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Catalytic Properties of 26 S and 20 S Proteasomes and Radiolabeling of MB1, LMP7, and C7 Subunits Associated with Trypsin-like and Chymotrypsin-like Activities*

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20 and 26 S proteasomes were isolated from rat liver. The procedure developed for the 26 S proteasome resulted in greatly improved yields compared with previously published methods. A comparison of the kinetic properties of 20 and 26 S proteasomes showed significant differences in the kinetic characteristics with certain substrates and differences in the effects of a protein substrate on peptidase activity. Observed differences in the kinetics of peptidylglutamyl peptide hydrolase activity suggest that the 26 S complex cannot undergo the conformational changes of 20 S proteasomes at high concentrations of the substrate benzyloxycarbonyl (Z) -Leu-Leu-Glu-β-naphthylamide. Various inhibitors that differentially affect the trypsin-like and chymotrypsin-like activities have been identified. Ala-Ala-Phe-chloromethyl (CH_2Cl) inhibits chymotrypsin-like activity assayed with succinyl (Suc) -Leu-Leu-Val-Tyr-AMC, but surprisingly not hydrolysis of Ala-Ala-Phe-7-amido-4-methylcoumarin (AMC). Tyr-Gly-Arg-CH_2Cl inhibits Suc-Leu-Leu-Val-Tyr-AMC hydrolysis as well as trypsin-like activity measured with *t*-butoxycarbonyl (Boc) -Leu-Ser-Thr-Arg-AMC, while Z-Phe-Gly-Tyr-diazomethyl (CHN_2) was found to inhibit only the two chymotrypsin-like activities. Radiolabeled forms of peptidyl chloromethane and peptidyl diazomethane inhibitors, [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl, [^{3}H]acetyl- and radioiodinated Tyr-Gly-Arg-CH_2Cl, and Z-Phe-Gly-Tyr-(^{125}I-CHN_2), have been used to identify catalytic components associated with each of the three peptidase activities. In each case, incorporation of the label could be blocked by prior treatment of the proteasomes with known active site-directed inhibitors, calpain inhibitor 1 or 3,4-dichloroisocoumarin. Subunits of labeled proteasomes were separated either by reverse phase-HPLC and SDS-polyacrylamide gel electrophoresis or by two-dimensional polyacrylamide gel electrophoresis followed by autoradiography/fluorography and immunoblotting with subunit-specific antibodies. In each case, label was found to be incorporated into subunits C7, MB1, and LMP7 but in different relative amounts depending on the inhibitor used, consistent with the observed effects on the different peptidase activities. The results strongly suggest a relationship between trypsin-like activity and chymotrypsin-like activity. They also help to relate the different subunits of the complex to the assayed multicatalytic endopeptidase activities.

The 20 S proteasome (multicatalytic proteinase complex) is a high molecular mass (700 kDa) multimeric proteinase that is found in all types of eukaryotic cells (1–4). It can be isolated by itself but also forms the catalytic core of the 26 S proteasome (5). Proteasomes are found both in the nucleus and cytoplasm and play a major role in both ubiquitin-dependent and ubiquitin-independent nonlysosomal pathways of intracellular protein turnover. They have also been implicated in the pathway by which antigens are processed for presentation by major histocompatibility complex (MHC)[1] class I molecules (reviewed in Refs. 6 and 7).

Proteasomes (20 S forms) are cylindrical particles that are composed of subunits of molecular mass of 20–35 kDa. Those isolated from archaebacteria (*Thermoplasma*) contain only two different types of subunit, which has made them a useful model for structural studies (8). Yeast proteasomes contain fourteen different subunits (9), but in animal cells there are additional non-essential subunits that are γ-interferon-inducible and contribute to antigen processing (*e.g.* Refs 10–13). All proteasomal subunits are encoded by members of the same gene family but they can be divided into two groups, α and β, based upon their similarity to either the α or β subunit of the archaebacterial proteasome. The structure forms a complex dimer made up of four rings with seven subunits in each ring (14).

Binding of regulatory complexes to the ends of the cylindrical structure can enhance the catalytic activity of proteasomes. 26 S proteasomes are composed of 20 S proteasome with 19 S (ATPase, PA700) complexes that contain many different subunits (15,16). Only the 26 S proteasome is able to degrade protein substrates such as polyubiquitinated proteins and ornithine decarboxylase in an ATP-dependent process. The peptidase activity of the 20 S proteasome is greatly enhanced upon binding of PA28 (11 S regulator), a 180-kDa hexamer consisting of two different subunits (17, 18). PA28 has been implicated in the antigen presentation pathway (19) and is inducible by γ-interferon.

The recent identification of a threonine residue as the catalytic nucleophile of proteasomes (20) has clearly shown that

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[1] The abbreviations used are: MHC, major histocompatibility complex; Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; CH_2Cl, chloromethyl; CHN_2, diazomethyl; AMC, 7-amido-4-methylcoumarin; nap, β-naphthylamide; Suc, succinyl; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

they represent a novel family of proteolytic enzymes. Eukaryotic proteasomes have multiple proteolytic activities that have been widely referred to as trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolase activities. Isolation of yeast mutants defective in proteasome chymotrypsin-like or peptidylglutamyl-peptide hydrolase activity has allowed identification of several β-type subunits of the yeast proteasome that influence catalytic activities (21–24), but even with the recently published structure of the yeast proteasome (25) many questions remain to be answered.

So far there is rather limited information available regarding functions of individual subunits of the mammalian complex where the situation is complicated by the additional β-type subunits. Three of the mammalian β-type subunits, as well as three non-essential γ-interferon-inducible subunits that can replace them, have putative catalytic N-terminal threonine residues, but the other four β subunits do not.

