# Isolation and Characterization of a Novel Endogenous Inhibitor of the Proteasome†

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ABSTRACT: A novel endogenous inhibitor of the proteasome (high molecular weight multicatalytic protease) has been isolated and characterized from human erythrocytes. After purification by ion-exchange and sizing chromatography, the inhibitor displayed a native molecular mass of approximately 200 kDa and contained a single subunit of 50 kDa with an isoelectric point of 6.9. Although the inhibitor noncompetitively blocks proteolysis of [*methyl*-$^{14}$C]-α-casein ($K_i$ = 7.1 × $10^{-8}$ M) and inhibits hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC, it did not affect hydrolysis of other peptide substrates, such as MeOSuc-Phe-Leu-Phe-MNA and Z-Ala-Arg-Arg-MNA. To further characterize the 50-kDa inhibitor, a monoclonal antibody (MI-8) was generated that showed specific binding upon Western blot analysis of both native PAGE and SDS–PAGE. Immunoprecipitation with MI-8 specifically removed inhibitor activity against the proteasome. The 50-kDa inhibitor is distinct from a previously described 40-kDa inhibitor of the proteasome (Murakami, K., & Etlinger, J. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7588–7592) on the basis of lack of cross-reactivity with MI-8 and dissimilar peptide digest patterns. It is suggested that these endogenous inhibitors may have a role in ATP/ubiquitin-dependent proteolysis and/or other cellular functions involving this protease.

Recently, considerable attention has focused on large multisubunit protease(s) active at neutral pH from the cytosol and nucleus of mammalian tissues as well as yeast and *Drosophila* (Orlowski, 1990; Hough et al., 1988; Rivett, 1989). Although these enzyme complexes have been referred to by various names including ingensin (Ishiura & Sugita, 1986), macropain (McGuire & DeMartino, 1986), multicatalytic protease (Hendil, 1988; Wilk & Orlowski, 1983; Dahlmann et al., 1985; Tsukahara et al., 1988), high molecular weight protease (Murakami & Etlinger, 1986, 1987), and proteasome (Arrigo et al., 1988; Matthews et al., 1989), evidence suggests that these proteases are closely related or identical in some cases. Typically these enzymes have an apparent molecular mass of 600–700 kDa, a sedimentation coefficient of 19–20S, and 10–14 electrophoretically distinct subunits in the 25–35-kDa range (Orlowski, 1990). There is evidence for at least one or two protease sites and at least two or more peptidase sites, which explains the term "multicatalytic" used by several investigators (Orlowski, 1990; Rivett, 1989). Electron microscopy reveals cylindrical structures consisting of stacks of four rings, each containing six subunits (Baumeister et al., 1988; Tanaka et al., 1988a). Immunological and structural evidence indicates that the proteasome is similar to or identical with the prosome, a particle suggested to function in the regulation of translation and RNA degradation (Arrigo et al., 1988; Falkenburg et al., 1988). Interestingly, proteasomes can be isolated in both an active and latent form (Falkenburg et al., 1988; McGuire et al., 1989). The latent enzyme can be activated by several means, including SDS[1] (0.04%) and polylysine (Dahlmann et al., 1985; Tanaka et al., 1988b), although the physiological significance of these various activated forms is unclear.

Several lines of evidence suggest that the proteasome is involved in ATP/ubiquitin-dependent proteolysis occurring in the cytosol (Tanaka et al., 1988a; Matthews et al., 1989; McGuire & DeMartino, 1989; Etlinger & Goldberg, 1977). For example, immunoprecipitation of proteasomes from reticulocyte (Tanaka & Ichihara, 1988), muscle (Matthews et al., 1989), and fibroblast (McGuire et al., 1989; McGuire & DeMartino, 1989) extracts completely inhibits ATP-dependent proteolysis while addition of latent proteasome restores this activity (McGuire & DeMartino, 1989). In addition, proteasomes associate with other factors in the presence of ATP to form a larger 26S complex that catalyzes ATP-dependent proteolysis (Eytan et al., 1989; Hough et al., 1987; Ishiura et al., 1989). Furthermore, proteasome-containing fractions sometimes exhibit ATP dependency, but the requirement for the nucleotide is labile, associated with a rise in ATP-independent activity (Etlinger et al., 1989; Driscoll & Goldberg, 1989). However, the active proteasome, in pure form, generally exhibits full activity in the absence of ATP (Hough et al., 1988; Rivett, 1989; Murakami & Etlinger, 1986; Etlinger et al., 1989; Dahlman et al., 1983). Perhaps an inhibited conformational state or coupling to a distinct inhibitory factor results in low proteolytic rates in the absence of ATP with derepression dependent on ATP (Etlinger et al., 1989).

Endogenous protein or polypeptide inhibitors have been described for many intracellular proteases including several cathepsins (Barret et al., 1986) and the calcium-dependent proteases (Suzuki et al., 1987; Parkes, 1986). However, little attention has been given to characterization of such inhibitors of the proteasome. Recently, a 240-kDa inhibitor containing 40-kDa subunits capable of blocking high molecular weight protease was purified from erythrocytes (Murakami & Etlinger, 1986). Although this protease resembled the proteasome, identity was not proven conclusively in these earlier studies.

We describe now a novel 50-kDa inhibitor of proteasomes that is distinct from the 40-kDa species on the basis of structural and immunological analysis. The 50-kDa compo-

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[1] Abbreviations: Suc, succinyl; AMC, 7-amino-4-methylcoumarin; MeO, methoxyl; Z, benzyloxycarbonyl; MNA, 4-methoxy-2-naphthylamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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nent inhibits degradation of protein noncompetitively but not the cleavage of several peptide substrates. The 50- and 40-kDa inhibitors may be involved in regulating an ATP-dependent proteolytic pathway and/or in blocking the activity of excess activated proteasome in the cell.

