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 pepididylglutamyl peptide hydrolyase 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-Glu-naphthylamide. Various inhibitors that differentially affect the trypsin-like and chymotrypsin-like activities have been identified. Ala-Ala-Phe-chloromethyl (CHCl) 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-CHCl 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 (CHN₂) was found to inhibit only two of the chymotrypsin-like activities. Radiolabeled forms of peptidyl chloromethane and peptidyl diazomethane inhibitors, [¹H]acetyl-Ala-Ala-Phe-CHCl, [¹H]acetylated and radiiodinated Tyr-Gly-Arg-CHCl, and Z-Phe-Gly-Tyr-(¹¹³I-CHN₂), 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-dichlorocoumarin. 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/fluoroimageography 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) class I molecules (reviewed in Refs. 6 and 7).

Proteasomes (20 S) 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 dimeric complex composed 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; CHCl, chloromethyl; CHN₂, 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 peptidylglycine α-amidating activities. Isolation of yeast mutants defective in proteasome chymotrypsin-like or peptidylglycine α-amidating 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 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 radio-labeled forms of three peptidyl chloromethane and peptidyl dioxazomethane 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-amiomethylcoumarin (AMC) was purchased from the Peptide Institute, Japan. Ala-Ala-Ala-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 in 100 mM sodium phosphate buffer, pH 7.0, with Iodobeads was carried out using iodobeads from Pierce (37). I-Tyr-Gly-Arg-CH_2Cl and I25-Tyr-Gly-Arg-CH_2Cl were prepared by incubating 3 mM NaI or 3 mM Na2125I (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-25I-NH_2 was prepared in a similar manner.

Acetylation of Ala-Ala-Phe-CH_2Cl and Tyr-Gly-Arg-CH_2Cl with acetamide (32) or [3H]acetic anhydride was carried out by the method of Rauber et al. (32). The acetylated products were purified and studied 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.

**Affinity Labeling of Proteasomes**—Affinity labeling of rat liver and bovine pituitary proteasomes was carried out as described in figure and table legends using iodobeads from Pierce (37). I-Tyr-Gly-Arg-CH_2Cl and I25-Tyr-Gly-Arg-CH_2Cl were preincubated with 3 mM NaI or 3 mM Na2125I (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. Radiolabeled Z-Phe-Gly-25I-NH_2 was prepared in a similar manner.

Acetylation of Ala-Ala-Phe-CH_2Cl and Tyr-Gly-Arg-CH_2Cl with acetamide (32) or [3H]acetic anhydride was carried out by the method of Rauber et al. (32). The acetylated products were purified and studied 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.

**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 0–55% 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-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 electrophorosed 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 immuno blot analysis (40). Radiolabeled subunits were also identified by their position on two-dimensional-PAGE gels by N-terminal sequence analysis.

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

**Tryptic Digestion and Protein Sequencing**—Labeled proteasomes or isolated subunits were subjected to carboxymethylation 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-AAA 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 phenylthiodyanthion 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 antisera reacted exclusively with subunit C7 in blots from two-dimension-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 (Table II). Ala-Ala-Phe-CH2Cl only partially inhibits 20 S proteasomes and, surprisingly, inhibits hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC by 20 S proteasomes but not that of Ala-Ala-Phe-AMC by proteasomes. 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.

**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-CH2Cl 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-CH2Cl 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-CH2Cl, Tyr-Gly-Arg-CH2Cl, and Z-Phe-Gly-Tyr-CH2N2 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).
Proteasomes were dialyzed against 50 mM Hepes/KOH, pH 7.5, at 4 °C prior to the experiments. Preincubations with unlabeled, radioiodinated or [3H]acetylated forms of Tyr-Gly-Ala-Arg-CH2Cl 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.

The rate of inactivation was found to be identical with Ala-Ala-Phe-CH2Cl and [3H]acetyl-Ala-Ala-Phe-CH2Cl, up to 2.5 mol of label were incorporated into each of the three peaks (Fig. 4B) as did pretreatment with 3,4-dichloroisocoumarin. The data obtained with [3H]acetyl-Ala-Ala-Phe-CH2Cl, 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 radioiodinated 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 [3H]acetyl-Ala-Ala-Phe-CH2Cl are shown in Table III. The labeling with iodinated and acetylated forms of Tyr-Gly-Arg-CH2Cl 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-CH2Cl 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 [3H]acetyl-Tyr-Gly-Ala-Arg-CH2Cl (Fig. 4, A and B) or [3H]acetyl-Tyr-Gly-Arg-CH2Cl, separation of proteasome subunits by reverse phase-HPLC showed one major peak of radioactivity and two minor ones (Fig. 4B). Pretreatment of proteasomes with 3,4-dichloroisocoumarin decreased incorporation of label into each of the three peaks (Fig. 4B) 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. 4A) were analyzed by SDS-PAGE, autoradiography, and immunoblot analysis. The major peak contained three protein bands (Fig. 4C). The lower molecular mass subunit (23 kDa) was shown by autoradiography to be the labeled one, and this

