Identification and Localization of a Cysteinyl Residue Critical for the Trypsin-like Catalytic Activity of the Proteasome†

Lawrence R. Dick,‡ Carolyn R. Moomaw,§ Bikash C. Pramanik,§ George N. DeMartino,‖ and Clive A. Slaughter*,‡,§

Department of Biochemistry, Howard Hughes Medical Institute, and Department of Physiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235

Received March 13, 1992; Revised Manuscript Received May 26, 1992

ABSTRACT: Chemical modification of the proteasome with *N*-ethylmaleimide (NEM) was performed for the purpose of identifying amino acid residues that play a role in the enzyme's proteolytic function. Modification of the proteasome with NEM specifically and irreversibly suppressed one of the three peptidase activities of the enzyme, viz., the "trypsin-like" activity. Leupeptin, a reversible competitive inhibitor of this activity, protected the activity from NEM inactivation, suggesting that NEM modifies a residue in the leupeptin binding site. Comparisons of enzyme samples labeled with [^{14}C]NEM either in the presence or in the absence of leupeptin allowed the identification of a proteasome subunit containing an NEM-modified, leupeptin-protected cysteinyl residue. The leupeptin protection experiments suggest that residues of this subunit contribute to the active site responsible for the proteasome's trypsin-like activity. This subunit was purified by reverse-phase high-performance liquid chromatography. Peptide mapping and N-terminal amino acid sequencing were employed to acquire information about the primary structure of the subunit, including the sequence surrounding the leupeptin-protected cysteinyl residue. The sequencing data suggest that this proteasome subunit is evolutionarily related to other proteasome subunits that have been sequenced, which show no homology to other known proteases. The assignment of a catalytic function to a member of the proteasome family supports the hypothesis that proteasome subunits represent a structurally and possibly mechanistically novel group of proteases.

The proteasome, also known as the multicatalytic protease complex or macropain, is a high molecular weight, multisubunit proteinase that has been found in all eukryotic cells examined (Orlowski, 1990). The enzyme is thought to be involved in general cytoplasmic protein turnover (Goldberg, 1992), and it recently has been suggested that the enzyme's proteolytic activity may have been recruited during evolution to function in the pathway for antigen presentation by class I major histocompatibility complex molecules (Brown et al., 1991; Glynne et al., 1991). Estimates of the proteasome's molecular weight by gel filtration chromatography have yielded values of approximately 650 000, and analysis of the particles by electron microscopy has revealed a distinctive cylinder-shaped morphology (Kopp et al., 1986). The particles are composed of subunits with molecular weights in the range of 25 000–35 000. With the exception of the archaebacterial proteasome (Dahlmann et al., 1989), which is composed of two types of subunits, proteasome particles from other sources have a complex subunit composition. For example, the subunits of the human erythrocyte enzyme represent the products of 13 or more different genes encoding polypeptides of different amino acid sequence (Tanaka et al., 1988; Lee et al., 1990).

Wilk and Orlowski (1980, 1983), who originally described the enzyme in bovine pituitaries, showed that it could cleave bonds on the carboxyl side of basic, hydrophobic, or acidic amino acid residues. These activities were designated as trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide-hyrolyzing, respectively. Further, these investigators observed that the three activities were differentially sensitive to effector molecules and proposed that three distinct catalytic centers are responsible for the three activities. The essential observations that led to this hypothesis have been repeated and extended by several other laboratories with enzyme isolated from a wide variety of sources. Although it is generally agreed that the proteasome is a multiproteinase complex, the number of distinct active sites and the particular combinations of substrates and effectors that may be used to operationally define these active sites are still matters of contention. Moreover, the recent work of Yu et al. (1991) indicates the presence of a fourth catalytic site, distinct from the three mentioned above, which may perform the initial proteolytic cleavages during proteasome-catalyzed degradation of protein substrates.

The complete primary structures of several proteasome subunits have been determined, and the results indicate that the subunits studied to date constitute a family of evolutionarily related proteins (Zwickl et al., 1991). However, these subunits lack homology to other proteases. This has impeded attempts to assign specific catalytic functions to particular subunits and to identify amino acid residues of functional importance. Heinemeyer et al. (1991) have employed genetics to identify a subunit of the yeast proteasome that is required for the chymotrypsin-like activity and that may contain residues in the corresponding catalytic center, although there are no sequence motifs in this subunit that allow it to be assigned to one of the four recognized mechanistic classes of proteolytic enzymes. Orlowski and Michaud (1990) have shown that treatment of the bovine pituitary proteasome with diisopropyl fluorophosphate selectively inhibits the chymotrypsin-like activity. Sato and Shiratsuchi (1990) reported a similar finding and also showed that diisopropyl fluorophosphate is preferentially incorporated into the smallest subunit of chicken

† This work was supported by the American Heart Association, Texas Affiliate, Inc., and by the Muscular Dystrophy Association.

* To whom correspondence should be addressed at the Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center.

‡ Department of Biochemistry.

§ Howard Hughes Medical Institute.

‖ Department of Physiology.

0006-2960/92/0431-7347$03.00/0

liver proteasome. The sequence of this chicken subunit has not yet been reported, so its relationship to the yeast subunit of Heinemeyer et al. (1991) is unknown. In the present work, we have performed chemical modification experiments with *N*-ethylmaleimide (NEM)[1] and have identified a subunit containing a cysteinyl residue critical for the trypsin-like enzymatic function. Furthermore, the data suggest that this residue is located in the corresponding catalytic center of the native enzyme complex.

MATERIALS AND METHODS

Enzyme Purification. Latent proteasome was purified from bovine erythrocytes, bovine heart, and human erythrocytes using previously published procedures (McGuire et al., 1989; Dick et al., 1991) with the exception that DEAE-Fractogel (E.M. Sciences, Gibbstown, NJ) was used in place of DEAE-Sephacel. Measurements of protein concentration and assessment of purity were performed as previously described (Dick et al., 1991).

Assay of Enzyme Activity. Proteasome-catalyzed hydrolysis of the peptide substrates Z-Val-Leu-Arg-MNA, Z-Leu-Leu-Glu-βNA, Suc-Leu-Leu-Val-Tyr-AMC, and Z-Gly-Gly-Leu-AMC was assayed by continuously monitoring the production of the reporter groups with a Perkin Elmer Model 650-10S fluorescence spectrophotometer. The wavelength settings used for the different reporter groups were as follows: MNA, excitation 340 nm, emission 425 nm; βNA, excitation 335 nm, emission 410 nm; AMC, excitation 380 nm, emission 460 nm. Hydrolysis of Z-Gly-Gly-Leu-pNA was assayed by continuously monitoring production of the reporter group by its absorption at 400 nm.