## Experimental Procedures

*Preparation of Endogenous Inhibitor.* All steps were carried out at 4 °C. Human erythrocytes were washed three times with isotonic saline and lysed with 3 volumes of 4 mM Tris-HCl (pH 7.2) containing 0.5 mM *p*-chloromercuribenzoate and 0.5 mM phenylmethanesulfonyl fluoride. Inclusion of these protease inhibitors was found to stabilize the 40-kDa inhibitor in previous studies (Murakami & Etlinger, 1986). After centrifugation at 18000*g* for 60 min, the supernatant was mixed with an equal volume of DEAE-Sephacel (Pharmacia) suspension (resin/buffer A 1:2) for 30 min. Buffer A contains 0.5 mM dithiothreitol, 0.5 mM $MgCl_2$, 20 mM KCl, and 20 mM Tris-HCl (pH 7.2). Unbound proteins were removed by washing with 3 volumes of buffer A. Protein was then eluted with 0.3 M KCl in buffer A and precipitated with 40% saturated ammonium sulfate. After dialysis against buffer A, protein was adsorbed on a DEAE-Sephacel column (2.5 × 7 cm) and eluted with a gradient of 20–300 mM KCl in 300 mL of buffer A. Fractions (5 mL) constituting the main peak of inhibitor activity were pooled and precipitated with ammonium sulfate at 50% saturation, followed by dialysis against buffer A containing 0.1 M KCl. The protein was then chromatographed at 5 mL/cm$^2$ per hour on a Bio-Gel A 0.5-m column (1.5 × 100 cm) equilibrated with the same buffer. Fractions (2 mL) were collected, and fractions containing inhibitor activity were pooled and concentrated by ultrafiltration with an Amicon Centricon with use of a PM-10 membrane under nitrogen. The inhibitor preparation was then applied to a Superose 6 HR 10/30 column (Pharmacia) equilibrated with the same buffer as above and chromatographed at a flow rate of 0.1 mL/min on a Pharmacia FPLC system. Fractions (0.5 mL) were collected to test inhibitor activity. Pure inhibitor was stored at −70 °C after concentration.

*Preparation of Proteasome (High Molecular Weight Multicatalytic Protease).* All steps were carried out at 4 °C. Human or rabbit erythrocytes were washed and lysed as described above but without inclusion of phenylmethanesulfonyl fluoride and *p*-chloromercuribenzoate. Lysate was dialyzed against several changes of buffer A (above) and mixed with an equal volume of DEAE-Sephacel, which was equilibrated with the same buffer (resin/buffer 1:2). After 0.5 h, the suspension was poured onto a Buchner funnel and washed extensively with buffer A. A protease fraction was obtained by batch elution with 0.4 M KCl, and the eluate was brought to 30% saturation with ammonium sulfate. The solution was then centrifuged at 16000*g* for 30 min, and the supernatant was brought to 75% saturation with ammonium sulfate and centrifuged as above. The supernatant was discarded, and the pellet was dialyzed against several changes of buffer A and applied to a column of DEAE-Sephacel (2.5 × 10 cm) equilibrated with buffer A. The column was then eluted with a linear gradient of 50–400 mM KCl in buffer A with fractions assayed for proteolytic activity. Active fractions were combined and concentrated in an Amicon Centricon on an XM-300 membrane under nitrogen. The protease was further purified on a Sephacryl S-300 HR column (1.5 × 100 cm) equilibrated with buffer A, with the active fractions being pooled, concentrated, and stored at −70 °C. SDS–PAGE indicated that this preparation contained greater than 99% proteasome subunits that migrated in the 25–35-kDa range.

*Assay of Protease and Inhibitor Activity.* Proteolytic activity was measured as the acid-soluble radioactivity released from [*methyl*-$^{14}$C]-α-casein. The reactions were carried out in a total volume of 50 μL containing 5 μmol of Tris-HCl (pH 8), 0.25 μmol of $MgCl_2$, 0.025 μmol of dithiothreitol, 5 μmol of KCl, 4.5 μg of labeled substrate (15000 cpm), and enzyme fraction. Trichloroacetic acid (10%) and carrier bovine serum albumin (final concentration 4 mg/mL) were added after 1 h of incubation at 37 °C. Following centrifugation, radioactivity in aliquots of the supernatant was counted in an LKB liquid scintillation counter. One unit of protease activity is defined as the amount of enzyme that will hydrolyze, to acid-soluble form, 1 μg of [*methyl*-$^{14}$C]-α-casein in 30 min at 37 °C. For kinetic analysis, standard assays were carried out for 30 min, resulting in less than 5% of the substrate degraded.

Peptidase activity was determined as the amount of free AMC or MNA released from fluorogenic peptide substrates obtained from Enzyme System Products. The reactions were performed as described above containing 0.4 mM fluorogenic peptides. Hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC was terminated after 30 min by quenching with 1 mL of cold absolute ethanol and measured at an excitation of 380 nm and emission of 460 nm. Hydrolysis of MeOSuc-Phe-Leu-Phe-MNA and Z-Ala-Arg-Arg-MNA was terminated with 1 mL of 0.1 M $Na_2B_4O_7$ (pH 9.0) and read at an excitation of 340 nm and emission of 425 nm.