Inhibitor studies with both rat liver and bovine pituitary proteasomes have shown that there are at least five distinct peptidase sites within the mammalian complex (*e.g.* Refs. 26–29). More recent efforts to investigate inhibitors of the different peptidase activities of proteasomes have not really clarified the situation, and it is still not clear how these measured activities relate to the many different subunits of the complex. One subunit, MB1(X), has been identified as the major target for inhibition by lactacystin (30). Identification of the catalytic components responsible for the known activities is essential for a further understanding of the multiple peptidase activities of this multifunctional complex.

In this study[2] we have investigated aspects of the the kinetic properties of 20 and 26 S proteasomes isolated from rat liver. To make a direct determination of catalytic subunits associated with the distinct peptidase activities, we have prepared radiolabeled forms of three peptidyl chloromethane and peptidyl diazomethane inhibitors of proteasomes (31) that differentially affect the trypsin-like activity and two chymotrypsin-like activities. The results demonstrate direct labeling of subunits associated with these activities.

EXPERIMENTAL PROCEDURES

Materials—Rats (Wistar) were obtained from the University of Leicester Biomedical Services Unit or at the University of Bristol. Boc-Leu-Ser-Thr-Arg-4-amidomethylcoumarin (AMC) was purchased from the Peptide Institute, Japan. Ala-Ala-Phe-AMC, succinyl-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-β-naphthylamide, trypsin, Ala-Ala-Phe-CH_2Cl and glutaraldehyde were from Sigma Chemical Co. Tyr-Gly-Arg-CH_2Cl and Z-Phe-Gly-Tyr-CHN_2 were prepared as described (31, 32). *N*-succinimidyl-3-(2-pyridyldithio)-propionate was from Boehringer Mannheim. 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc) was obtained from Life Technologies, Inc. Na^{125}I (specific activity 2170 Ci/mmol) and [^{3}H]acetic anhydride (specific activity 5 Ci/mmol) were purchased from Amersham.

Purification of 20 and 26 S Proteasomes—20 S proteasomes were purified from fresh or frozen rat livers as described (27). For the purification of 26 S proteasomes, the early steps were based on a published procedure (33) but later steps were designed to improve the yield. Briefly, 100 g rat livers were homogenized in 3–4 volumes of buffer A (20 mM Tris/HCl, pH 7.0, containing 5 mM ATP, 1 mM mercaptoethanol, 0.1 mM EDTA, and 20% (v/v) glycerol). After removing cell debris from the homogenate by centrifugation at 100,000 × *g* for 60 min, the 26 S proteasome was sedimented by centrifugation at 140,000 × *g* for 6 h. The pellet was resuspended in buffer A and incubated with 20 ml of Biogel hydroxylapatite equilibrated in buffer A for 30 min. Unbound material was concentrated using an Amicon XM 50 membrane and layered on 6 sucrose gradients (10–30% sucrose in buffer A without glycerol, 31 ml each) and spun for 16 h at 113,000 × *g*. Fractions containing 26 S proteasome activity were pooled, concentrated, and loaded onto a Superose-6 column (Pharmacia 16/50, 50 mg of protein per run) equilibrated with 20 mM Tris/HCl, pH 7.5, containing 2 mM ATP, 1 mM mercaptoethanol, 100 mM NaCl, and 10% (v/v) glycerol. Active fractions were pooled and then applied to a Resource Q column (Pharmacia Biotech Inc., 1 ml) equilibrated with 20 mM Tris/HCl, pH 7.5, containing 1 mM mercaptoethanol and 10% (v/v) glycerol. The enzyme was eluted using a linear gradient from 0 to 500 mM NaCl in the equilibration buffer. Active fractions were concentrated and applied to a Superose-6 gel filtration column (Pharmacia 10/30) equilibrated in the same buffer as the earlier gel filtration step. All steps apart from those involving fast protein liquid chromatography were performed at 0–4 °C. The purified 26 S proteasome preparation was dialyzed against buffer A containing 1 mM dithiothreitol instead of mercaptoethanol and stored either on ice or at −20 °C.

The purity of proteasome preparations was judged by SDS-PAGE (34), and protein concentrations were determined by the Bradford method (35) using bovine serum albumin as standard.

Proteasome Assays—Peptidase activities of 20 S proteasomes were assayed with synthetic peptide substrates in 50 mM Hepes buffer, pH 7.5 or 8.0, as described previously (27). For the continuous assays, data were analyzed using FluSys software kindly provided by Rawlings and Barrett (36). Trypsin-like activity was assayed with 50 μM Boc-Leu-Ser-Thr-Arg-AMC, chymotrypsin-like activities were assayed with 50 μM Ala-Ala-Phe-AMC and 50 μM Suc-Leu-Leu-Val-Tyr-AMC, and peptidylglutamyl peptide hydrolase activity was assayed with Z-Leu-Leu-Glu-nap as substrates. Inhibitor studies with 20 and 26 S proteasomes were carried out by assaying peptidase activities after preincubation of enzyme with inhibitor for 30 min at 25 °C. Further details are given in figure and table legends.

Radiolabeling of Ala-Ala-Phe-CH_2Cl, Tyr-Gly-Arg-CH_2Cl, and Z-Phe-Gly-Tyr-CHN_2—Iodination of the inhibitors containing tyrosine was carried out using iodobeads from Pierce (37). I-Tyr-Gly-Arg-CH_2Cl and ^{125}I-Tyr-Gly-Arg-CH_2Cl were prepared by incubating 3 mM NaI or 3 mM Na^{125}I (1 mCi), respectively, in a 1 mM solution of Tyr-Gly-Arg-CH_2Cl in 100 mM sodium phosphate buffer, pH 7.0, with Iodobeads according to the manufacturer instructions. The reaction was stopped after 15 min by the removal of the solution from the Iodobeads. Radioiodinated Z-Phe-Gly-Tyr-CHN_2 was prepared in a similar manner.