Inactivation by NEM. Following purification, the enzyme buffer was exchanged with 50 mM phosphate (Na^+), pH 7.4, either by dialysis or by ultrafiltration to obtain a final enzyme concentration of 0.2–0.4 mg/mL; 90 μL of this solution was mixed with 10 μL of a freshly prepared NEM stock solution (50 mM) to give a final NEM concentration of 5 mM. At selected intervals, 10 μL of the enzyme solution was mixed with 1 mL of 50 mM Tris-HCl, pH 8.0, and 1 mM β-mercaptoethanol containing one of the four peptide substrates at the desired concentration (see figure legends). The enzyme–substrate mixture was transferred to a cuvette, and the progress curve for hydrolysis was monitored for 3–6 min. Prior to assay, the substrate solution was warmed to 30 °C, and the cell holder of the spectrophotometer was maintained at this temperature by a circulating water bath. The 100-fold dilution of the NEM and the molar excess of β-mercaptoethanol achieved upon mixing the enzyme and substrate solutions quench further reaction of NEM with the enzyme. The initial slopes of the progress curves were used as measures of reaction velocity.

Assays for leupeptin protection from NEM inactivation are described in the legend of Table I. Progress curves for Z-Val-Leu-Arg-MNA hydrolysis by leupeptin-bound enzyme exhibited a lag phase that persisted for >20 min (data not shown). Presumably this reflects the slow dissociation of this tightly bound inhibitor (Mason, 1990) upon dilution of the enzyme–leupeptin complex into the substrate solution. No lag in the approach to steady state was observed in progress curves for leupeptin-protected, NEM-modified enzyme after dialysis (see Table I), indicating that dialysis was effective in removing leupeptin.

Labeling of the Proteasome with [^{14}C]NEM. [^{14}C]NEM, purchased from New England Nuclear (E. I. du Pont de Nemours and Co. Inc., Wilmington, DE), was supplied at a concentration of 2.5 mM in 0.5 mL of pentane with a specific activity of 85 dpm/pmol. The pentane solution was layered on top of 250 μL of water in a 5-mL conical bottom vial. The open vial was placed in a fume hood, and the pentane was allowed to evaporate (approximately 2 h). The aqueous 5 mM solutions of [^{14}C]NEM obtained in this way were stable for at least 10 days when stored at 4 °C as judged by the efficiency of incorporation of radiolabel into protein.

The enzyme samples used for labeling with [^{14}C]NEM were prepared in 50 mM phosphate (Na^+) buffer, pH 7.4, at a concentration of 3–5 mg/mL; 100 μL of enzyme was mixed with 25 μL (5 μCi) of [^{14}C]NEM to give a final NEM concentration of 1 mM. After 1 h at room temperature, the reaction was quenched by adding a 4-fold molar excess of β-mercaptoethanol. Unincorporated [^{14}C]NEM, which represented ≥97% of the total radioactivity in the samples, was removed by gel filtration on a 2-mL disposable column of Sephadex G-50 equilibrated with phosphate buffer. A total of 15 fractions of 2 drops (approximately 90 μL) were collected, and the peak of radiolabeled protein was identified by subjecting 5 μL of each fraction to liquid scintillation analysis in 5 mL of liquid scintillation cocktail (Aquasol-2, New England Nuclear). The enzyme elutes in the void volume, so it was easy to judge when to terminate flow through the column, thus minimizing the volume of radioactive waste. For ^{14}C labeling of the enzyme in the presence of leupeptin, the enzyme samples were mixed with leupeptin at a final concentration of 20 μM and allowed to equilibrate for ≥1 h before addition of NEM. The reactions of samples with or without leupeptin were staggered for 30 min, which allowed sufficient time to complete the gel filtration step.

SDS–PAGE and Radioanalytic Imaging. ^{14}C-Labeled enzyme samples were subjected to SDS–PAGE in 12.5%, 1-mm-thick gels. Protein bands were visualized by staining with Coomassie Blue. The gel was dried on filter paper, and an Ambis radioanalytic imaging system (Ambis Systems, Inc., San Diego, CA) was used to measure β^- particle emission from the dried gel. Use of the 0.8 × 3.2 mm resolution plate and a scan time of 20 h yielded the image in Figure 2. The total number of β^- particles detected in the area of the proteasome bands was approximately 10 times greater than the background detected in an equivalent area on an unused portion of the gel. Each gel lane contained 17 μg of protein with a total activity of 35 000 cpm. Molecular weight values were estimated with low-range SDS–PAGE standards (Bio-Rad Laboratories, Richmond, CA).

Separation of Subunits by HPLC. Reverse-phase HPLC separations of proteasome subunits were performed using a 6 × 150 mm Shodex RSpak D4-613 column run on a Waters (Millipore Inc., Milford, MA) HPLC system. Chromatography was performed at 40 °C in 0.05% (v/v) trifluoroacetic acid in water at a flow rate of 0.75 mL/min. Elution of the protein was achieved with a gradient of acetonitrile begun 10 min after injection. The gradient program included two phases which differed in slope: 10–20 min, 0–25% acetonitrile; 20–145 min, 25–42% acetonitrile. The chromatograms shown in

[1] Abbreviations: NEM, *N*-ethylmaleimide; DEAE, diethylaminoethyl; MNA, 4-methoxy-2-naphthylamine; βNA, β-naphthylamine; Suc, succinyl; AMC, 7-amino-4-methylcoumarin; pNA, *p*-nitroaniline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin; IB_{ox}, oxidized B chain of bovine insulin; PIR, National Biomedical Research Foundation Protein Identification Resource; Swiss-Prot, Swiss-Prot protein sequence data bank.

Figure 3 were obtained from samples that contained approximately 50 μg of total protein. For preparative isolation of subunits, samples containing as much as 500 μg of protein were employed without any significant loss of resolution. The absorbance of the column eluant was monitored at 214 nm, and the UV chromatograms were digitized, stored, and integrated using a Waters Model 840 chromatography data station. For quantitation of ^{14}C associated with the chromatographic peaks, the individual peaks (typically 1–3 mL) were collected manually into scintillation vials, lyophilized, and counted in 10 mL of liquid scintillation cocktail.

Peptide Mapping. Fractions isolated using the HPLC protocol described above were dried in a vacuum centrifuge. Typically, the fractions contained 5–20 μg of protein in 2 mL of acetonitrile/water with 0.05% TFA. Before fractions were dried, 2 μL of 10% (w/v) Lubrol PX detergent was added to prevent adsorption of the polypeptide to the surface of the tube. Tests of this procedure with the radiolabeled polypeptides were performed, and it was found that, provided the detergent was present during the drying process, virtually 100% of the radiolabel could be recovered by dissolving the pellet in 75 μL of deionized distilled water. The samples were buffered with 25 μL of 0.2 M Tris-HCl, pH 7.9; 0.2 μg of chymotrypsin was added and the digestion proceeded for 20 h at 37 °C. After digestion, the samples were acidified with 100 μL of 1% (v/v) TFA and stored frozen for subsequent analysis.