To measure inhibitor activity, aliquots of proteasome (0.5 unit) and inhibitor-containing fractions were combined in the assay mixture as described above without addition of labeled substrate. After 30 min on ice, substrate was added and the reaction was carried out at 37 °C for 30 min. The acid-soluble radioactivity released from the substrate was measured as described above. One unit of inhibitor represents the amount of inhibitor that results in 50% inhibition of 0.5 unit of proteasome activity in the above assay.

*Peptide Mapping.* Peptide maps were obtained according to a procedure that is similar to that described previously (Cleveland, 1983). Purified inhibitors were subjected to SDS–PAGE on 12% gels (see below) and stained with 0.1% Coomassie Blue, 50% methanol, and 10% acetic acid. Individual inhibitor bands were excised with a razor blade and equilibrated against 10 mL of buffer B (1.68% Tris base, pH 6.8, 10% glycerol, 0.1% SDS, 1 mM EDTA, and 0.3% 2-mercaptoethanol) for 1 h. Each gel slice was then pushed in to the bottom of a sample well of a second-dimension gel consisting of a 4.5% stacking gel on a 15% resolving gel. Spaces around slices were filled with buffer B. *Staphylococcus aureus* V8 and papain at varying concentrations (0–50 ng) as indicated were diluted with buffer B, loaded over each slice, and electrophoresed at 60 V until bromphenol blue cleared the stacking gel. At that time the voltage was increased to 150 V, and electrophoresis was continued until the dye front reached the bottom of the gel. Peptide maps were visualized by Coomassie Blue staining.

*Preparation of Monoclonal Antibody to 50-kDa Inhibitor.* (*a*) *Immunization.* BALB/c mice were immunized with the 50-kDa inhibitor isolated from human erythrocyte. Protein was diluted with sterile phosphate-buffered saline and emulsified in an equal volume of Freund's adjuvant (complete for initial injections, incomplete for subsequent injections). For each injection (ip), 100 μg of protein in a total volume of 0.3 mL was used per mouse. The mice are injected over a period of 6–8 weeks, at 3–4-week intervals. Three days prior to

fusion, mice were injected with 50 μg of protein (ip) in a total volume of 0.3 mL. Two days after the injection, mice were bled by retroorbital puncture and the sera were titered. The mouse with the highest titer was used for the fusion on the next day.

(*b*) *Hybridoma Production.* Fusions are carried out essentially as described by Kohler and Milstein (1975). Mice were killed by cervical dislocation, and the spleens were aseptically removed and minced finely. Spleen cells were washed in several changes of serum-free Dulbecco's-modified Eagle's medium. Erythrocytes were lysed with cold 0.17 M ammonium chloride on ice for 10 min. After an additional wash, unlysed cells were resuspended and counted (approximately $100 \times 10^6$ cells/spleen). Mouse myeloma cells (P3UI, furnished by Dr. Thomas Easton) in log-phase growth were washed with serum-free medium. A lymphocyte:myeloma ratio of 3–4:1 was used. The cells were combined and pelleted, and 1 mL of PEG-1000 (50% solution) was added slowly. After incubation with PEG for 8 min, the PEG was diluted with medium and the cells were slowly resuspended. The cells were diluted to $5 \times 10^6$ myeloma cells/mL in Iscove's-supplemented HAT selection medium (containing 100 μM hypoxanthine, 10 μM aminopterin, and 30 μM thymidine)/20% fetal bovine serum and were plated in 96-well plates. Cell supernatants were tested for reaction to antigen in RIA (see below), and positive wells were expanded and cloned by limiting dilution. Cells were frozen in medium containing 40% fetal bovine serum and 10% dimethyl sulfoxide in liquid nitrogen. Pristane-treated BALB/c mice were used to generate ascites fluid.

*Radioimmunoassay.* Antigen in PBS was dried on wells of PVC plates at 1 μg of protein/well. Nonspecific binding was blocked by incubation of wells with 2% BSA in PBS for 1 h followed by addition of 50 μL of monoclonal antibody (MI-8) with incubation for 30 min at room temperature. The wells were washed several times with PBS/BSA/0.05% Tween-20, and $^{125}$I-labeled goat anti-mouse Ig ($10^5$ cpm/50 μL/well, diluted with PBS/BSA/Tween) was added to the wells and incubated for 30 min. After extensive washing with PBS/BSA/Tween, wells were excised and counted in an LKB γ counter.

*Western Blots.* Proteins were first subjected to SDS–PAGE or native PAGE and then electrophoretically transferred to nitrocellulose paper. A lane was stained for total protein with amido black to assess transfer efficiency. Nitrocellulose was incubated in blocking solution (see RIA above) at 4 °C overnight. Blots were incubated for 1 h in blocking solution containing monoclonal antibody MI-8 followed by extensive washing with PBS/BSA/Tween over a period of at least 1 h. Blots were then incubated with a second antibody, $^{125}$I-labeled goat anti-mouse Ig (diluted to $(2–3) \times 10^5$ cpm/mL) for 1 h at room temperature and washed as above. Blots were dried, affixed to 3-mm chromatography paper, and exposed to Kodak X-Omat X-ray film at −70 °C in cassettes with intensifying screens.