Acetylation of Ala-Ala-Phe-CH_2Cl and Tyr-Gly-Arg-CH_2Cl with acetic anhydride or [^{3}H]acetic anhydride was carried out by the method of Rauber *et al.* (32). The acetylated products were lyophilized and purified by reverse phase-HPLC on a Vydac C18 column (4.6 × 250 mm) with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. HPLC analysis yielded one major peak of absorbance at 274 nm that contained >70% of the eluted radioactivity. The identity of the products was confirmed by electrospray mass spectrometry (kindly performed by G. Eaton, Department of Chemistry, University of Leicester).

Affinity Labeling of Proteasomes—Purified 20 S proteasomes (0.5 mg/ml) were incubated with 100 μM ^{125}I-Tyr-Gly-Arg-CH_2Cl or Z-Phe-Gly-Tyr(^{125}I-CHN_2) in 50 mM sodium phosphate buffer, pH 7.0, for 2 h at 20 °C. Excess ^{125}I and unbound inhibitor were removed using two successive Sephadex G-25 PD10 columns (Pharmacia), and the enzyme was concentrated to 3–5 mg/ml using a Centricon 30 concentrator (Amicon). In experiments designed to investigate the blocking of enzyme labeling by pretreatment with known active site-directed inhibitors, proteasomes (0.1 mg/ml) were preincubated with 3,4-dichloroisocoumarin (5 μM) or calpain inhibitor 1 (10 μM) in 50 mM sodium phosphate buffer, pH 7.0, for 30 min at 20 °C. Labeling with [^{3}H]acetyl-Tyr-Gly-Arg-CH_2Cl (50 μM) was performed in the same way as described for the iodinated inhibitor except that incubations with labeled inhibitor were for 2.5 h. Labeling with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl was carried out as described in figure and table legends.

Analysis of Labeled Subunits—Labeled proteasome samples (50 μg) were analyzed by two-dimensional polyacrylamide gel electrophoresis (38) followed by autoradiography or fluorography of dried or electroblotted gels using Kodak X-Omat AR film. Labeled proteasome subunits were also identified by reverse phase-HPLC. Samples were loaded onto a Vydac C4 column (250 × 4.6 mm), and subunits were eluted with a flow rate of 0.3 ml/min and a gradient of 0–35% acetonitrile in 0.1% trifluoroacetic acid for 10 min followed by 35–55% acetonitrile plus 0.1% trifluoroacetic acid in 2.5 h. Radiolabeled peaks were identified by counting fractions in a γ or scintillation counter as appropriate.

The labeled peaks from the HPLC were lyophilized to dryness, redissolved in SDS-PAGE sample buffer, run on SDS-PAGE gels, and then electroblotted on to nitrocellulose (39). Blotted proteins were visualized with Ponceau S, and labeled bands were identified by autoradiography/fluorography (usually 2 weeks at −70 °C) followed by immunoblot analysis (40). Radiolabeled subunits were also identified by their position on two-dimensional-PAGE gels by N-terminal sequence anal-

[2] Part of this work was carried out in the Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK.

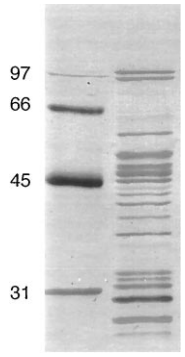

FIG. 1. **SDS-PAGE of purified 26 S proteasomes.** 26 S proteasomes (5 μg) were separated on 12.5% gels, and protein was visualized by silver staining (45).

ysis for unblocked subunits (41) and by immunoblot analysis (42).

Tryptic Digestion and Protein Sequencing—Labeled proteasomes or isolated subunits were subjected to carboxacetamidation prior to digestion with trypsin (5% w/w) in 50 mM Tris/HCl, pH 7.2, for 6 h. The tryptic peptides were purified by reverse phase-HPLC using a Vydac C18 column with a flow rate of 0.5 ml/min and a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid over 2 h. Purified labeled peptides were covalently attached to Sequelon-AA membranes (Millipore Corp.) following the manufacturer instructions. Sequence analysis of peptides was carried out by automated Edman degradation using an Applied Biosystems model 470A gas-phase sequencer equipped with a model 120A phenylthiohydantoin amino acid analyzer (41).

Antibodies and Immunoblotting—Polyclonal antibodies against a C7-derived peptide (GYELSPTAAANFTRC) corresponding to residues 72–85 of rat and human C7 with an added cysteine residue) were kindly provided by Dr. K. B. Hendil (University of Copenhagen) (14). The antiserum reacted exclusively with subunit C7 in blots from two-dimensional-PAGE gels of purified proteasomes (42). Other subunit-specific polyclonal antibodies used include those raised in rabbits against recombinant rat LMP7 (43) and monoclonal antibodies as described by Hendil and co-workers (42). Polyclonal antibodies against human MECL1 (44) were kindly provided by G. Foss. Immunoblotting was carried out as described previously (40).

RESULTS

Purification and Kinetic Properties of the 26 S Proteasome—The purification procedure developed for 26 S proteasomes resulted in apparently homogeneous preparations as judged by nondenaturing PAGE and showed subunit patterns on SDS-PAGE (Fig. 1), similar to 26 S proteasomes isolated from other sources (15, 16). The yield of 26 S proteasome was typically 1–3 mg from 100 g of rat liver compared with 0.5 mg from 500 g of rat liver reported by Ugai *et al.* (33).

