Properties of subunits of the multicatalytic proteinase complex revealed by the use of subunit-specific antibodies

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The multicatalytic proteinase (MCP) is a high-molecular-mass non-lysosomal proteinase that gives rise to a characteristic pattern of bands of molecular mass 22–34 kDa on SDS/PAGE gels. Isoelectric-focusing gels of the enzyme purified from rat liver show 16 bands with isoelectric points in the range of pH 5–8.5. Two-dimensional PAGE gels reveal that there are more than the previously reported 13 polypeptides associated with the MCP from rat liver and show a pattern of 15–20 major spots and several minor ones, similar to that of MCP isolated from some other sources. Possible relationships between the different polypeptides were investigated by immunoblot analysis of electrophoretically purified proteinase subunits with affinity-purified subunit-specific antibodies as well as antibodies raised against individual denatured subunits of the complex. The results demonstrate that many of the major polypeptide components of the MCP complex are antigenically distinct. Moreover comparison of immunoreactive material in crude cell extracts with that in purified MCP preparations has shown that the polypeptides are not derived from a smaller number of higher-molecular-mass subunits. Also, individual subunits have the same apparent molecular mass in a variety of rat tissues, suggesting close similarity between MCPs of different tissues. The highest concentrations of MCP subunits occur in liver and kidney. Gel-filtration analysis of crude extracts has demonstrated that MCP polypeptides are also associated with a higher-molecular-mass complex, which may be the 26 S proteinase that has been implicated in the degradation of ubiquitin–protein conjugates.

The multicatalytic proteinase (MCP) complex (otherwise referred to as the proteasome) is a high-molecular-mass proteinase (700 kDa) that is found in a wide variety of eukaryotic cells (for reviews see refs. [1] and [2]). The complex may have other functions in addition to its proposed role in non-lysosomal pathways of intracellular protein degradation [1,2]. It appears, from electron microscopy, to have a hollow cylindrical structure [3], and has several characteristics in common with a number of other cylindrical particles, including 'ring-type' particles, cylinder particles and ribonucleoprotein particles called prosomes. These particles appear to be closely related [4–6], but there is only a small amount of a specific RNA associated with the rat liver MCP [7].

MCP can degrade some protein substrates [8]. It catalyses cleavage of peptide bonds on the carboxy side of basic, hydrophobic or acidic amino acid residues, and these three distinct proteolytic activities can conveniently be assayed independently with appropriate synthetic peptide substrates [9,10]. The activities of purified MCP are generally found not to be stimulated by ATP [1], but the complex has been implicated in ubiquitin- and ATP-dependent as well as ubiquitin-independent pathways of non-lysosomal protein degradation [1,2,11–13].

The enzyme is composed of many different types of subunit. When analysed by SDS/PAGE, it gives rise to a characteristic pattern of bands all having molecular masses in the range 22–34 kDa. However, the precise subunit composition of MCP has not been well defined. Tanaka et al. [14] have suggested, on the basis of their separation by reverse-phase h.p.l.c., that there are 13 components of the rat liver proteinase. However, by two-dimensional PAGE we find that there are more different polypeptides associated with the complex consistent with the number of polypeptides associated with MCP from a variety of other sources, although there are clearly some species differences. Since little was known about the possible relationships between the many spots observed on two-dimensional gels, the purpose of the present study was to investigate such relationships by using an immunochemical approach. Although the proteinase has now been isolated from many different sources and its occurrence in different tissues has been investigated with antibodies raised against the native complex [15,16], detailed immunochemical studies of the subunit composition have not been reported. It has been suggested that MCP may share structural components with a higher-molecular mass ubiquitin–protein-conjugate-degrading proteinase complex [17].

Polyclonal antibodies raised against the intact rat liver proteinase complex [18], like those raised against the enzyme isolated from other sources [11,15,19], do not recognize all of the proteinase subunits separated on SDS/PAGE gels. The antigenic response to the complex was successfully increased by treating it with dinitrobenzenesulphonic acid (DNBS), and antibodies were also raised against some individual isolated subunits of the complex. Questions concerning the size and tissue distribution of subunits and the relationships between them were addressed by using affinity-purified subunit-specific antibodies. Analysis of the immunoreactivity of the subunits revealed that many of the 20 or so polypeptides observed on two-dimensional polyacrylamide gels are antigenically distinct. Moreover, these polypeptides are not derived from a small number of higher-molecular-mass subunits. They appear to be present in similar ratios and of the same size in several tissues of the rat. The subunits are present in rat liver associated both with the MCP complex and with an even larger complex.