*Immunoprecipitation of Inhibitor Activity.* A total of 50 μL of 50% protein A/agarose was washed three times with PBS and then resuspended in 250 μL of blocking solution (5% powder milk in PBS); 3 μL of rabbit anti-mouse IgG (Ab 4.0 mg/mL, from Cappel) was added to the beads followed by incubation at room temperature for 2 h. The beads were washed five times with PBS after incubation and resuspended in 250 μL of blocking solution containing different amounts of monoclonal antibody MI-8. After another incubation carried out as above, the immunoadsorbents were washed 10 times with PBS. Purified inhibitor was then added to the beads and incubated at 4 °C overnight. Inhibitor activity in the supernatant was determined as previously described. Beads, with adsorbed inhibitor, were washed 10 times with PBS/Tween. Inhibitor was extracted by adding equal volumes of 2 × SDS sample solubilizing buffer and boiling at 100 °C for 2 min. The supernatant was subjected to Western blot analysis. Control assays were performed with a monoclonal antibody of irrelevant specificity (mAb-B6 against 39-kDa vaccinia virus core protein, a gift from Dr. M. Esteban).

*Protein Determination.* Protein was determined by the Bradford method with BSA as a standard (Bradford, 1976).

*Electrophoresis.* SDS–PAGE was carried out in the slabs according to the method of Laemmli (1970). Inhibitor and proteasome preparations were dissolved with 2× solubilizing buffer and resolved on 12.5% gels. Molecular mass standards used were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (22 kDa), lysozyme (14 kDa), and myosin (200 kDa).

Native PAGE gels were run according to the procedure described above for denaturing gels with the following changes: SDS was removed from all buffers, protein was prepared in 10% sucrose (no solubilizing buffer), and bromphenol blue was included as a tracking dye.

Isoelectric focusing was performed according to the method of O'Farrell (1975) with modifications as indicated. The gel solution (10 mL) contained 5.5 g of urea dissolved in 1.33 mL of acrylamide stock solution (28.38% w/v acrylamide and 1.62% bisacrylamide), 3.0 mL of NP-40 (20%), 0.5 mL of ampholine (pH 5–7), 0.24 mL of ampholine (pH 4–6), 0.05 mL of ampholine (pH 3–10), and 1.96 mL of $H_2O$. After polymerization, gels were prerun with the upper reservoir containing 20 mM NaOH and the lower reservoir containing 10 mM $H_3PO_4$ according to the following schedule: 0.5 h at 200 V, 1 h at 300 V, and 1 h at 400 V. Inhibitor, in lysis buffer containing 9.5 M urea, 3% NP-40, 2% ampholines (1.6% pH 5–7, 0.4% pH 3–10), and 5% β-mercaptoethanol, was then loaded on the gel and run with the same buffer at 450 V for 20 h. To determine isoelectric point, gel slices were soaked in water individually overnight and the pH of each was measured. The gel containing sample was incubated in buffer solution (0.0635 M Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, and 5% β-mercaptoethanol) for 30 min. The second-dimension analysis was performed by SDS–PAGE as described above.

## Results

*Resolution of Two Distinct Proteasome Inhibitors.* To assay proteasome inhibitory activity, purified proteasomes displaying the characteristic subunits in the 25–35-kDa range were prepared from human erythrocytes (Figure 1). The 100000*g* erythrocyte supernatant fraction was adsorbed to DEAE-Sephacel and eluted at 0.3 M KCl. Inhibitory activity against degradation of [*methyl*-$^{14}$C]-α-casein could not be measured in crude cell-free extract or in 0.3 M KCl batch eluate presumably due to the presence of protease activities in these preparations. However, activity inhibiting casein hydrolysis by proteasome was detected after ammonium sulfate precipitation since protease activity in the 0.3 M KCl batch eluate remains soluble in 40% ammonium sulfate (Table I). Proteins were rechromatographed by elution with a 0.02–0.3 M KCl gradient (Figure 1). A broad peak of activity was detected at approximately 0.2 M KCl. Although proteasome was removed during the previous purification steps, it should be noted that proteasome elution on DEAE-Sephacel overlaps with most of the inhibitor activity although the peak of pro-

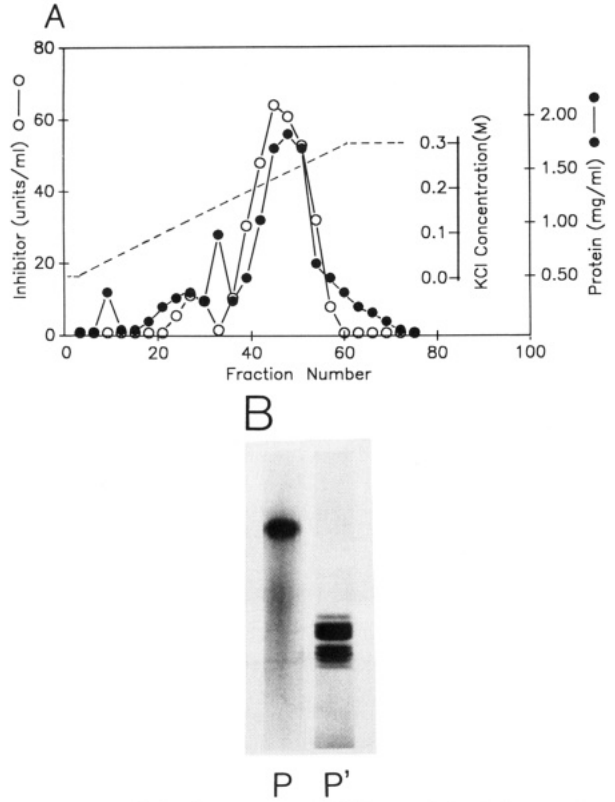


FIGURE 1: Analysis of proteasome inhibitor activity by ion-exchange chromatography. (A) Human erythrocyte lysate, after prior batchwise elution with 0.3 M KCl from a DEAE-Sephacel column (2.5 × 10 cm), was reapplied to the same column and eluted with a linear gradient of 20–300 mM KCl as described under Experimental Procedures. Inhibitor activity against hydrolysis of [*methyl*-$^{14}$C]-$\alpha$-casein was tested with 25 $\mu$L of every three fractions using 0.5 unit of purified proteasome. (B) The purity of the proteasome preparation was determined by native PAGE (5%) as shown in lane P and SDS–PAGE (12.5%) as shown in lane P'.