The rate of hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC and Boc-Leu-Ser-Thr-Arg-AMC by 26 S proteasome preparations was found to be approximately ten times that of 20 S proteasomes with a similar K_m value (58 μM for Suc-Leu-Leu-Val-Tyr-AMC). The specific activity with Ala-Ala-Phe-AMC was not very different between the 20 and 26 S proteasomes. However, there was a striking difference in the peptidylglutamyl peptide hydrolase activity. We have previously shown that rat liver 20 S proteasomes have two activities: LLE1 with a K_m value for Z-Leu-Leu-Glu-nap of 0.07 mM and LLE2 which shows positive co-operativity with a $K_{0.5}$ value of 0.28 mM (46). The 26 S proteasomes by comparison displayed Michaelis-Menten kinetics over a wide range of concentrations of this substrate (Fig. 2) with a K_m value of 0.1 mM and no distinct LLE2 activity.

Another striking difference between 20 and 26 S proteasome preparations is the effect of substrate protein on the rate of hydrolysis of synthetic peptide substrate. Casein inhibits hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-nap, and to a lesser extent Boc-Leu-Ser-Thr-Arg-AMC by 20 S proteasomes but slightly stimulates the hydrolysis of these substrates by 26 S proteasomes (Table I).

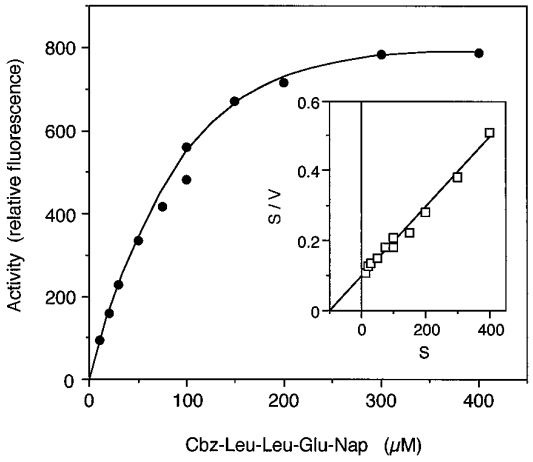

FIG. 2. **Peptidylglutamyl peptide hydrolase activity of 26 S proteasomes.** Enzyme activities of the purified 26 S proteasomes (0.5 μg/assay) were assayed in 50 mM Hepes/KOH, pH 8.0, incubated for 20 min at 37 °C. From the *inset*, a K_m value of 0.1 mM was obtained.

TABLE I

Effect of casein on peptidase activities of 20 and 26 S proteasomes

Assays were performed as described under "Experimental Procedures" using 0.5 and 2 μg of enzyme for 26 and 20 S proteasome, respectively. The substrate concentration was 40 μM for LSTR (Boc-Leu-Ser-Thr-Arg-AMC), LLVY (Suc-Leu-Leu-Val-Tyr-AMC), and AAF (Ala-Ala-Phe-AMC). LLE (Z-Leu-Leu-Glu-nap) was used at 0.4 mM for the 26 S proteasome and at 0.1 mM for the 20 S proteasome. The casein concentration was 0.1 mg/ml. Values are given as the average of two separate experiments, each performed in triplicate.

Proteasomes	Activity with casein added (% of control without added casein) with substrate			
	LSTR	LLVY	AAF	LLE
26 S	116.3 ± 3.5	119.1 ± 3.0	113.0 ± 2.8	115.8 ± 1.7
20 S	68.9 ± 1.9	33.7 ± 1.6	102.0 ± 2.5	52.1 ± 4.1

Inhibitor Characteristics of 20 and 26 S Proteasomes—Activated forms of proteasomes have been reported to be more sensitive to inhibition by some compounds. The effects of a variety of peptidyl aldehyde, chloromethane, and diazomethane inhibitors were tested on both 20 S and 26 S proteasome preparations to determine whether the higher specific activity of 26 S proteasomes meant that they could also be inhibited more rapidly. In general, this was found not to be the case, and the effect of inhibitors was similar for 20 and 26 S proteasomes (Table II). Ala-Ala-Phe-CH_2Cl only partially inhibits 20 S proteasomes and, surprisingly, inhibits hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC but not that of Ala-Ala-Phe-AMC by proteasomes (31). Both the rate and extent of inactivation with Ala-Ala-Phe-CH_2Cl was identical for 20 and 26 S proteasomes (Fig. 3), and in neither case did further additions of inhibitor cause further inhibition of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis.

The radiolabeled forms of Ala-Ala-Phe-CH_2Cl, Tyr-Gly-Arg-CH_2Cl, and Z-Phe-Gly-Tyr-CHN_2 were prepared since these are potential affinity labels for identification of subunits associated with the different activities. They inhibited the trypsin-like and chymotrypsin-like activities to different extents while the peptidylglutamyl peptide hydrolase activity was unaffected or even slightly stimulated by these reagents. Since there appeared to be no significant differences in reactivity with 20 and 26 S proteasomes, 20 S preparations were used for all of the studies with radiolabeled inhibitors because of their relative ease of purification and greater stability compared with 26 S proteasomes.

Little difference was observed in the inhibition of proteasome activities by radiolabeled and unlabeled forms of the peptidyl chloromethanes and peptidyl diazomethane inhibitors (Table II).