Digestion mixtures were subjected to reverse-phase HPLC on a 2.1 × 100 mm Aquapore RP-300 column (Applied Biosystems Inc., Foster City, CA) at 40 °C in 0.05% (v/v) TFA in water at a flow rate of 0.25 mL/min. Elution was performed with a 120-min linear gradient of 0–70% acetonitrile begun 10 min after injection. For measurements of peptide-associated radiolabel (Figure 6), 1.5-min (375 μL) fractions were collected in scintillation vials and subjected to liquid scintillation counting in 5 mL of cocktail. For some experiments, the chymotryptic peptides were further purified by subjecting them to reverse-phase HPLC on a 2.1 × 100 mm Aquapore RP-300 column at 40 °C in 0.1% (w/v) ammonium acetate in water at a flow rate of 50 μL/min. The column was run on an Applied Biosystems Inc. Model 130A HPLC. Elution was performed with a 100-min linear gradient of 0–70% acetonitrile/0.1% (w/v) ammonium acetate, begun 20 min after injection.

N-Terminal Amino Acid Sequencing. Peptides were subjected to automated Edman degradation on an Applied Biosystems Inc. Model 470A amino acid sequencer with a Model 120A PTH-amino acid analyzer, using standard manufacturer's programming and chemicals. Pyridylethylation of cysteinyl residues was performed by the method of Andrews and Dixon (1987).

Fast Atom Bombardment Mass Spectrometry. Peptides were prepared in thioglycerol by adding 3 μL of thioglycerol to the HPLC column fractions (typically 25–200-μL volume) and drying in a vacuum centrifuge until only the small drop of thioglycerol remained. Spectra were obtained with a VG 30-250 quadrupole mass spectrometer using xenon as the reagent gas.

RESULTS

NEM Inactivation of Proteasome Activity. Treatment of the proteasome with NEM resulted in a time-dependent loss of the enzyme's ability to hydrolyze Z-Val-Leu-Arg-MNA (Figure 1A). The half-time for inactivation was inversely proportional to NEM concentration over the range of 1–10 mM (data not shown), $k_{obs/[I]} = 0.5\ s^{-1}\ M^{-1}$. Treatment of the enzyme with NEM for 10 min or longer, conditions that yielded ≥80% inactivation of Z-Val-Leu-Arg-MNA-hydrolyzing (trypsin-like) activity, had no effect on the proteasome's ability to hydrolyze Z-Leu-Leu-Glu-βNA or Suc-Leu-Leu-Val-Tyr-AMC (Figure 1A). Z-Gly-Gly-Leu-AMC hydrolysis was also inactivated by NEM treatment, but at a much slower than that that observed for Z-Val-Leu-Arg-MNA (Figure 1A). Both Rivett (1989) and Wilk and Orlowski (1980) have obtained results with NEM that are qualitatively similar to those shown in Figure 1A as regards the relative specificity of this reagent for the trypsin-like activity. In previous work on proteasome-catalyzed degradation of a model polypeptide substrate, the oxidized B chain of bovine insulin (IB_{ox}) (Dick et al., 1991), cleavage at one of the six major sites, Gln_4-His_5, was found to be carried out in the same catalytic center as that which cleaves Z-Val-Leu-Arg-MNA. In the present study, NEM-treated proteasome was found to lose the ability to cleave IB_{ox} at Gln_4-His_5, but cleavage at the other sites was undiminished (data not shown). These data further illustrate the specificity of NEM inactivation for the trypsin-like catalytic center.

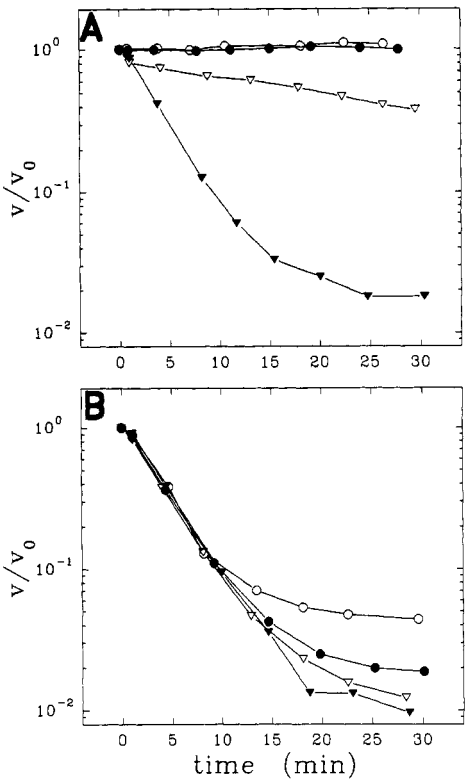

FIGURE 1: NEM modification of the proteasome differentially affects peptidase activities of the enzyme. Fractional activity remaining (ordinate, V/V_0) is plotted against the duration of the NEM modification reaction (abscissa). The assay protocol is described under Materials and Methods. (A) NEM inactivation kinetics for hydrolysis of Z-Leu-Leu-Glu-βNA (○), Suc-Leu-Leu-Val-Tyr-AMC (●), Z-Gly-Gly-Leu-AMC (▽), and Z-Val-Leu-Arg-MNA (▼). The substrate concentration was 50 μM in each experiment. (B) NEM inactivation kinetics were determined for the Z-Val-Leu-Arg-MNA-hydrolyzing (trypsin-like) activity utilizing substrate concentrations of 50 (▼), 100 (▽), 200 (●), and 400 μM (○) in the assays. The K_M for Z-Val-Leu-Arg-MNA estimated from the V_0 values measured in this experiment was ≥350 μM.

During the first 10–15 min of exposure to NEM, 90% of the Z-Val-Leu-Arg-MNA-hydrolyzing activity was lost in an apparent first-order process (Figure 1B), consistent with a single-hit mechanism. However, the first-order plots (Figure 1B) showed curvature after longer times of exposure to the reagent, suggesting that a small fraction of the activity is

Table I: Leupeptin Protects the Trypsin-like Activity of the Proteasome from NEM Inactivation[a]

substrate	sp act. (% of control)[b] minus leupeptin	plus leupeptin
Z-Leu-Leu-Glu-βNA	71 ± 4[c]	84 ± 4
Suc-Leu-Leu-Val-Try-AMC	82 ± 5	67 ± 4
Z-Gly-Gly-Leu-pNA	59 ± 6	56 ± 5
Z-Val-Leu-Arg-MNA	7.4 ± 0.4	42 ± 2

[a] Proteasome was reacted with 5 mM NEM at 30 °C either in the absence (minus) or in the presence (plus) of 20 μM leupeptin. The reactions were quenched after 20 min with β-mecaptoethanol, and the enzyme samples were dialyzed to remove leupeptin (see Materials and Methods). [b] Protein concentrations and peptidase activities of the enzyme samples were measured, and the data are expressed as the percent specific activity relative to an untreated enzyme sample. [c] The values are the mean ± SD for three measurements.

insensitive to NEM modification. This "NEM-insensitive" Z-Val-Leu-Arg-MNA-hydrolyzing activity was approximately 5% of the total in assays performed at 400 μM substrate concentration after 30 min of NEM exposure (Figure 1B). The residual activity may be due to partial inhibition by NEM of the catalytic center that hydrolyzes Z-Val-Leu-Arg-MNA. Alternatively, the residual activity may represent hydrolysis of this substrate by an NEM-insensitive catalytic center of the enzyme. Each of these interpretations has different implications as regards the functional role of the NEM-modified residue (see Discussion).