EXPERIMENTAL

Materials

Frozen rat livers were obtained from North East Biomedical (Denham, Middx., U.K.). Proteinase substrates Ala-Ala-Phe-NH-Mec and Z-Leu-Leu-Glu-NH-Nap, pI markers, Coomassie Brilliant Blue R, Ponceau S, 4-chloro-1-naphthol, pig anti-(rabbit IgG) antibody–peroxidase conjugate and Tween 20 were purchased from Sigma Chemical Co. Boc-Leu-Ser-Thr-Arg-NH-Mec was from the Peptide Institute (Osaka, Japan). Protein A–Sepharose was purchased from Pharmacia, acrylamide and SDS were from Serva, and ampholytes and urea (AristaR grade) were from BDH Chemicals. Other reagents for PAGE including molecular-mass markers were purchased from Bio-Rad Laboratories. DNBS and tetramethylbenzidine were from Aldrich Chemical Co. Nitrocellulose sheets (0.2 μm) were from Schleicher and Schuell and dried milk powder (Marvel) was from Cadbury. Pig anti-(rabbit IgG) antibody and rabbit PAP [a soluble complex of horseradish peroxidase and rabbit anti-(horseradish peroxidase) antibody] were obtained from Dakopatts (High Wycombe, Bucks., U.K.), and e.l.i.s.a. plates were from Dynatech. Phosphate-buffered salt tablets (Dulbecco's modified medium without Ca^{2+} and Mg^{2+}) were purchased from Flow Laboratories.

Proteinase purification

MCP was purified from fresh or frozen rat livers as described previously [10] or by a modification of that method in which the agarose–hexylamine step was replaced by a Mono Q 10/10 (Pharmacia) ion-exchange step. The column was equilibrated with 20 mM-Tris/HCl buffer, pH 7.2, containing 0.1 M-KCl and the proteinase was eluted by applying a linear gradient of 0.1–0.5 M-KCl in buffer. With the use of this modified procedure the hydroxyapatite chromatography step of the earlier method [10] was found to be unnecessary.

Proteinase assay

The three distinct activities of the proteinase [9,10] were assayed with Ala-Ala-Phe-NH-Mec, Boc-Leu-Ser-Thr-Arg-NH-Mec and Z-Leu-Leu-Glu-NH-Nap in 0.1 M-Hepes buffer, pH 7.5 or 8.0, as described previously [10]. Protein concentrations were determined by the Bradford method [20] with the reagent from Bio-Rad Laboratories, with BSA as standard.

PAGE

SDS/PAGE was carried out by the method of Laemmli [21] with a 15 % separating gel. Samples were prepared by heating for 2–3 min at 90 °C in the presence of SDS and 2-mercaptoethanol. Isoelectric focusing (IEF) and two-dimensional PAGE was carried out by the method of O'Farrell [22] as modified by Duncan & Hershey [23]. IEF rods were stained for protein with Coomassie Blue after being soaked overnight in 10 % (w/v) (and then being washed with 1 %, w/v) trichloroacetic acid.

Preparation of antisera against intact MCP

Polyclonal antibodies were raised in New Zealand White rabbits. For antibodies against the native MCP, booster injections (100 μg of protein mixed with Freund's incomplete adjuvant) were given subcutaneously at 2-week intervals starting 1 month after the first subcutaneous injection (200 μg of protein emulsified with Freund's complete adjuvant). The same rabbit was injected subsequently with MCP complex that had been treated with DNBS to make it more immunogenic [24]. This treatment, which introduces dinitrophenyl (Dnp) groups on to the surface of the protein, had no detectable effect on the molecular mass of the complex and resulted in less than 30 % loss of proteolytic activity measured with Ala-Ala-Phe-NH-Mec.

Antibodies raised against individual subunits

Proteinase for the first injection (200 μg) was dissociated by heating for 5 min at 95 °C in the presence of 10 % (w/v) SDS/10 mM-dithiothreitol and then dialysed against 10 mM-Hepes buffer, pH 7.5, before being mixed with Freund's complete adjuvant. For subsequent injections at 2-week intervals, slices of IEF gels containing a total of 50–100 μg of a single polypeptide band (see Fig. 1) were washed with phosphate-buffered saline (PBS) and then sonicated with roughly equal volumes of PBS and Freund's incomplete adjuvant.