TABLE II

Effect of unlabeled and radiolabeled forms of peptidyl chloromethane inhibitors on peptidase activities of rat liver proteasomes

Proteasomes were dialyzed against 50 mM Hepes/KOH, pH 7.5, at 4 °C prior to the experiments. Preincubations with unlabeled, radioiodinated or [^{3}H]acetylated forms of Tyr-Gly-Arg-CH_2Cl were carried out for 1 h at 20 °C, using 0.1 mg/ml proteasomes with 0.1 mM inhibitor. Preincubations with the Ala-Ala-Phe-chloromethane inhibitors was carried out for 1 h at 25 °C with 10 μM inhibitor. At the end of the preincubations, enzyme samples were diluted into substrate solutions and assays were carried out as described under "Experimental Procedures." Abbreviations for substrates are the same as in Table I. Control incubations were carried out in the absence of affinity label. All data are the average of results from at least two separate experiments each carried out in duplicate.

Inhibitor		Activity (% of control) with substrate			
		LLVY	AAF	LSTR	LLE
Tyr-Gly-Arg-CH_2Cl	26 S	43	73	36	84
	20 S	35	80	10	100
[^{3}H]acetyl-Tyr-Gly-Arg-CH_2Cl	20 S	45	95	75	105
^{125}I-Tyr-Gly-Arg-CH_2Cl	20 S	34	97	55	125
Ala-Ala-Phe-CH_2Cl	26 S	49	94	103	98
	20 S	51	97	96	92
[^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl	20 S	47	93	83	95
Z-Phe-Gly-Tyr-CHN_2	26 S	28	58	86	103
	20 S	47	64	102	102

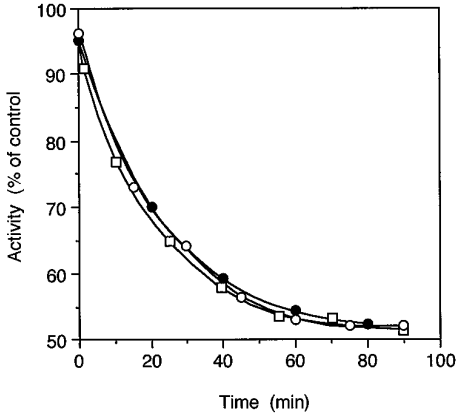

FIG. 3. **Inhibition of proteasomes by Ala-Ala-Phe-CH_2Cl.** 20 S proteasomes (0.5 mg/ml (*round symbols*)) and 26 S proteasomes (0.2 mg/ml (*square symbols*)) were incubated with 10 μM Ala-Ala-Phe-CH_2Cl (*open symbols*) or [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl (*solid symbols*) in 50 mM Hepes/KOH, pH 7.5, at 25 °C. Samples were removed at given intervals and assayed using 50 μM Suc-Leu-Leu-Val-Tyr-AMC, incubated for 15 min at 37 °C. Values are expressed as percent of control activity in samples containing no inhibitor.

The rate of inactivation was found to be identical with Ala-Ala-Phe-CH_2Cl and [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl (Fig. 3). Tyr-Gly-Arg-CH_2Cl, which might be predicted to be a specific inhibitor for trypsin-like activity because of the arginine residue in the P1 position, was found to inhibit trypsin-like activity and also chymotrypsin-like activity measured with Suc-Leu-Leu-Val-Tyr-AMC but not that assayed with Ala-Ala-Phe-AMC (Table II). Two radiolabeled forms of Tyr-Gly-Arg-CH_2Cl were prepared: ^{125}I-Tyr-Gly-Arg-CH_2Cl and [^{3}H]-acetyl-Tyr-Gly-Arg-CH_2Cl. Both of these reagents were found to inhibit the same activities as the unmodified peptidyl chloromethane as might be expected, but in each case the modified inhibitor was less effective than Tyr-Gly-Arg-CH_2Cl itself (Table II). Also, with the radiolabeled forms of the inhibitor, the inactivation of chymotrypsin-like activity was slightly greater than that of the trypsin-like activity (Table II). Z-Phe-Gly-Tyr-CHN_2, unlike the peptidyl chloromethane reagents used, inhibited Ala-Ala-Phe-AMC hydrolysis as well as affected other activities to a lesser extent (Table II).

TABLE III

Effect of active site-directed inhibitors on the incorporation of label after treatment with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl

20 S proteasomes (0.1 mg/ml) were incubated either with 20 μM 3,4-dichloroisocoumarin in 50 mM Hepes/KOH, pH 7.5, at 25 °C for 1 h or with 100 μM calpain inhibitor 1 in 50 mM Hepes/KOH, pH 7.5, at 37 °C for 30 min. Excess 3,4-dichloroisocoumarin was removed by overnight dialysis against 50 mM Hepes, pH 7.5. Control samples were incubated in the absence of inhibitor. [^{3}H]Acetyl-Ala-Ala-Phe-CH_2Cl (10 μM) was added, and the reaction mixture was incubated at 25 °C for 1 h. Proteasomes were removed immediately before and after the incubations and assayed with Suc-LLVY-AMC, incubating for 10 min at 37 °C. The labeled enzyme was applied to a PD10 column (Sephadex G-25M, 5 cm) equilibrated with 50 mM Hepes/KOH, pH 7.5, in order to remove excess labeled inhibitor. The protein concentration and radioactivity was measured in the eluent to determine the stoichiometry of labeling. Results are the mean of three separate experiments.