Leupeptin Protection from NEM Inactivation. Leupeptin, a peptide aldehyde that contains a C-terminal arginal residue, is a specific competitive inhibitor of the trypsin-like activity and is believed to occupy that catalytic center (see Discussion). Inhibition by leupeptin of proteasome-catalyzed hydrolysis of small peptide substrates containing P_1 arginyl residues (e.g., Z-Val-Leu-Arg-MNA) differentiates and operationally defines the trypsin-like activity of the enzyme (Wilk & Orlowski, 1983). In order to test the hypothesis that the site of NEM modification that results in loss of the trypsin-like activity is located in the leupeptin binding site, we determined the ability of leupeptin to protect the trypsin-like activity from NEM inactivation. The results are shown in Table I. Recovery of the trypsin-like actvity from NEM-modified, leupeptin-protected enzyme was approximately 6-fold higher than that from unprotected enzyme, whereas approximately equal amounts of three other peptidase activities were recovered from both protected and unprotected enzyme. Although no loss of the activities hydrolyzing Z-Leu-Leu-Glu-βNA or Suc-Leu-Leu-Val-Tyr-AMC was apparent in the 30-min period shown in Figure 1A, a partial loss of these activities did occur during the 2 days of dialysis required to remove leupeptin (Table I). Two observations suggest that this could be explained by instability of the NEM-modified enzyme. First, the SDS–PAGE pattern observed for NEM-modified enzyme was altered after dialysis, suggesting that partial denaturation and autolysis had occurred (data not shown). Second, whereas a low concentration of SDS (0.04%) stimulates activity of the unmodified enzyme when assayed with Z-Leu-Leu-Glu-βNA, Suc-Leu-Leu-Val-Tyr-AMC, or Z-Gly-Gly-Leu-AMC, the NEM-modified enzyme is rapidly inactivated by the detergent (data not shown). We also compared the abilities of unprotected and leupeptin–protected, NEM-modified enzyme samples to fragment IB_{ox}. The leupeptin-protected enzyme retained the ability to cleave IB_{ox} at Gln_4-His_5 (data not shown). The results obtained with both the peptide substrates and IB_{ox} suggest that NEM inactivates the trypsin-like activity of the proteasome by modifying a residue or residues in a leupeptin binding site.

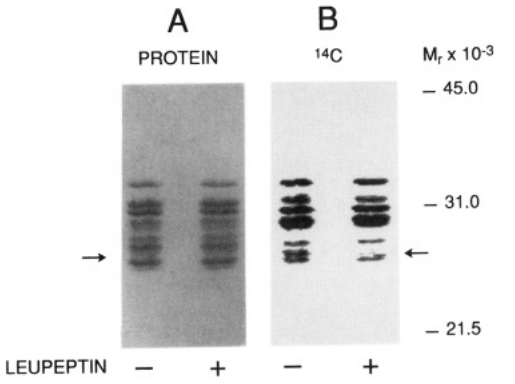

FIGURE 2: Identification of a subunit that leupeptin protects from labeling with [14C]NEM. Proteasome labeled with [14C]NEM either in the absence (–) or in the presence (+) of leupeptin was fractionated by SDS–PAGE, and the gel was analyzed by Coomassie Blue staining (A) and radioanalytic imaging (B). Arrows indicate the position of the leupeptin-protected band. This band stains faintly with Coomassie Blue but becomes clearly visible upon purification (Figure 3D).

Identification and Isolation of an NEM-Modified, Leupeptin-Protected Subunit. Comparisons of proteasome labeled with [14C]NEM in the presence and absence of leupeptin were performed in order to identify subunits of the enzyme complex that leupeptin could protect from NEM modification. SDS–PAGE analysis of the samples is shown in Figure 2. Staining of the gel with Coomassie Blue revealed a group of 10 bands corresponding to polypeptide subunits of the enzyme complex (Figure 2A). Radioanalytic imaging of the gel (Figure 2B) revealed a band that had incorporated less 14C in the leupeptin-protected sample than it had in the unprotected sample (see arrow in Figure 2). Quantitative analysis of this image indicated that the peak intensity of this band in unprotected enzyme was 50% higher than in protected enzyme. Although comparisons of the other radiolabeled bands revealed some smaller differences of ≤12% in peak intensity, the difference observed in the band indicated by the arrow (Figure 2) was the only difference consistently observed in three independent experiments. Labeling resulted in the incorporation of approximately 2.2×10^3 cpm/μg of protein in the absence of leupeptin. Given a molecular weight of 6.5×10^5 for the proteasome and 85 dpm/pmol for the specific activity of the [14C]NEM, the level of enzyme-associated 14C corresponds to an average of ≥17 NEM-modified sites per enzyme particle. In the presence of leupeptin, the total incorporation of radiolabel was 6–7% lower than in the absence of leupeptin, suggesting that an average of one site per enzyme particle was protected by the ligand.

Samples of enzyme that had been labeled with [14C]NEM in the presence or absence of leupeptin were next analyzed by reverse-phase HPLC. Figure 3A,B shows a comparison of the polypeptide maps of the two samples. The chromatograms are indistinguishable and virtually identical to the chromatogram observed for unmodified enzyme (data not shown). The individual peaks were manually collected from the HPLC, and the amount of 14C in each fraction was measured by liquid scintillation counting. The specific radioactivity of the polypeptide in peak 13 was approximately 1.5-fold higher in unprotected than in leupeptin-protected enzyme samples (Figure 3C). Peak 13 showed variable electrophoretic mobility when analyzed by SDS–PAGE. Values for the apparent molecular weight ranged from 26 700 to 27 700, with a mean value of 27 200 corresponding to the pattern shown in Figure 3D. Similar variability from experiment to experiment in electrophoretic mobility was also observed in the leupeptin-protected subunit from unfractionated [14C]NEM-labeled pro-