Table I: Purification of 50-kDa Inhibitor from Human Erythrocytes[a]

| purification step | total protein (mg) | total units | sp act. (units/mg) |
|---|---|---|---|
| lysate[b] | 14,700 | – | – |
| batch DEAE[b] | 784 | – | – |
| ammonium sulfate 0–40% saturation | 172.5 | 1897 | 11 |
| DEAE gradient | 88 | 3784 | 43 |
| Bio-Gel A 0.5 | 3.5 | 2639 | 754 |
| Superose FPLC[c] | 1.1 | 891 | 810 |

[a] Purification of the 50-kDa inhibitor started with 220 mL of packed human erythrocytes as described under Experimental Procedures. Specific activities shown are for inhibition of casein hydrolysis by purified proteasome. One unit of inhibitor activity is expressed as 50% inhibition of 0.5 unit of proteasome activity. [b] No apparent inhibitor activity was detected due to the presence of proteasome activity. [c] The significant loss of unit value is the result of separation of the 40-kDa inhibitor from this preparation (see text).

teasome activity is at a slightly higher KCl concentration (0.26–0.27 M). Inhibitory fractions were pooled and further purified according to size on a Bio-Gel A 0.5-m column (Figure 2). Most of the proteasome inhibitory activity eluted after the bulk of the remaining red cell proteins, indicating that inhibition due to general red cell protein is at most a minor contribution. The principal Bio-Gel column activity peak contained two major components as resolved by native PAGE (Figure 3). This preparation was subjected to Superose 6 FPLC, which resolved two active fractions with estimated sizes of 200 and 240 kDa, respectively, each displaying a single major band on native PAGE (Figure 3A, lanes 2 and 3).

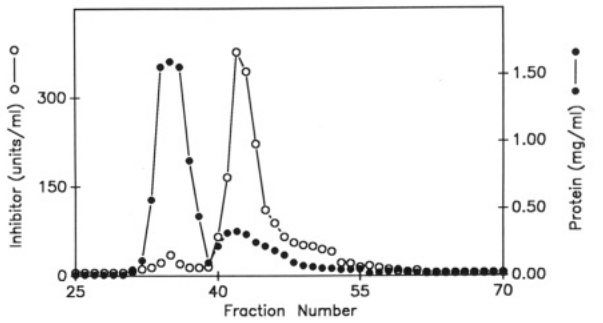


FIGURE 2: Molecular exclusion chromatography of inhibitor. Fractions constituting the predominant peak of inhibitory activity from the DEAE-Sephacel column were concentrated by ammonium sulfate precipitation followed by dialysis against buffer A containing 0.1 M KCl. Inhibitor was applied to a Bio-Gel A 0.5-m column (1.5 × 100 cm) equilibrated with the same buffer. Inhibitor activity was assayed in 5-$\mu$L fraction aliquots using 0.5 unit of proteasome and [*methyl*-$^{14}$C]-$\alpha$-casein as substrate as described.

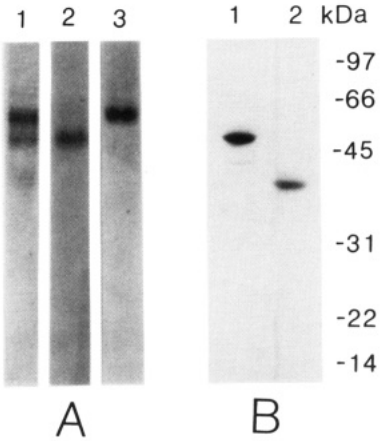


FIGURE 3: Native PAGE and SDS–PAGE analysis of purified inhibitors. Inhibitors were purified as described under Experimental Procedures. (A) Lane 1 contains the active fraction from the Bio-Gel column. Lanes 2 and 3 contain active fractions from the Superose 6 column, representing the 50-kDa inhibitor and the 40-kDa inhibitor, respectively. (B) SDS–PAGE (12.5%) of proteins eluted from parallel unstained native gels: lane 1 contains the 50-kDa inhibitor eluted from lane 2 in panel A whereas lane 2 contains the 40-kDa inhibitor eluted from lane 3 in panel A.

When eluted proteins were subjected to SDS–PAGE, the 240-kDa species displayed a 40-kDa subunit as previously reported (Murakami & Etlinger, 1986), whereas the 200-kDa inhibitor consisted of a single polypeptide of 50 kDa (Figure 3B). Isoelectric focusing, as described under Experimental Procedures, revealed isoelectric points of 6.8 for the 40-kDa inhibitor and 6.9 for the 50-kDa species.

To determine if the 40- and 50-kDa inhibitors are structurally related, limited peptide digests were carried out. Peptide maps obtained by digestion with papain (type III) and *Staphylococcus* V8 of the two inhibitors generated completely distinct fragments on SDS–PAGE (Figure 4). Therefore, the 50-kDa inhibitor appears to be a new proteasome inhibitor with a primary sequence distinct from the previously described 40-kDa component.