![Table II](image)

**Table II**

| Inhibitor                  | Activity (% of control) with substrate |
|----------------------------|---------------------------------------|
|                            | LLVY | AAF | LSTR | LLE |
| Tyr-Gly-Arg-CH2Cl          | 26 S | 43  | 73   | 36  | 84  |
| [3H]acetyl-Tyr-Gly-Arg-CH2Cl| 20 S | 35  | 80   | 10  | 100 |
| 125I-Tyr-Gly-Arg-CH2Cl     | 20 S | 34  | 97   | 55  | 125 |
| Ala-Ala-Phe-CH2Cl          | 26 S | 49  | 94   | 103 | 98  |
| [3H]acetyl-Ala-Ala-Phe-CH2Cl| 20 S | 51  | 97   | 96  | 92  |
| [3H]acetyl-Ala-Ala-Phe-CH2Cl| 20 S | 47  | 93   | 83  | 95  |
|                          | 26 S | 28  | 58   | 86  | 103 |
| Z-Phe-Gly-Tyr-CHN2         | 20 S | 47  | 64   | 102 | 102 |

**Table III**

| Pretreatment with active site-directed inhibitor | Activity of proteases (% of control) | Stoichiometry of labeling by [3H]acetylAAFCK | % Labeling blocked by pretreatment with active site-directed inhibitor |
|------------------------------------------------|--------------------------------------|-----------------------------------------------|---------------------------------------------------------------------|
| 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 [3H]acetylated inhibitors. With [3H]acetyl-Tyr-Gly-Arg-CH2Cl, up to 2.5 mol of label were incorporated per mole of enzyme, whereas for [3H]acetyl-Ala-Ala-Phe-CH2Cl, 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).
subunit was identified as RC7 by Western blot analysis using polyclonal antibodies specific for this subunit (Fig. 4C). The specificity of the antibodies had been checked against purified 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. 4C). Labeling with [3H]acetyl-Ala-Ala-Phe-CH2Cl 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 [3H]-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 [3H]acetyl-Tyr-Gly-Arg-CH2Cl (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 [3H]acetyl-Ala-Ala-Phe-CH2Cl and [125I]-Tyr-Gly-Arg-CH2Cl, the major spots were MB1, LMP7, and C7, whereas with Z-Phe-Gly-Tyr-(125I-CH2Cl), which also inhibited activity measured with Ala-Ala-Phe-AMC, these subunits were labeled in addition to one other subunit (Fig. 5D). 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 proteasine. Despite these differences in catalytic properties of 20 and 26 S proteasomes, their reactivity with 3,4-dichloroisocoumarin and various peptide aldehydes, peptideyl chloromethane, and peptideyl diazomethane inhibitors was found to be very similar.

The observed decrease in the rate of reaction with peptideyl 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 archaeabacterial 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 in all 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).

REFERENCES

1. Rivett, A. J. (1993) Biochem. J. 291, 1–10
2. Rubin, D. M., and Finley, D. (1995) Curr. Biol. 5, 854–858
3. Hochstrasser, M. (1995) Curr. Opin. Cell Biol. 7, 215–223
4. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–857
5. Rechsteiner, M., Hoffman, L., and Dubiel, W. (1993) J. Biol. Chem. 268, 6065–6068
6. Goldberg, A. L., and Rock, K. L. (1992) Nature 357, 375–379
7. Tanaka, K., Tanahashi, N., Tsuromi, C., Yokota, K. Y., and Simbara, N. (1997) Adv. Immunol. 64, 1–38
8. Lowe, J., Stock, D., Jasp, B., Zwickl, P., Baumeister, W., and Huber R. (1995) Science 268, 529–530
9. Heinemeyer, W., Trondle, N., Albrecht, G., and Wolf, D. H. (1994) Biochemistry 53, 12229–12237
10. Belich, M. P., Glynne, R. J., Senger, G., Sheer, D., and Trowsdale, J. (1994) Curr. Biol. 4, 779–786
11. Fruh, K., Gassen, M., Wang, K., Bujard, H., Peterson, P. A., and Yang, Y. (1994) EMBO J. 13, 3236–3244
12. Fehling, H. J., Swat, W., Laplace, C., Kuhn, R., Rajewsky, K., Muller, U., and von Boehn, H. (1994) Science 265, 1234–1236
13. Sibille, C., Gould, K., Willard-Gallo, K., Thomson, S., Rivett, A. J., Powis, S., Butcher, G. W., and DeHartseiser, F. (1995) Curr. Biol. 5, 923–930
14. Kopp, F., Hendil, K. B., Dahlmann, B., Kristensen, P., Soheb, A., and Uerkvitz, W., (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2939–2944
15. Hoffman, L., Pratt, G., and Rechsteiner, M. (1996) J. Biol. Chem. 267, 801–804
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22362–22368