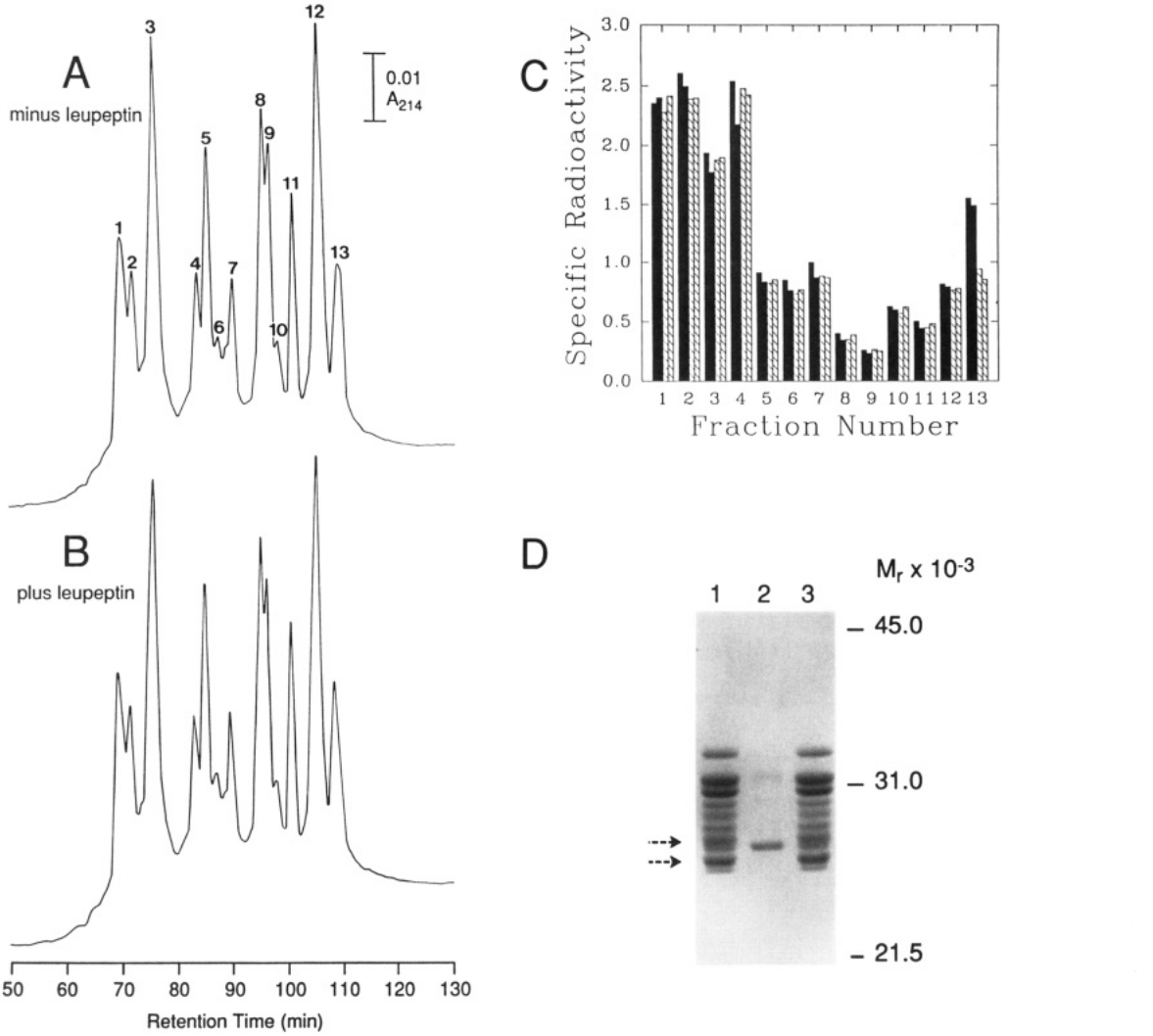

FIGURE 3: Isolation of an NEM-modified, leupeptin-protected proteasome subunit. Proteasome samples labeled with [^{14}C]NEM were subjected to reverse-phase HPLC as described under Materials and Methods. Panels A and B show chromatograms of UV absorption at 214 nm obtained for unprotected and leupeptin-protected samples, respectively. HPLC column fractions were collected according to the numbering in panel A, and the amounts of ^{14}C in each fraction were determined. (C) The specific radioactivities of the fractions were compared for enzyme samples labeled in the presence (hatched bars) or absence (black bars) of leupeptin. The ordinate values were calculated as the cpm for a given fraction divided by the integrated intensity of the corresponding peak in the UV chromatogram. The histogram includes data from two independent experiments. (D) SDS–PAGE of the polypeptide contained in peak 13 derived from 100 μg of proteasome (lane 2) compared with whole enzyme (lanes 1 and 3, 20 μg of proteasome each). The bands were visualized by staining the gel with Coomassie Blue. Arrows indicate the range of variability in electrophoretic migration of the polypeptide in peak 13 (see text).

teasome. Although the reason for this variability has not yet been determined, the subunit showed highly reproducible behavior on reverse-phase HPLC which allowed it to be identified and purified for subsequent analysis.

Identification of the NEM-Modified, Leupeptin-Protected Residue. The polypeptide corresponding to peak 13, hereafter designated "subunit 13", was isolated from unmodified, NEM-modified, and NEM-modified, leupeptin-protected enzyme and subjected to digestion with chymotrypsin. Reverse-phase chymotryptic peptide maps are shown in Figure 4. A prominent peak in the peptide map of unmodified subunit 13 (peak A) was decreased in intensity in the peptide maps of NEM-modified samples. Also, two additional peaks (B and C) appeared only in the peptide maps of the NEM-modified samples. Peak A was higher and peaks B and C were lower relative to the remaining peaks in the leupeptin-protected sample than in the unprotected sample. These results suggest that the leupeptin-protected residue is associated with the region of subunit 13 sequence from which peptides A, B, and C are derived.

The material eluting with peak A was collected and re-purified by reverse-phase HPLC using solvents buffered with ammonium acetate (see Materials and Methods). Automated Edman degradation resulted in the identification of a residue in all but 2 of the first 11 cycles (Table II). Cycles 2 and 9 contained no detectable residue. Analysis of the peptide by fast atom bombardment mass spectrometry (Figure 5) provided a value for the mass of the protonated molecular ion that was consistent with the presence of the amino acids identified by sequencing plus two cysteinyl residues, which normally would not have been detected as PTH amino acids during Edman degradation of nonalkylated peptides. The masses of several fragment ions were consistent with the sequence determined by Edman degradation (Figure 5, inset). The identification of cysteine at positions 2 and 9 was confirmed by S-alkylation with vinylpyridine which resulted in identification of the PTH derivative of (pyridylethyl)cysteine at cycles 2 and 9 upon subsequent sequence analysis.

Sequencing of peak C indicated that more than one peptide was present. Nevertheless, the most abundant PTH amino

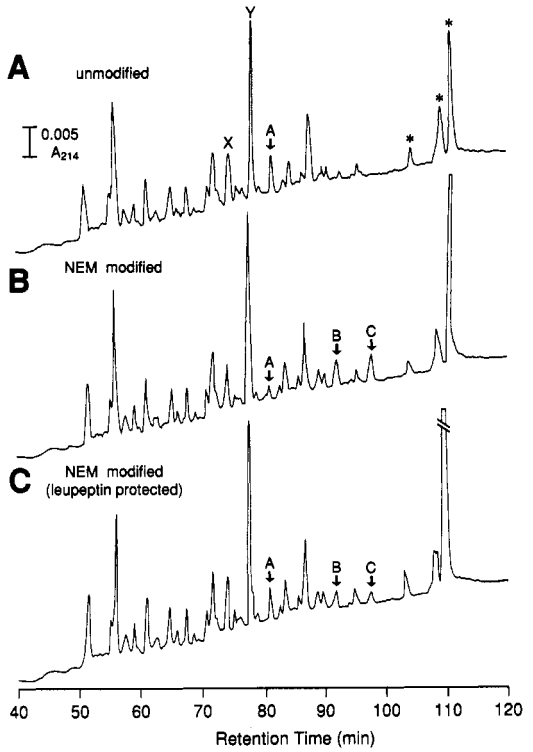

FIGURE 4: Reverse-phase chymotryptic peptide maps of the NEM-modified, leupeptin-protected proteasome subunit. Subunit 13 was isolated from (A) unmodified proteasome, (B) NEM-modified proteasome, and (C) NEM-modified, leupeptin-protected proteasome and subjected to digestion with chymotrypsin. The peptides generated were analyzed by reverse-phase HPLC. The arrows and letters designate various peptides discussed in the text and Table II. Asterisks designate peaks due to Lubrol PX detergent which was added after isolation of subunit 13 by HPLC to improve yields during dry-down prior to digestion (see Materials and Methods).