*Selectivity and Mode of Inhibition for the 50-kDa Inhibitor.* Increasing concentrations of 50-kDa inhibitor resulted in complete inhibition of $\alpha$-casein proteolysis with an apparent $K_i = 7.1 \times 10^{-8}$ M (Figure 5). To ascertain the mode of inhibition for the 50-kDa inhibitor, activity was measured at different substrate concentrations. With increasing concentration of $\alpha$-casein, inhibition was not abolished. Lineweaver–Burk plots indicate an apparent noncompetitive mode of inhibition with a reduction in $V_{max}$ and no change in $K_m$ (not

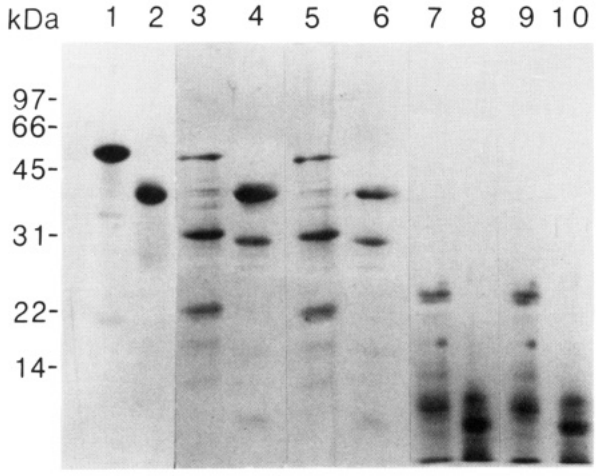


FIGURE 4: Peptide digests of the 40- and 50-kDa inhibitors. The 50-kDa inhibitor (lanes 1, 3, 5, 7, and 9) and the 40-kDa inhibitor (lanes 2, 4, 6, 8, and 10) were each digested by *Staphylococcus aureus* V8 and papain (type III). Proteolysis was carried out in the 4.5% stacking gel followed by electrophoresis of the digest through a 15% resolving gel. The peptide maps were visualized by Coomasie Blue staining. Lanes 1 and 2 contain undigested 40- and 50-kDa inhibitors. Lanes 3 and 4 were obtained after digestion with 10 ng of V8, lanes 5 and 6 with 50 ng of V8, lanes 7 and 8 with 10 ng of papain, and lanes 9 and 10 with 50 ng of papain.

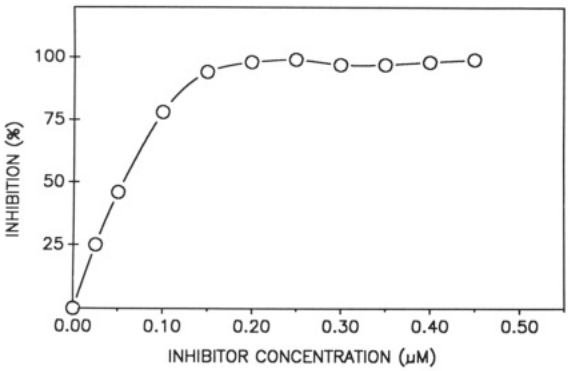


FIGURE 5: Effect of the 50-kDa inhibitor concentration on proteasome activity. Increasing amounts of 50-kDa inhibitor were added to a fixed amount of proteasome (1.6 μg) and assayed for protease activity using [*methyl*-$^{14}$C]-α-casein as substrate.

shown). Consistent with these results, inhibitor is resistant to proteolysis under the standard assay conditions for at least 30 min as determined by SDS–PAGE. Since the proteasome is known to have multiple active sites, several peptide substrates were compared to α-casein. The 50-kDa inhibitor did not affect degradation of MeOSuc-Phe-Leu-Phe-MNA and Z-Ala-Arg-MNA (Table II). However, hydrolysis of one chymotryptic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC, was inhibited (Table II).

*Characterization of a Monoclonal Antibody to the 50-kDa Inhibitor.* A monoclonal antibody (MI-8) of the IgG subtype was produced in hybridomas after immunizing a BALB/c mouse with purified 50-kDa inhibitor. MI-8 was generated in mouse ascites fluid and purified by Affi-Gel blue chromatography. Western blot analysis indicated that MI-8 specifically binds to a single 50-kDa band in crude inhibitor although these partially purified fractions displayed several bands on native and SDS gels (Figure 6, lanes 3A and 1B). The purified 40-kDa inhibitor did not cross-react with MI-8 (Figure 6, lane 4A).

Although MI-8 did not directly block inhibitor activity when added in the presence of protease (not shown), evidence that this antibody recognizes a functional 50-kDa proteasome inhibitor was obtained from immunoprecipitation. An immunoadsorbent was prepared by coupling MI-8 to rabbit anti-mouse IgG/protein A/agarose complex and incubating with inhibitor. After removal of adsorbed antigen, inhibitor in the supernatant was assayed against proteasome. Inhibitor activity could be completely removed by this procedure (Figure 7A). In contrast, with monoclonal antibody of irrelevant specificity, no loss of inhibitor activity was detected (Figure 7A). Adsorbed inhibitor was separated by SDS–PAGE followed by Western blot analysis using MI-8, indicating that the 50-kDa inhibitor was specifically immunoprecipitated by MI-8 in parallel with the removal of inhibitory activity (Figure 7B). Thus, these results indicate that the 50-kDa component recognized by MI-8 has inhibitory activity against proteasome although the antibody is not directly antifunctional.

