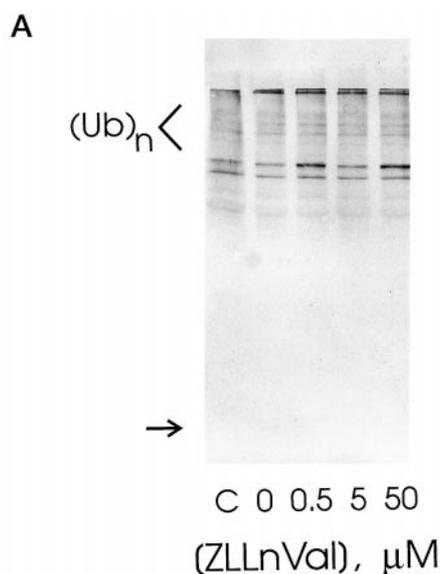

FIG. 6. **Inhibition of degradation of polyubiquitinylated proteins in yeast lysate by ZLLnVal.** Yeast lysate samples were incubated with ATP and various concentrations of ZLLnVal as described under "Experimental Procedures." *Panel A*, Western blot probed with anti-ubiquitin polyclonal antibody. The *arrow* shows the mobility of a bovine ubiquitin sample electrophoresed in the same gel. $(Ub)_n$ = high molecular mass polyubiquitinylated proteins. C = control extract heated in SDS sample buffer without prior incubation with ATP. Other *lanes* = 0, 0.5, 5, and 50 μM ZLLnVal in the incubation mixture. *Panel B*, percent protection against loss of polyubiquitinylated high molecular mass proteins by various concentrations of ZLLnVal. The $(Ub)_n$ portion of the blot in *panel A* and blots from three other independent experiments were scanned by densitometry to detect degradation of these proteins and protection by ZLLnVal. Significantly different from control by Student's unpaired t test ($p = 0.036$, $n = 4$) is indicated by *. *Error bars* = S.E.

more limited body of studies, it has been noted that hydrophobic peptide aldehydes are much poorer inhibitors of the proteasome when protein substrates are employed (6). In addition, the structurally related inhibitor, ZLLY-DMK, a diazomethyl ketone with little activity against proteasomal caseinolytic (21) or hydrophobic peptidohydrolase (33) activities, shares at least one anticipated property of cell permeant proteasome inhibitors; it is a highly potent inhibitor of cell cycle progression through G_1 phase, and it leads to stabilization of p53 tumor suppressor protein in late G_1 (9). In the present report, effects

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 *S. cerevisiae* 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. Lactacystin was selective for the proteasome, having no apparent calpain inhibitory activity (Fig. 3), and it should be useful for establishing intracellular proteolytic events requiring the proteasome. Lactacystin has recently been employed in [3H]tyrosine pulse-chase studies to demonstrate that proteolysis of the short-lived protein fraction in several cell types is dependent on participation of the proteasome (34). The utilization of lactacystin in combination with more calpain-selective inhibitors should provide a valuable approach for assessing the involvement of these two proteinase systems in various cellular processes requiring discrete proteolysis of specific regulatory proteins. Moreover, it was recently demonstrated that lactacystin had a modest peak effect on inhibition of WI-38 fibroblast progression through late G_1 phase, and that addition of saturating concentrations of lactacystin had no apparent influence on the ability of ZLLY-CHN$_2$ to further inhibit progression (35). Thus, a calpain-like proteolytic activity appears to be involved in this important cell cycle transition.