Characterization of antibodies

IgG preparations were obtained by Protein A–Sepharose chromatography of antisera as described previously [10]. All characterization of the antibodies was carried out with the purified IgG preparations, with pre-immune IgG prepared from normal rabbit serum as a control.

Ouchterlony double-diffusion experiments were carried out in 1 % (w/v) agarose gels in PBS. Tests for effect on proteolytic activities and for precipitation of the proteinase were carried out as described previously [10] by incubating proteinase with IgG for 2 h on ice and then assaying proteinase activity before and after centrifugation to precipitate immune complexes.

Antisera were also characterized by e.l.i.s.a. Micro-titre plates (96-well) were coated by using a 1 μg/ml solution of the proteinase in 20 mM-Tris/HCl buffer, pH 9.0. BSA (1 %, w/v, in PBS) was used as the block. After incubation with the primary antibodies, washing with PBS containing 0.1 % Tween 20, and incubation with goat anti-(rabbit IgG) antibody–peroxidase conjugate, plates were developed with tetramethylbenzidine [25]. Anti-subunit antibodies were also tested for binding to native MCP by incubating with the proteinase for 2 h at 4 °C, passing the solution over a small (0.2 ml bed volume) Protein A–Sepharose column and assaying the eluate for proteinase activity.

Immunoblotting

MCP subunits separated by SDS/PAGE or two-dimensional PAGE were electrophoretically transferred to nitrocellulose sheets [26] by means of the Bio-Rad Trans-blot apparatus with 25 mM-Tris/192 mM-glycine/10 % (v/v) methanol as transfer buffer. Transferred proteins were detected with 0.2 % Ponceau S stain in 3 % (w/v) trichloroacetic acid. Non-specific binding sites on the nitrocellulose were blocked with blocking solution I [10 % (w/v) Marvel, 10 % (v/v) glycerol, 1 M-glucose and 0.01 % (v/v) Tween 20 in PBS] or II [3 % (w/v) Marvel and 0.01 % (v/v) Tween 20 in PBS]. The nitrocellulose was then incubated overnight with a rabbit anti-proteinase IgG preparation (usually 1/200 dilution of 2–3 mg/ml solution).

After the nitrocellulose had been washed with PBS, rabbit antibody was allowed to react (1 h, room temperature) with a 1:750 dilution of pig anti-(rabbit immunoglobulin) antibody in blocking solution II. The nitrocellulose was washed again with PBS before incubation for 1 h with a 1/750 dilution of rabbit PAP in blocking solution II. The nitrocellulose was washed with Tris-buffered saline (TBS) before detection of immunoreactive material with 4-chloro-1-naphthol [1 mg/ml in 20 % (v/v) methanol/80 % (v/v) TBS] and H_2O_2 (0.01 %, v/v).

Affinity purification of antibodies

When necessary (see the Results section), IgGs were affinity-purified with the use of proteinase subunits separated by SDS/PAGE and blotted on to nitrocellulose. Strips of nitrocellulose containing the immunoreactive subunit of interest were incubated with the appropriate IgG preparation overnight. They

were then washed with TBS before elution of the bound IgGs with 0.2 M-glycine, pH 2.3. The resulting solution was neutralized with Tris base.

Gel filtration of rat liver extracts

Fresh rat liver (Wistar) was perfused with 50 mM-Hepes buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol and then homogenized in 5 vol. of the same buffer. After centrifugation at 20000 g for 20 min and then at 100000 g for 1 h, the resulting soluble extract was used for gel-filtration experiments. Gel filtration was carried out on f.p.l.c. equipment with a Superose 6 column (Pharmacia) and 50 mM-potassium phosphate buffer, pH 7.0, containing 0.1 M-KCl. In some cases ATP (0.5 mM) and MgCl$_2$ (5 mM) were added to the extract, 1 mM-ATP and 5 mM-MgCl$_2$ were included in the gel-filtration buffer, and the extract was incubated with 5 mM-ATP at 37 °C for 15 min before gel filtration.

RESULTS

Subunit composition of the MCP

SDS/PAGE of the MCP complex isolated from rat liver gives rise to up to 12 bands (depending on the resolution of the gels) all having apparent molecular masses in the range 22–34 kDa. The 34 kDa polypeptide appears to be susceptible to proteolytic cleavage, and the amount of this polypeptide can vary in different preparations. It is difficult to obtain a clear separation of all the polypeptide components of the complex by SDS/PAGE. Better separation of MCP subunits can be obtained by IEF (Fig. 1) or two-dimensional PAGE (Fig. 2). On IEF rod gels 14–16 major bands were observed with pI values ranging from pH 5 to 8.5, and two-dimensional PAGE gels show up to 20 major poly-peptides. The pattern of the major spots on two-dimensional gels is highly reproducible from one preparation to another but relative amounts of some of the spots do vary.