Pretreatment with active site in directed inhibitor	Activity of proteasomes (% control)	Stoichiometry of labeling by [^{3}H]acetylAAFCK	% Labeling blocked by pretreatment with active site-directed inhibitor
		mol/mol	
None	100	1.6	0
3,4-Dichloroisocoumarin	8	0.1	93
Calpain inhibitor 1	9	0.3	81

Labeling of Proteasomes and Protection by Known Active Site-directed Inhibitors—The stoichiometry of labeling was determined with the [^{3}H]acetylated inhibitors. With [^{3}H]acetyl-Tyr-Gly-Arg-CH_2Cl, up to 2.5 mol of label were incorporated per mole of enzyme, whereas for [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl, where only partial inhibition was achieved, 1.6 mol/mol were incorporated (Table III). The site-specific nature of the modifications were investigated by determining rates of inactivation by inhibitors in the presence of substrates and by measuring incorporation of label following pretreatment of proteasomes with inhibitors known to react at active sites (8, 20, 25).

For all of the radiolabeled inhibitors studied, the incorporation of radiolabel paralleled the loss of peptidase activity. Also, incorporation of radiolabel into proteasomes with any of the labeled inhibitors used was effectively blocked by prior treatment with an active site-directed inhibitor, calpain inhibitor 1, or 3,4-dichloroisocoumarin. The data obtained with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl are shown in Table III. The labeling with iodinated and acetylated forms of Tyr-Gly-Arg-CH_2Cl was also reduced following pretreatment of proteasomes with 3,4-dichloroisocoumarin (see separation of radiolabeled subunits below). Moreover, the rate of inactivation by Tyr-Gly-Arg-CH_2Cl was decreased in the presence of the synthetic peptide substrate Boc-Leu-Ser-Thr-Arg-AMC (data not shown). These results suggest that although the inhibitors react slowly and complete inhibition cannot easily be achieved, they do bind at substrate/inhibitor binding sites and are not reacting nonspecifically under the conditions used for the labeling experiments.

Identification of Labeled Subunits—The radiolabeled inhibitors were used to determine the subunits that are involved in chymotrypsin-like and trypsin-like activities. Following labeling with ^{125}I-Tyr-Gly-Arg-CH_2Cl (Fig. 4, *A* and *B*) or [^{3}H]acetyl-Tyr-Gly-Arg-CH_2Cl, separation of proteasome subunits by reverse phase-HPLC showed one major peak of radioactivity and two minor ones (Fig. 4*B*). Pretreatment of proteasomes with 3,4-dichloroisocoumarin decreased the incorporation of label into each of the three peaks (Fig. 4*B*) as did pretreatment with Pefabloc, (4-(2-aminoethyl)-benzenesulfonylfluoride) for selective inhibition of trypsin-like activity (28) (data not shown). The fractions from the HPLC run of the labeled proteasomes (Fig. 4*A*) were analyzed by SDS-PAGE, autoradiography, and immunoblot analysis. The major peak contained three protein bands (Fig. 4*C*). The lower molecular mass subunit (23 kDa) was shown by autoradiography to be the labeled one, and this