Table II: Substrate Specificity of Proteasome and 50-kDa Inhibitor[a]

| substrates | proteasome act. (units/mg) −inhibitor | proteasome act. (units/mg) +inhibitor | inhibition (%) |
|---|---|---|---|
| Suc-Leu-Leu-Val-Tyr-AMC | 47 | 20.7 | 56 |
| MeOSuc-Phe-Leu-Phe-MNA | 5 | 5 | 0 |
| Z-Ala-Arg-Arg-MNA | 12 | 11.8 | 0 |
| [*methyl*-$^{14}$C]-α-casein | 155 | 59 | 62 |

[a] One unit of proteasome is expressed as 1 nmol of MNA (or AMC) produced per minute or 1 μg of [*methyl*-$^{14}$C]-α-casein hydrolyzed in 30 min at 37 °C. Samples of 1.6 μg of proteasome and 0.8 μg of 50-kDa inhibitor were used for assay. Replicate determinations yielded values agreeing to within ±10% ($n$ = 3).

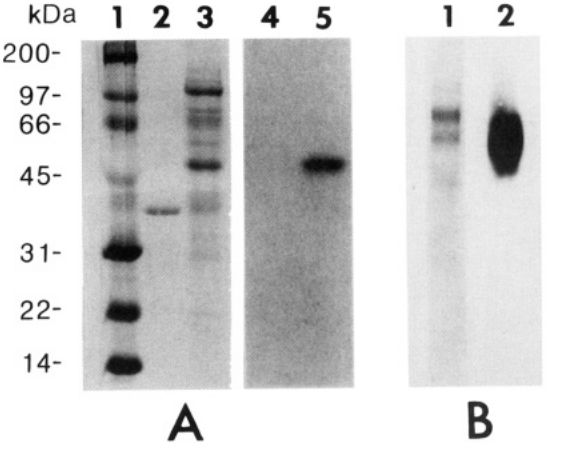


FIGURE 6: Binding of monoclonal antibody (MI-8) to the 50-kDa inhibitor by Western blot analysis. (A) Crude 50-kDa inhibitor from the DEAE-Sephacel chromatography step shown above (lane 3, see Experimental Procedures) and purified 40-kDa inhibitor (lane 2) were analyzed by 12.5% SDS–PAGE. Lanes 4 and 5 are radioautographs of proteins electrophoresed in parallel and stained with MI-8 after transfer to nitrocellulose membrane. Lane 1 contains molecular mass markers as indicated. (B) The pooled inhibitor active fraction from the Bio-Gel column shown above (lane 1) was analyzed by 7.5% native PAGE. A radioautograph (lane 2) was obtained as above.

## Discussion

These studies have revealed a previously uncharacterized endogenous inhibitor of the proteasome in erythrocytes. The inhibitor is a protein of 200 kDa and is composed of a single 50-kDa subunit with an isoelectric point of 6.9. A specific monoclonal antibody against the 50-kDa inhibitor does not cross-react with a previously described 40-kDa inhibitor of this protease (Murakami & Etlinger, 1986). Likewise, in other studies, a polyclonal antibody to the 40-kDa inhibitor does not cross-react with the 50-kDa inhibitor (G. Guo and J. D. Etlinger, manuscript in preparation). In addition, peptide digest mapping displayed different patterns for the two inhibitors.

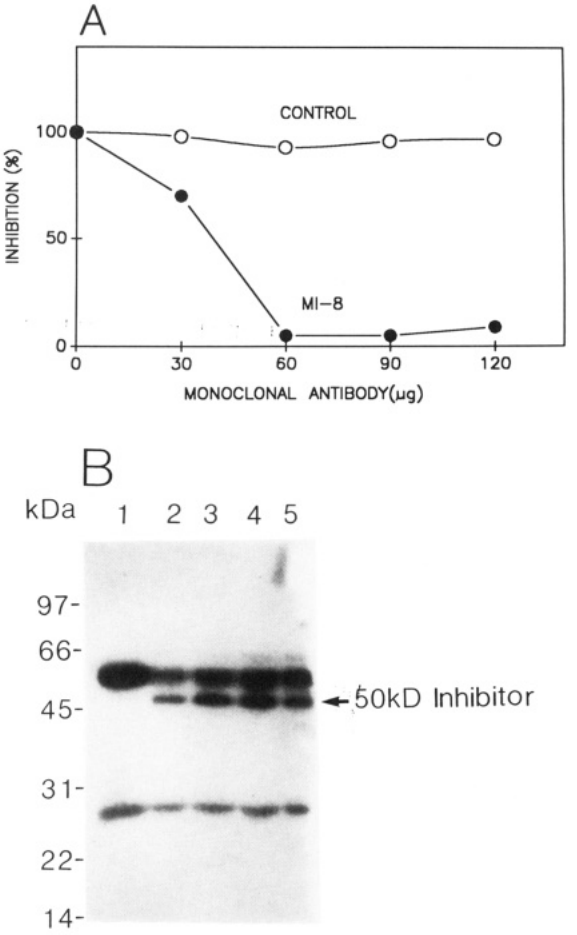


FIGURE 7: Immunoprecipitation of inhibitor activity (A) and 50-kDa protein (B). Purified inhibitor was incubated with adsorbent suspension containing MI-8 (●) or a monoclonal antibody of irrelevant specificity (○) as described under Experimental Procedures. After centrifugation, the supernatant was assayed for inhibitor activity against purified proteasomes. Proteins extracted from the MI-8 immunoadsorbent were subjected to 12.5% SDS–PAGE (B). After electrophoresis, proteins were transferred to a nitrocellulose membrane and autoradiograms were obtained as described under Experimental Procedures. Lanes 1, 2, 3, 4, and 5 corresponding to data points of 0, 30, 60, 90, and 120 μg in panel A.