Characterization of anti-MCP and anti-Dnp-MCP antibodies

Polyclonal antibodies raised against the purified native rat liver MCP (anti-MCP antibodies) precipitate the complex, and Ouchterlony double-diffusion experiments carried out with purified proteinase gave rise to a single precipitin band (results not shown). The antibodies have no effect on proteolytic activities measured with small synthetic peptide substrates and even have rather little effect on protein degradation. It is therefore possible to demonstrate precipitation of immune complexes by assaying activity before and after centrifugation as described previously [10].

Analysis of anti-MCP IgG preparation by immunoblotting of SDS/PAGE gels showed two protein bands of 30 kDa and 29 kDa to contain the major immunoreactive polypeptides (results not shown, but see legends to Figs. 5 and 6). Three other protein bands of lower molecular mass were found to be weakly immunoreactive and were detected with anti-MCP antibodies when blots were developed with higher antibody concentrations or with the use of a less stringent blocking solution.

Repeated injections of native MCP over a 6-month period failed to improve significantly the titre and/or affinity of the anti-MCP antibodies raised. In an attempt to increase the antigenicity of the MCP complex, some proteinase was treated with DNBS and injected into the same rabbit as that used to raise the anti-MCP antibodies. The resulting IgG preparations (referred to as anti-Dnp-MCP antibodies) were more effective than anti-MCP IgGs in precipitating the enzyme and recognized more of the MCP polypeptides but otherwise showed broadly similar characteristics to the anti-MCP antibodies. One interesting difference between the two antibody preparations was the partial

blocking of proteolytic activity measured with Ala-Ala-Phe-NH-Mec as substrate by anti-Dnp-MCP antibodies (Fig. 3). However, this effect was probably non-specific, and proteolytic activity measured with the other two substrates was unaffected. Immuno-blots of SDS/PAGE gels showed most bands to be immuno-
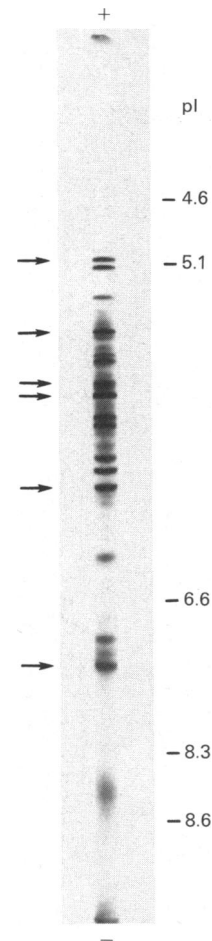

Fig. 1. IEF of the MCP isolated from rat liver

IEF rod of the proteinase showing the position of pI markers. Arrows indicate the position of bands taken for immunization of rabbits to raise anti-subunit antibodies as described in the Experimental section.
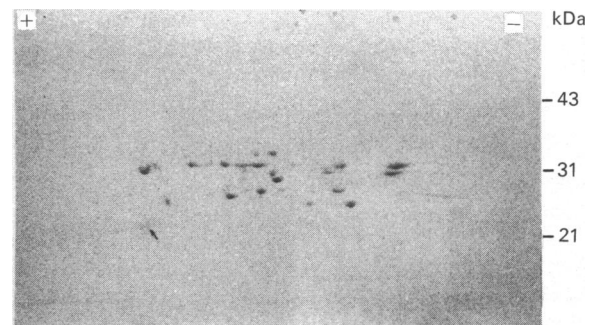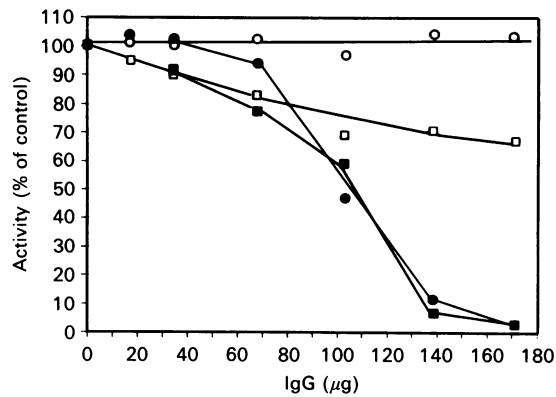

Fig. 2. Two-dimensional PAGE of the purified MCP

Characteristic pattern obtained when purified rat liver MCP is run on two-dimensional polyacrylamide gels and stained for protein with Coomassie Blue. The arrow indicates the position of the 22 kDa polypeptide, the relative amount of which varies in different preparations.