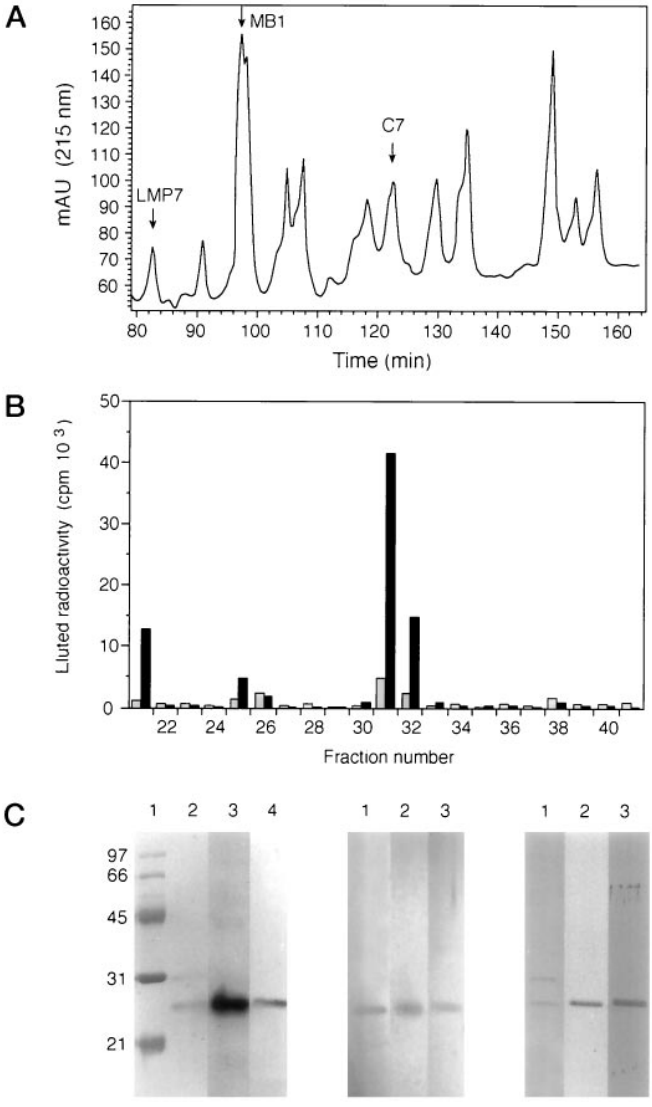

FIG. 4. **HPLC analysis of radiolabeled proteasome subunits and the effect of active site-directed inhibitors on labeling of proteasomes.** *A*, proteasomes were treated with ^{125}I-Tyr-Gly-Arg-CH_2Cl, and subunits were separated by reverse phase-HPLC using a Vydac C4 column as described under "Experimental Procedures." *Arrows* indicate the elution positions of labeled polypeptides (see *panel B*), which were identified as LMP7, MB1, and C7 (see *panel C*). The same protein elution profile was obtained for proteasomes labeled with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl. *B*, radioactivity determined by counting 4-min fractions (*dark bars*) from the HPLC separation of subunits shown in *panel A*. *Shaded bars* indicate radioactivity in fractions obtained from samples of proteasomes that were pretreated with 3,4-dichloroisocoumarin to inhibit chymotrypsin-like and trypsin-like activity prior to modification with ^{125}I-Tyr-Gly-Arg-CH_2Cl. *C*, identification of the major radiolabeled subunits from proteasomes labeled with ^{125}I-Tyr-Gly-Arg-CH_2Cl and [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl. Samples from radiolabeled subunit peaks identified by HPLC analysis were run on SDS-PAGE gels and blotted on to nitrocellulose. *Left panel*, *lane 1*, molecular mass standards stained with Ponceau S; *lane 2*, fraction 31 (see *panel B*) of ^{125}I-Tyr-Gly-Arg-CH_2Cl treated proteasomes stained with Ponceau S; *lane 3*, autoradiograph of *lane 2*; *lane 4*, Western blot with anti-C7 antibodies. *Middle* and *right panels*, fractions from peaks obtained after labeling with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl where C7 was only a minor labeled component. *Middle panel, lane 1*, fraction 25 stained with Ponceau S; *lane 2*, autoradiograph of *lane 1*; *lane 3*, Western blot with anti-MB1 antibodies. *Right panel, lane 1*, fraction 21, stained with Ponceau S; *lane 2*, autoradiograph of *lane 1*; *lane 3*, Western blot with anti-LMP7 antibodies.

subunit was identified as RC7 by Western blot analysis using polyclonal antibodies specific for this subunit (Fig. 4*C*). The specificity of the antibodies had been checked against purified

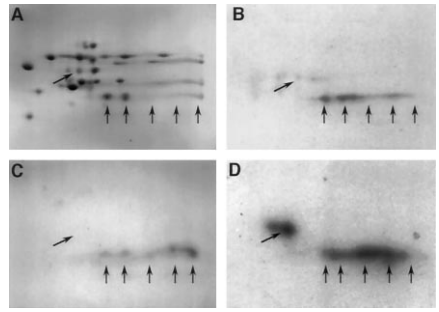

FIG. 5. **Two-dimensional PAGE analysis of radiolabeled subunits.** Proteasome subunits were separated by two-dimensional PAGE. *A*, proteasomes blotted onto nitrocellulose and stained with Ponceau S. *Arrows* indicate the position of labeled subunits shown in the *lower panels*. The lower molecular weight subunits were identified as, from *left* to *right*, C7 (*2 spots*), LMP7, and MB1. *B*, autoradiogram of proteasomes treated with ^{125}I-Tyr-Gly-Arg-CH_2Cl. *C*, proteasomes treated with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl. *D*, proteasomes treated with Phe-Gly-Tyr(^{125}I-CH_2Cl).

proteasome subunits separated on two-dimensional-PAGE gels. The two other labeled components were identified as MB1 and LMP7 by immunoblot analysis using appropriate subunit-specific antibodies (Fig. 4*C*). Labeling with [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl gave the same three labeled peaks, but in this case C7 was a minor labeled peak (data not shown, but see below).

Many attempts were made to purify labeled tryptic peptides from proteasome preparations labeled with [^{3}H]-acetylated inhibitors for sequence analysis by Edman degradation to try to determine the modified residues. However, because of the complexity of the mixture of peptides generated either from whole proteasomes or because of the poor recoveries of isolated subunits, it proved very difficult to isolate pure peptide and to obtain sufficient material to provide reliable amino acid sequence data. However, in each case when a tryptic digest of the major radiolabeled subunit of proteasomes labeled with [^{3}H]acetyl-Tyr-Gly-Arg-CH_2Cl (C7) was run on a C18 reverse phase column, one labeled peak was obtained consistent with the idea that a single site of the subunit was modified.

All of the results obtained by HPLC analysis were confirmed by two-dimensional-PAGE (Fig. 5) so that the relative labeling could be assessed. Identification of the position of proteasome subunits on two-dimensional-PAGE gels was achieved by N-terminal sequence analysis and by immunoblotting. With [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl and ^{125}I-Tyr-Gly-Arg-CH_2Cl, the major spots were MB1, LMP7, and C7, whereas with Z-Phe-Gly-Tyr-(^{125}I-CHN_2), which also inhibited activity measured with Ala-Ala-Phe-AMC, these subunits were labeled in addition to one other subunit (Fig. 5*D*). From its position on two-dimensional-PAGE gels, this subunit could possibly be MECL1, but we were unable to identify it directly by immunoblot analysis because of the lack of cross-reactivity with the antibodies to human MECL1.

DISCUSSION

The purification procedure developed for the 26 S proteasome resulted in higher yields than reported previously (33). 26 S proteasome preparations were found to have greater activity with synthetic peptide substrates than did the 20 S proteasomes as found by others (15,16). However, unlike 20 S proteasomes, they did not show the activation and sigmoidal substrate concentration dependence at high concentrations of the Z-Leu-Leu-Glu-nap substrate, and they lacked the LLE2 3,4-dichloroisocoumarin-inhibited peptidylglutamyl peptide hydrolase activity of the 20 S complex (46). This may be explained by the inability of 26 S complexes to undergo the large conformational changes of the 20 S proteasome that are associated with

the LLE2 activity (47). The other difference found in the catalytic properties of the two types of proteasomes was in the effect of protein substrates on peptidase activities. The greater effects of casein on peptidase activities of 20 S rather than 26 S proteasomes was surprising and not easily explained since casein is a substrate for both forms of the proteinase. Despite these differences in catalytic properties of 20 and 26 S proteasomes, their reactivity with 3,4-dichloroisocoumarin and various peptide aldehyde, peptidyl chloromethane, and peptidyl diazomethane inhibitors was found to be very similar.