Table II: Amino Acid Sequencing of Subunit 13 Peptide Fragments[a]

Peptides containing a leupeptin-protected cysteinyl residue	
ct A	I C S L D L I G Ⓒ P M . . .
ct B	I G Ⓒ P M V T D D F V V . . .
ct C	I C S L D L I G Ⓒ P M V T D D F V V S G T . . .
Additional subunit 13 sequences	
CNBr1	(M)G D R L Y I G L A G L A T D V Q T V A Q R L K F R L N L Y E L K E G . . .
	t1 t2
	t3
CNBr2	(M)K G K N X V A I A A D R R F G I Q A Q
CNBr3	(M)V T T D F Q K I F P . . .
	t4
CNBr4	C(M)V A N L L Y E K R F G P Y Y T E P V I A G L D P . . .
	ctX ctY
CNBr5	(M)G V I V H I I E K D K I T T R T L K A R . . .
CNBr6	(M)D P E X L F E T I S Q A . . .

[a] Sequences of peptides generated from subunit 13 by chymotrypsin (ct A, B, C, X, Y; see Figure 4), trypsin (t1–4), and cyanogen bromide (CNBr1–6). The circled C denotes the leupeptin-protected cysteinyl residue in ct A, B, or C. M in parentheses denotes the methionine assumed to be located N-terminal to the first residue of each CNBr peptide. Note that 15 peptides are displayed here in 6 stretches of sequence that have been assembled by the method of overlaps.

acids detected in the first 11 cycles of Edman degradation matched the sequence of peptide A (Table II). As with peptide A, no signal from the cysteine at cycle 2 was recorded. However, PTH amino acid analysis for cycle 9 recorded a

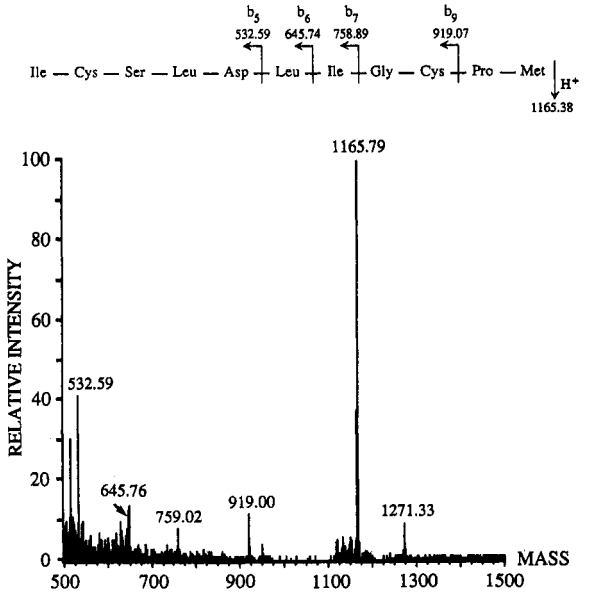

FIGURE 5: Analysis of chymotryptic peptide A by fast atom bombardment mass spectrometry. The inset shows the sequence of peptide A along with the calculated masses of the protonated molecular ion and several fragments that were observed in the spectrum.

unique doublet with a retention time between the PTH derivatives of proline and methionine. Smyth and Colman (1991) have interpreted these two signals as the two stereoisomers of NEM-modified cysteine. The sequence of peptide C extended beyond the C-terminus of peptide A (the 11th residue) for at least 10 residues, suggesting that NEM modification of the cysteine at position 9 hinders chymotryptic cleavage after the methionine at position 11 (Table II).

N-Terminal sequence analysis of peak B also yielded a mixture of two or more PTH amino acids at each cycle of Edman degradation. No single sequence was clearly most abundant, but comparison with the sequences of fragments A and C suggested that one of the peptides in peak B has a sequence identical to that beginning with the seventh residue of peptide A or C. This suggests that NEM modification promotes cleavage by chymotrypsin after the leucyl residue at position 6 in peptides A and C.

Fractions collected from chymotryptic peptide maps of ^{14}C-labeled subunit 13 were analyzed by liquid scintillation counting. The results are expressed in Figure 6 as the fraction of the total radioactivity associated with each peak. Prior to peptide mapping, an aliquot of each sample was subjected to liquid scintillation counting so that the recovery of radioactive material from the HPLC column could be determined. Virtually 100% of the ^{14}C injected into the column was recovered within the range of retention times shown. Figure 6 shows that the fraction of radioactivity associated with peaks B and C is lower in leupeptin-protected samples than in unprotected samples and that the fractions associated with other peaks are correspondingly higher, a result concordant with the identification of the leupeptin-protected cysteinyl residue above. Among the other radiolabeled components, the prominent one designated peak X (Figures 4 and 6) was subjected to amino acid sequence analysis (Table II) and was found to contain a peptide with a sequence different from peptides A, B, or C. The sequence begins with a cysteinyl residue, which like the cysteinyl residue in peptides A, B, and C is susceptible to NEM labeling. However, this cysteine was not protected from labeling by leupeptin. Other radioactive peaks in Figure 6 have not yet been sequenced, but

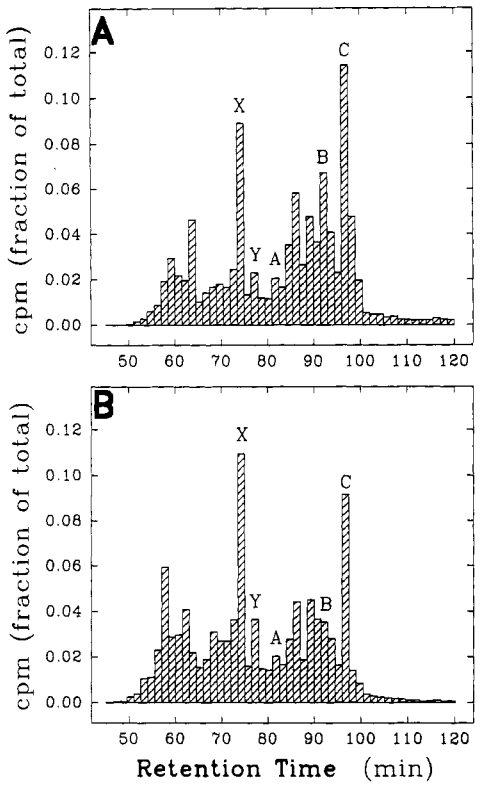

FIGURE 6: Reverse-phase chymotryptic peptide maps of [^{14}C]NEM-labeled subunit 13. Subunit 13 was isolated from proteasome that had been labeled with [^{14}C]NEM either in the absence (A) or in the presence (B) of leupeptin. Chymotryptic peptide mapping was performed as in Figure 4. Fractions from the HPLC column were collected, and the amount of ^{14}C in each fraction was determined. Each bar corresponds to one fraction. The ordinate values on the histogram were calculated by dividing the cpm found in each fraction by the total cpm that eluted from the column. The letter designations of various column fractions indicate their correspondence to peaks observed in the UV chromatograms of Figure 4.

presumably contain peptides that have one or the other of these three cysteinyl residues, or have other cysteines yet to be identified.