Thus, the 50-kDa component does not resemble the 40-kDa high molecular mass protease inhibitor on the basis of several distinct structural and immunological properties.

The 50-kDa inhibitor was able to completely block degradation of casein (Figure 5) by an apparent noncompetitive mode of inhibition resulting in a reduction in $V_{max}$ with no change in $K_m$. The $K_i$ of $7.1 \times 10^{-8}$ M is similar to that noted previously (Murakami & Etlinger, 1986) for the 40-kDa inhibitor ($8.3 \times 10^{-8}$ M). Although it is useful to compare the kinetics of inhibition to other protein inhibitors, the kinetic analysis should be interpreted with caution because of the complexity of the proteasome system. For example, several active sites may be involved either simultaneously or sequentially to form acid-soluble products of protein substrates. Thus, the present analysis may not rule out a competitive mode of inhibition with respect to a particular active site involved in casein hydrolysis.

Interestingly, calpastatin, the endogenous inhibitor of calpain, displays a similar $K_i$ of $7.2 \times 10^{-8}$ M with respect to protein substrate (Murachi, 1989). As discussed previously (Murakami & Etlinger, 1986), other red cell proteins can act as competitive substrates but show much lower specific activity relative to the noncompetitive 40- and 50-kDa inhibitors (Figure 2). Furthermore, we estimate (from Table I) the concentration of the 50-kDa inhibitor in red cells to be approximately $5 \times 10^{-8}$ M, the same order of magnitude as the apparent $K_i$ consistent with a physiological function for this protein (Table I). These results, together with evidence indicating that the inhibitor is not itself a substrate under the assay conditions used in the present studies, suggest that this factor may not bind directly to the protease active site(s) but instead acts in an allosteric fashion or by steric hindrance.

The inhibitor did not affect the breakdown of two synthetic peptide substrates, suggesting that different sites are responsible for protein degradation (Hough et al., 1988; Rivett, 1989; Arrigo et al., 1988). However, hydrolysis of one chymotryptic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC, was reduced by the 50-kDa inhibitor (Table II). The active site responsible for hydrolysis of this peptide may be different from the casein proteolytic site on the basis of lack of competition of α-casein with respect to peptide hydrolysis. Therefore, the 50-kDa inhibitor specifically inhibits a protease active site and one of the peptidase sites on this multicatalytic enzyme. Interestingly, like the 40-kDa inhibitor (Murakami & Etlinger, 1986), 50-kDa inhibitor did not affect the activity of several other proteases, including trypsin, chymotrypsin, and papain, although it was able to inhibit calpain (G. Guo, X. Li, and J. D. Etlinger, manuscript in preparation).

Several lines of evidence implicate the proteasome in ATP/ubiquitin-dependent proteolysis. In addition, it has been shown that the 19S proteasome can associate with other undefined factors in the presence of ATP to form a 26S protease that exhibits ATP-dependent degradation of ubiquitin/protein conjugates (Eytan et al., 1989; Hough et al., 1987). Thus, the proteasome appears to be a major protease complex responsible for hydrolytic steps in ATP-dependent proteolysis. The 50-kDa inhibitor is not, however, a component of the latent proteasome or the 26S complex although variable amounts of endogenous inhibitors can contaminate preparations of proteasome contributing to the apparent latency of these activities. In addition, the 50-kDa inhibitor does not inhibit the activity of the 26S complex even though it inhibits isolated 19S proteasome activity (unpublished observations). Apparently, other components such as the 40-kDa inhibitor within the larger complex directly or indirectly prevent binding of the 50-kDa inhibitor to proteasome sites (X. Li and J. D. Etlinger, manuscript in preparation).

We speculate that additional ATP-dependent pathways as well as different pools of proteasomes may exist in cells resulting from a dynamic equilibrium between 19S and 26S proteasome. Previous studies suggested that proteasome inhibition can be derepressed by an ATP/ubiquitin-dependent mechanism (Murakami & Etlinger, 1987; Etlinger et al., 1989; Speiser & Etlinger, 1983). Such apparent modulation was produced by the combination of crude fractions and was consistent with derepression of proteolysis caused by ubiquitination of inhibitor or possibly by kinases, phosphatases, or ubiquitinated protein substrates that could act allosterically to affect inhibitor/protease interaction. Although such mechanisms have not been proven, it is possible that multiple ATP/ubiquitin-dependent pathways as well as ATP/ubiquitin-independent pathways function in the cell (Orlowski, 1990; Matthews, 1989). Modulation of activated proteasome/inhibitor interaction could serve as an alternative pathway that would be responsive to ubiquitin levels and not necessarily require conjugation to protein substrates. In addition, it has been shown that proteasomes are responsible for the degradation of certain oxidized proteins in a process that does not

require ATP or ubiquitin (Rivett, 1989; Pacific et al., 1989). The endogenous inhibitors could function in the regulation of this ATP-independent activity and/or act as scavengers to block irreversibly activated proteasome that may accumulate in cells.

Demonstration of a role for the 50-kDa inhibitor in a particular proteolytic pathway will require a reagent that is able to selectively block inhibitor function or remove inhibitor from a proteolytic system. MI-8 has not been useful in this regard since the epitope recognized by this antibody appears to be involved, at least indirectly, in protease binding to inhibitor. Therefore, although MI-8 recognizes native as well as denaturated inhibitor, it does not bind to inhibitor in the presence of proteasome (unpublished observations). Hopefully suitable antifunctional reagents will become available so that the physiological functions of these proteasome inhibitors can be clarified.

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