The observed decrease in the rate of reaction with peptidyl chloromethane and diazomethane inhibitors in the presence of a substrate as well as blocking of incorporation of radiolabel by prior treatment of proteasomes with known active site-directed inhibitors, 3,4-dichloroisocoumarin, or calpain inhibitor I support the view that these reagents react at active/substrate-binding sites and are therefore suitable for identification of subunits associated with measured peptidase activities of proteasomes.

It has recently become clear, following determination of the three-dimensional structure (8,25) and site-directed mutagenesis of the archaebacterial proteasome (20), that the β subunits play a catalytic role and that the N-terminal threonine residue is the catalytic nucleophile. However, not all of the eukaryotic β-type subunits have a putative catalytic threonine residue. In animal cells, the three γ-interferon-inducible subunits (including LMP7 and MECL1) as well as the subunits they can replace (including MB1) all have the N-terminal threonine and are believed to be catalytic. MB1(X) has previously been identified as the target for the proteasome inhibitor lactacystin (30).

The results of the labeling studies presented here add to what is known about catalytic subunits of mammalian proteasomes. The three chosen inhibitors affected different peptidase activities and gave rise to distinct labeling patterns of proteasome subunits separated by HPLC or by two-dimensional-PAGE. Tyr-Gly-Arg-CH_2Cl was the most effective of the peptidyl chloromethane inhibitors tested previously on the trypsin-like activity of rat liver proteasomes (27). This reagent as well as the radioiodinated and [^{3}H]acetylated forms were found to inhibit both trypsin-like activity and chymotrypsin-like activity assayed with Suc-Leu-Leu-Val-Tyr-AMC. The labeled subunits were C7, MB1, and LMP7. [^{3}H]acetyl-Ala-Ala-Phe-CH_2Cl, which inhibited only the activity assayed with Suc-Leu-Leu-Val-Tyr-AMC, labeled the same three subunits, but labeling of C7 was only minor. With Z-Phe-Gly-Tyr-(^{125}I-CHN_2), which inhibited the two chymotrypsin-like activities but not the trypsin-like activity, the major labeled subunit was a different one of higher molecular mass, which was tentatively identified as MECL1 from its position on two-dimensional-PAGE gels (44). The labeled subunits are all β-type subunits that are located in the inner rings of the cylindrical structure. The labeled subunits identified with the different inhibitors demonstrate that there is overlapping specificity between the different peptidase activities of the complex. In particular, the results presented strongly suggest that there is an interaction between trypsin-like and chymotrypsin-like activities, and this view is supported by the very recent structural data for the yeast proteasome (25).

The predominant labeling of C7 with an affinity label for trypsin-like and chymotrypsin-like activity is interesting because this subunit does not possess a putative catalytic N-terminal threonine residue and is N-terminally blocked (41). Subunit C10 has previously been implicated in mammalian proteasome trypsin-like activity from sequence information obtained using an indirect labeling approach with ^{14}C-labeled *N*-ethylmaleimide (48). However, it is possible that the labeled cysteine could affect activity without being part of a catalytic subunit, and we found no specific labeling of RC10 with our affinity labels. Interestingly, C7 is the yeast homologue of the yeast Pre2 subunit, mutants in which affect chymotrypsin-like activity (22).

Alignment of the different β-type proteasome subunit sequences shows that there are only three glycine residues and two aspartic acid residues that are absolutely conserved (49). Chloromethane inhibitors usually alkylate histidine residues in serine proteases, but there is no obviously conserved histidine residue in proteasome subunits. However, other amino acid residues could be modified by peptidyl chloromethanes, and the blocking of reactivity of radiolabeled inhibitors by 3,4-dichloroisocoumarin and calpain inhibitor 1 suggests site-specific modification. An alignment of C7 subunit sequences with that of PRE1 and the *Thermoplasma* proteasome β subunit shows that relatively few residues are absolutely conserved in this group, and of these, Val-20, Lys-41, Gly-141 (*Thermoplasma* β subunit numbering) are also highly conserved in related eubacterial sequences (50). The lysine residue, Lys-33 of the mature β subunit is essential for activity in the *Thermoplasma* proteasome (20). The equivalent lysine residue in C7 could be modified by a peptidyl chloromethane, but there are also some fairly well conserved aspartic acid residues that could react with this type of reagent in a site-specific manner.

The arrangement of subunits within 20 S proteasomes has recently been determined both from cross-linking studies of the mammalian enzyme (14) and from the structure determination of the yeast proteasome (25). Subunits C7 and MB1 are located adjacent to each other both within each ring and also across on the neighboring β ring. It is interesting that these two subunits are the mammalian homologues of the yeast Pre1 and Pre2 subunits, mutations which were found to affect chymotrypsin-like activity (21,22). The γ-interferon-inducible LMP7 subunit is believed to replace MB1 (10) and presumably occupies the same position within the complex. Replacement by and differences in the levels of the γ-interferon-inducible subunits are known to affect peptidase activities of proteasomes (*e.g.* Ref. 51). However, because of the close interaction between different subunits, it is easy to imagine that modification of any subunit can potentially affect activities at different sites within the proteasome complex. This underlines the importance of the direct labeling approach used here to define subunits involved in catalytic activities that have previously only been well defined by their substrate and their overlapping inhibitor specificity (26, 27, 52–54).

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Catalytic Properties of 26 S and 20 S Proteasomes and Radiolabeling of MB1, LMP7, and C7 Subunits Associated with Trypsin-like and Chymotrypsin-like Activities

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