Relationship of Subunit 13 to Other Proteasome Subunits. Further amino acid sequence information was acquired for subunit 13 to assist in elucidating its relationship to other proteasome subunits. Samples of subunit 13 were subjected to digestion with trypsin or cleavage with cyanogen bromide, and the resulting fragments were isolated by reverse-phase HPLC. Four tryptic peptides and six cyanogen bromide peptides were sequenced, and the results are shown in Table II. These sequences encompass approximately 60% of the primary structure of subunit 13, assuming a molecular weight of 27 200. Lee et al. (1990) employed two-dimensional gel electrophoresis to isolate and partially characterize several proteasome subunits. The spots in their electrophoretograms were given Greek letter designations. The partial amino acid sequence information acquired in the course of the present work indicates that subunit 13 corresponds to the spot designated as θ (pI = 6.7) in that work.

No identical matches were obtained in a computer-aided comparison of the subunit 13 peptides with the PIR and Swiss-Prot amino acid sequence data bases. Likewise, comparisons with other proteasome subunit sequences not yet contained in the data bases revealed no identical matches. However, one of the peptides (CNBr4, Table II) was found to be similar to proteasome subunit C5 from rat (Tamura et al., 1990) and from yeast (Lee et al., 1992). An alignment is presented in Figure 7. Proteasome subunits hitherto sequenced constitute

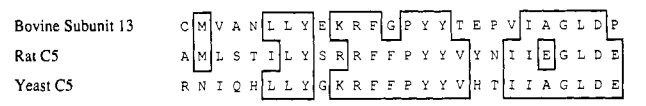

FIGURE 7: Similarity between a peptide from bovine subunit 13 and sequences from rat and yeast subunit C5. CNBr fragment 4 (see Table II) is aligned with residues 117–142 from rat C5 (Tamura et al., 1991) and with residues 110–135 from yeast C5 (Lee et al., 1992).

a family of evolutionarily related polypeptides (Tamura et al., 1991; Zwickl et al., 1991). The similarity of CNBr4 to a region of C5 suggests that subunit 13 represents a member of this same family. However, none of the other peptide sequences (Table II) showed similarity to previously sequenced proteasome subunits, suggesting that subunit 13 may have diverged from other members of the family more than these family members have diverged from one another. Subunit C5 also shows a relatively high degree of divergence from the remainder of the family (Tamura et al., 1991).

A preliminary investigation of the evolutionary conservation of subunit 13 was conducted by isolating a subunit of the human red blood cell enzyme that shows similar chromatographic behavior to subunit 13 from bovine blood on reverse-phase HPLC (Figure 3A). The chymotryptic peptide map of this human proteasome subunit was nearly identical to the peptide map of bovine subunit 13 shown in Figure 4A. One of the chymotryptic fragments was identical in retention time to fragment A and showed the same sequence and mass spectrum (Figure 5), suggesting that the overall primary structure of this polypeptide, including the region surrounding the leupeptin-protected cysteinyl residue, has been evolutionarily conserved in mammals.

DISCUSSION

We have found that leupeptin, a reversible inhibitor of the proteasome's trypsin-like activity, can protect that activity from inactivation by NEM modification. The trypsin-like activity was first defined by Wilk and Orlowski (1983) as the activity of the proteasome from bovine pituitary that catalyzes the hydrolysis of Z-D-Ala-Leu-Arg-βNA and D-Ala-Phe-Leu-Arg-βNA. This activity was distinguished from a chymotrypsin-like activity that hydrolyzes Z-Gly-Gly-Leu-pNA, and also from an activity that hydrolyzes Z-Leu-Leu-Glu-βNA, by its susceptibility to inhibition by leupeptin. Inhibition by leupeptin of proteasome-catalyzed hydrolysis of small peptide substrates containing P_1 arginyl residues has since been found to be a characteristic feature of all eukaryotic proteasomes tested. Furthermore, leupeptin's inhibitory effect has been consistently observed to be specific for peptide substrates containing P_1 arginyl residues as opposed to peptide substrates with other P_1 residues frequently employed to assay proteasome activity, e.g., Suc-Leu-Leu-Val-Tyr-AMC and Gly-Ala-Ala-Phe-AMC (Tanaka et al., 1986; Rivett, 1989).

The mechanism by which leupeptin inhibits the trypsin-like activity of the proteasome is unknown. However, it is generally assumed that the mechanism is analogous to leupeptin inhibition of trypsin or papain (Schultz et al., 1989) in which leupeptin acts competitively by occupying the catalytic center. We have identified a subunit of proteasome that is protected from NEM modification by bound leupeptin. Under the hypothesis that the leupeptin binding site is a substrate binding site, the data suggest that residues of subunit 13 contribute to the active site responsible for the proteasome's trypsin-like activity. Although the simplest model for proteasome enzymatic function assumes that there is a one to one correspondence of active sites to subunits, a reasonable

alternative which is also compatible with the present data is that a single active site could be formed by quaternary interactions, i.e., the juxtaposition of two or more subunits within the proteasome complex. Heinemeyer et al. (1991) have suggested this as one possible explanation for their finding that mutations of two different yeast proteasome subunits both result in a loss of the chymotrypsin-like activity.

We also have found evidence that a relationship of homology exists between subunit 13 and previously sequenced proteasome subunits. These constitute a family that shows no similarity to other proteases (DeMartino et al., 1991). Because the list of subunits which have hitherto been sequenced remains incomplete, the homology of these subunits to one another has not precluded the possibility that the subunits which mediate catalysis belong to a different family, possibly one which is related to known serine or cysteine proteinases, while the subunits whose sequences are known act in some regulatory role. The present data suggest that at least one catalytic subunit, that responsible for the trypsin-like activity, is indeed a member of the proteasome family and therefore lend support to the hypothesis that this family represents a structurally and perhaps mechanistically novel class of proteolytic enzymes.