16. Kanayama, H.-O., Tamura, T., Ugaí, S., Kagawa, S., Tanahashi, N., Yoshimura, T., Tanaka, K., and Ichihara, A. (1992) Eur. J. Biochem. 206, 567–578

17. Chu-Ping, M., Slaughter, C. A., and DeMartino, G. N. (1992) J. Biol. Chem. 267, 10515–10523

18. Dubiel, W., Pratt, G., Ferrell, K., and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22369–22377

19. Dick, T. P., Ruppert, T., Groettrup, M., Kloetzel, P. M., Kuehn, L., Koszinowski, U. H., Stevanovic, S., Schild, H., and Rammensee, H. G. (1996) Cell 86, 253–262

20. Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R., and Baumeister, W. (1995) Science 268, 579–582

21. Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C., and Wolf, D. H. (1991) EMBO J. 10, 555–562

22. Hilt, W., Enenkel, C., Gruhler, A., Singer, T., and Wolf, D. H. (1993) J. Biol. Chem. 268, 3479–3486

23. Heinemeyer, W., Gruhler, A., Mohrle, V., Mahe, Y., and Wolf, D. H. (1993) J. Biol. Chem. 268, 5115–5120

24. Enenkel, C., Lehman, H., Kipper, J., Gückel, R., Hilt, W., and Wolf, D. H. (1994) FEBS Lett. 341, 193–196

25. Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtuler, M., Bartunik, H. D., and Huber, R. (1997) Nature 386, 463–471

26. Orlowski, M., Cardozo, C., and Michaud, C. (1993) Biochemistry 32, 1563–1572

27. Rivett, A. J., Savory, P. J., and Djaballah, H. (1994) Methods Enzymol. 244, 331–350

28. Djaballah, H., Harness, J. A., Savory, P. J., and Rivett, A. J. (1992) Eur. J. Biochem. 209, 629–634

29. Figuerido-Pereira, M. E., Chen, W.-E., Yuan, H. M., and Wilk, S. (1995) Arch. Biochem. Biophys. 317, 69–78

30. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) Science 268, 726–731

31. Savory, P. J., Djaballah, H., Angliker, H., Shaw, E., and Rivett, A. J. (1993) Biochem. J. 296, 691–695

32. Rauber, P., Wikström, P., and Shaw, E. (1988) Anal. Biochem. 168, 259–264

33. Ugaí, S. I., Tamura, T., Tanahashi, N., Takai, S., Komi, N., Chung, C. H., Tanaka, K., and Ichihara, A. (1993) J. Biochem. 113, 754–768

34. Laemmli, U. K. (1970) Nature 227, 680–685

35. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

36. Rawlings, N. D., and Barrett, A. J. (1990) Comput. Appl. Biosci. 6, 118–119

37. Markwell, M. A. K. (1982) Anal. Biochem. 125, 427–432

38. Duncan, B., and Hershey, J. W. B. (1984) Anal. Biochem. 138, 144–150

39. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354

40. Rivett, A. J., and Sweeney, S. T. (1991) Biochem. J. 278, 171–177

41. Lilley, K. S., Davison, M. D., and Rivett, A. J. (1990) FEBS Lett. 262, 327–329

42. Kristensen, P., Johnsen, A. H., Uerkvitz, W., Tanaka, K., and Hendil, K. B. (1994) Biochem. Biophys. Res. Commun. 205, 1785–1789

43. Palmer, A., Rivett, A. J., Thomson, S. T., Hendil, K. B., Butcher, G. W., Fuertes, G., and Knecht, E. (1996) Biochem. J. 316, 401–407

44. Larsen, F., Solheim, J., Kristensen, T., Kolsto, A. B., and Prydz, H. (1993) Hum. Mol. Genet. 2, 1589–1595

45. Blum, H., Beier, H., and Gross, H. J. (1987) Electrophoresis 8, 93–99

46. Djaballah, H., and Rivett, A. J. (1992) Biochemistry 31, 4133–4141

47. Djaballah, H., Rowe, A. J., Harding, S. E., and Rivett, A. J. (1993) Biochem. J. 292, 857–862

48. Dick, L. R., Moomaw, C. R., Pramanik, B. C., DeMartino, G. N., and Slaughter, C. A. (1992) Biochemistry 31, 7347–7355

49. Rivett, A. J., Mason, G. F., Thomson, S., Pike, A. M., Savory, P. J., and Murray, R. Z. (1995) Mol. Biol. Rep. 21, 35–41

50. Lupas, A., Zwickl, P., and Baumeister, W. (1994) Trends Biochem. Sci. 19, 535–534

51. Gaszynska, M., Goldberg, A. L., Tanaka, K., Hendil, K. B., and Rock, K. L. (1996) J. Biol. Chem. 271, 17275–17280

52. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761–771

53. Vinitsky, A., Cardozo, C., Sepp-Lorenzino, L., Michaud, C., and Orlowski, M. (1994) J. Biol. Chem. 168, 29860–29866

54. Cardozo, C., Chen, W.-E., Wilk, S. (1996) Arch. Biochem. Biophys. 334, 113–120
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