REFERENCES

1. Miyamoto, S., Chiao, P. J., and Verma, I. M. (1994) *Mol. Cell. Biol.* **14,** 3276–3282
2. Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994) *Cell* **77,** 53–62
3. King, R. W., Deshaies, R. J., Peters, J.-M., and Kirschner, M. W. (1996) *Science* **274,** 1652–1658
4. Earnshaw, W. C. (1995) *Trends Cell Biol.* **5,** 217–220
5. Na, S., Chuang, T.-H., Cunningham, A., Turi, T. G., Hanke, J. H., Bokoch, G. M., and Danley, D. E. (1996) *J. Biol. Chem.* **271,** 11209–11213
6. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) *Cell* **78,** 761–771
7. Mellgren, R. L., Shaw, E., and Mericle, M. T. (1994) *Exp. Cell Res.* **215,** 164–171
8. Walker, G., Pfeilschifter, J., and Kunz, D. (1997) *J. Biol. Chem.* **272,** 16679–16687
9. Zhang, W., Lu, Q., Xie, Z.-J., and Mellgren, R. L. (1997) *Oncogene* **14,** 255–263
10. Kubbutat, M. H. G., and Vousden, K. H. (1997) *Mol. Cell. Biol.* **17,** 460–468
11. Dietrich, C., Bartsch, T., Schanz, F., Oesch, F., and Wieser, R. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10815–10819
12. Zhou, M., Wu, X., and Ginsberg, H. N. (1996) *J. Biol. Chem.* **271,** 24769–24775
13. Milligan, S. A., Owens, M. W., and Grisham, M. B. (1996) *Arch. Biochem. Biophys.* **335,** 388–395
14. Drexler, H. C. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 855–860
15. Meerovitch, K., Wing, S., and Goltzman, D. (1997) *J. Biol. Chem.* **272,** 6706–6713
16. Bush, K. T., Goldberg, A. L., and Nigam, S. K. (1997) *J. Biol. Chem.* **272,** 9086–9092
17. Bokkala, S., and Joseph, S. K. (1997) *J. Biol. Chem.* **272,** 12454–12461
18. Lopes, U. G., Erhardt, P., Yao, R., and Cooper, G. M. (1997) *J. Biol. Chem.* **272,** 12893–12896
19. Mortensen, E. R., Marks, P. A., Shiotani, A., and Merchant, J. L. (1997) *J. Biol. Chem.* **272,** 16540–16547
20. Mellgren, R. L. (1990) *Biochim. Biophys. Acta* **1040,** 28–34
21. Mellgren, R. L., Song, K., and Mericle, M. T. (1993) *J. Biol. Chem.* **268,** 653–657
22. Mellgren, R. L. (1988) *Biochem. Biophys. Res. Commun.* **150,** 170–176
23. Mellgren, R. L., Repetti, A., Muck, T. C., and Easly, J. (1982) *J. Biol. Chem.* **257,** 7203–7209
24. Hadari, T., Warms, J. V. B., Rose, I. A., and Hershko, A. (1992) *J. Biol. Chem.* **267,** 719–727
25. Mahaffey, D., Yoo, Y., and Rechsteiner, M. (1993) *J. Biol. Chem.* **268,** 21205–21211
26. Blake, M. S., Johnstone, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem.* **136,** 175–179
27. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* **268,** 726–731
28. Dick, L. R., Cruikshank, A. A., Grenier, L., Melandri, F. D., Nunes, S. L., and Stein, R. L. (1996) *J. Biol. Chem.* **271,** 7273–7276
29. Jentsch, S. (1992) *Annu. Rev. Genet.* **26,** 179–207
30. Lee, D. H., and Goldberg, A. L. (1996) *J. Biol. Chem.* **271,** 27280–27284
31. Griscavage, J. M., Wilk, S., and Ignarro, L. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3308–3312
32. Lee, H.-W., Smith, L., Pettit, G. R., Vinitsky, A., and Smith, J. B. (1996) *J. Biol. Chem.* **271,** 20973–20976
33. Savory, P. J., Djaballah, H., Angliker, H., Shaw, E., and Rivett, A. J. (1993) *Biochem. J.* **296,** 601–605
34. Craiu, A., Gaczynska, M., Akopian, T., Gramm, C. F., Fenteany, G., Goldberg, A. L., and Rock, K. L. (1997) *J. Biol. Chem.* **272,** 13437–13445
35. Mellgren, R. L. (1997) *Biochem. Biophys. Res. Commun.* **236,** 555–558