Assuming that the leupeptin-protected cysteinyl residue is located in the substrate binding site, the question arises as to whether this residue functions in the catalytic mechanism. If this residue were involved in the catalytic mechanism in a manner analogous to the essential cysteinyl residues of cysteine proteases, then its modification by NEM would be expected to completely abolish its activity. However, this expectation contrasts with the results shown in Figure 1B, which revealed a small fraction of "NEM-insensitive" Z-Val-Leu-Arg-MNA-hydrolyzing activity. Two kinds of models can explain this observation. The first assumes that a structurally and functionally homogeneous population of catalytic centers is responsible for all of the trypsin-like activity in the unmodified enzyme. The modification of a catalytic center by NEM may increase K_M or decrease V_{max}, but may not completely inactivate the catalytic center. This model implies that the cysteinyl residue is not essential for catalysis. The second kind of model recognizes that the population of catalytic centers that hydrolyzes Z-Val-Leu-Arg-MNA may not be homogeneous. The proteasome can indeed be purified in two functionally distinct forms, termed latent and active (Tanaka et al., 1986). The latent form is unable to degrade large protein substrates, but can hydrolyze small peptide substrates (McGuire et al., 1989). Treatment of latent proteasome with SDS can reversibly activate the proteolytic activity (McGuire et al., 1989) and reversibly stimulate the peptidase activity (Tanaka et al., 1989). The biochemical basis for these phenomena is unclear, but presumably they reflect conformational alterations of the proteasome, giving reason to suspect that there may be some degree of heterogeneity in the enzyme samples. Thus, the NEM-insensitive activity may represent a subpopulation of catalytic centers that is inaccessible to NEM or that contains the cysteinyl residue in an unreactive form, e.g., bound to a metal ion. An additional alternative is that the Z-Val-Leu-Arg-MNA hydrolysis that remains after NEM treatment occurs at a different active site, e.g., an active site that catalyzes hydrolysis of one of the other substrates shown in Figure 1A. In contrast to the first model, neither of the latter two models would exclude the possibility that the leupeptin-protected cysteinyl residue functions in the catalytic mechanism. Distinguishing between these alternatives will require further study, possibly involving alternative sulfhydryl-modifying reagents.

Since the primary structures of proteasome subunits have been conserved over large evolutionary distances (Zwickl et al., 1991) and, with the exception of the archaebacterial proteasome (Dahlmann et al., 1989), a trypsin-like activity conforming to the functional criteria that we have employed is a constant feature of the enzyme regardless of its source, it is likely that homologues of this subunit exist in other species. The hypothesis that the cysteinyl residue we have identified plays an essential role in catalytic function leads to the prediction that it will be conserved in these homologues. In a more general sense, we expect that the residues essential for catalysis, whatever their nature, are conserved in proteasomes. A comparison of the primary structure of subunit 13 with its putative homologues from evolutionarily distant species (e.g., yeast, *Drosophila*) may aid in identifying those residues and thus help to elucidate the proteasome's catalytic mechanism.

ACKNOWLEDGMENT

We thank Kevin Kuehn for technical support in isolating the proteasome, J. Royce Potter for secretarial and graphics assistance, and the Waters Chromatography Division of Millipore for the use of a Shodex RSpak D4-613 column to separate subunits of the proteasome by reverse-phase HPLC. We are also grateful to Drs. Lou Hersh and Mike McGuire for critical reading of the manuscript.

REFERENCES

Andrews, P. C., & Dixon, J. E. (1987) *Anal. Biochem. 161*, 524–528.

Brown, M. G., Driscoll, J., & Monaco, J. J. (1991) *Nature 353*, 355–357.

Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R., & Baumeister, W. (1989) *FEBS Lett. 251*, 125–131.

DeMartino, G. N., Orth, K., McCullough, M. L., Lee, L. W., Munn, T. Z., Moomaw, C. R., Dawson, P. A., & Slaughter, C. A. (1991) *Biochim. Biophys. Acta 1079*, 29–38.

Dick, L. R., Moomaw, C. R., DeMartino, G. N., & Slaughter, C. A. (1991) *Biochemistry 30*, 2725–2734.

Glynne, R., Powis, S. H., Beck, S., Kelly, A., Kerr, L.-A., & Trowsdale, J. (1991) *Nature 353*, 357–360.

Goldberg, A. L. (1992) *Eur. J. Biochem. 203*, 9–23.

Heinemeyer, W., Kleinschmidt, J. A., Saidowski, J., Escher, C., & Wolf, D. H. (1991) *EMBO J. 10*, 555–562.

Kopp, F., Steiner, R., Dahlmann, B., Kuehn, L., & Reinauer, H. (1986) *Biochim. Biophys. Acta 872*, 253–260.

Lee, L. W., Moomaw, C. R., Orth, K., McGuire, M. J., DeMartino, G. N., & Slaughter, C. A. (1990) *Biochim. Biophys. Acta 1037*, 178–185.

Lee, D. H., Tanaka, K., Tamura, T., Chung, C. H., & Ichihara, A. (1992) *Biochem. Biophys. Res. Commun. 182*, 452–460.

Mason, R. W. (1990) *Biochem. J. 265*, 479–484.

McGuire, M. J., McCullough, M. L., Croall, D. E., & DeMartino, G. N. (1989) *Biochim. Biophys. Acta 995*, 181–186.

Orlowski, M. (1990) *Biochemistry 29*, 10289–10297.

Orlowski, M., & Michaud, C. (1989) *Biochemistry 28*, 9270–9278.

Rivett, A. J. (1989) *J. Biol. Chem. 264*, 12215–12219.

Sato, S., & Shiratusuchi, A. (1990) *Biochim. Biophys. Acta 1041*, 269–272.

Schultz, R. M., Varma-Nelson, P., Ortiz, R., Kozlowski, K. A., Orawski, A. T., Pagast, P., & Frankfater, A. (1989) *J. Biol. Chem. 264*, 1497–1507.

Smyth, G. E., & Colman, R. F. (1991) *J. Biol. Chem. 266*, 14918–14925.

Tamura, T., Tanaka, K., Kumatori, A., Yamada, F., Tsurumi, C., Fujiwara, T., Ichihara, A., Tokunaga, F., Aruga, R., & Iwanaga, S. (1990) *FEBS Lett. 264*, 91–94.

Tamura, T., Lee, D. H., Osaka, F., Fujiwara, T., Shin, S., Chung, C. H., Tanaka, K., & Ichihara, A. (1991) *Biochim. Biophys. Acta 1089*, 95–102.
Tanaka, K., Ii, K., Ichihara, A., Waxman, L., & Goldberg, A. (1986) *J. Biol. Chem. 261*, 15197–15203.
Tanaka, K., Yoshimura, T., Ichihara, A., Ikai, A., Nishigai, M., Morimoto, Y., Sato, M., Tanaka, N., Katsube, Y., Kameyama, K., & Takagi, T. (1988) *J. Mol. Biol. 203*, 985–996.
Tanaka, K., Yoshimura, T., & Ichihara, A. (1989) *J. Biochem. (Tokyo) 106*, 495–500.
Wilk, S., & Orlowski, M. (1980) *J. Neurochem. 35*, 1172–1182.
Wilk, S., & Orlowski, M. (1983) *J. Neuochem. 40*, 842–849.
Yu, B., Pereira, M. E., & Wilk, S. (1991) *J. Biol. Chem. 266*, 17396–17400.
Zwickl, P., Lottsspeich, F., Dahlmann, B., & Baumeister, W. (1991) *FEBS Lett. 278*, 217–221.