Fig. 3. Proteolytic activities of the MCP after incubation with anti-Dnp-MCP IgG

Proteinase was incubated with various amounts of IgG for 2 h on ice. Assays were carried out before (○ and □) and after (● and ■) centrifugation for 5 min to precipitate immune complexes. Activity was measured with Boc-Leu-Ser-Thr-Arg-NH-Mec (○ and ●) and Ala-Ala-Phe-NH-Mec (□ and ■) as substrate.
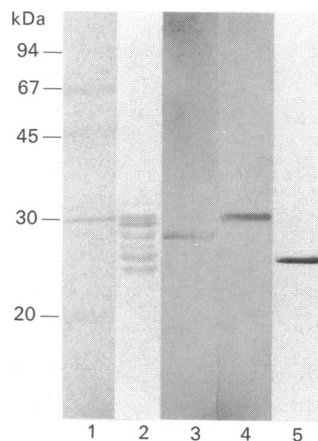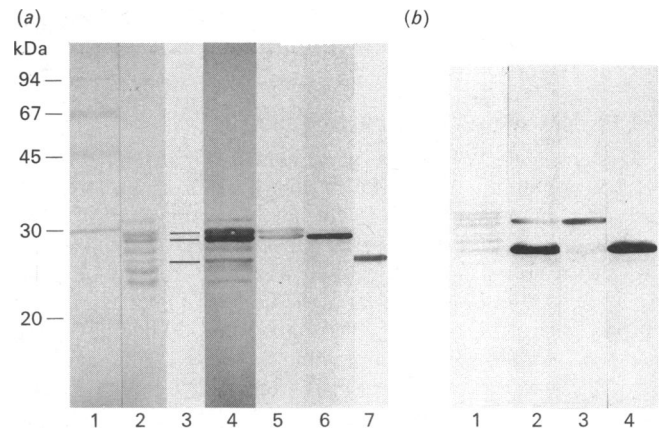

Fig. 4. Immunoblot analysis of antibodies raised against isolated subunits of the MCP complex

Nitrocellulose blot of SDS/PAGE gel: lane 1, molecular-mass markers stained with Ponceau S; lane 2, MCP (5 μg) stained with Ponceau S; lanes 3–5, immunoblot analysis with antibodies raised against isolated subunits, anti-B (lane 3), anti-C (lane 4) and anti-D (lane 5), with blocking solution II as described in the Experimental section.


Fig. 5. Immunoblot analysis of the proteinase with anti-Dnp-MCP and anti-E antibodies and with affinity-purified antibodies against individual polypeptides

(a) Anti-Dnp-MCP antibodies. Nitrocellulose blot from an SDS/PAGE gel with molecular-mass standards in lane 1 and 5 μg of purified MCP in each of lanes 2, 4, 5, 6 and 7. Lanes 1 and 2 are stained for protein with Ponceau S. Lane 3 indicates the three major bands detected by immunoblot with anti-Dnp-MCP the results of which are shown in lane 4, and lanes 5–7 show immunoblots with affinity-purified IgGs for each of the three bands in lane 3 respectively. The top two of these three bands are the ones that are most immunoreactive with anti-MCP antibodies. (b) Anti-E antibodies. Immunoblot as described above with MCP in lane 1 stained with Ponceau S, in lane 2 detected with anti-E antibodies, and in lanes 3 and 4 detected with affinity-purified antibodies against each of the top and bottom immunoreactive bands shown in lane 2 respectively. Blocking solution II was used in each experiment and other details were as described in the Experimental section.

reactive (Fig. 5a, lane 4), the two strongest of these being the same as the principal immunoreactive bands with the anti-MCP antibodies.

Preparation and characterization of antisera against individual subunits of the complex

Because immunoblotting with the anti-Dnp-MCP IgG preparations showed that several polypeptides were still not immunoreactive, polyclonal antisera were also raised against individual denatured subunits to allow further immunochemical characterization of the complex. MCP subunits were separated by IEF, and bands towards the acid end of the pI range (see Fig. 1) were chosen for raising antibodies. The antibodies raised were analysed by immunoblots of SDS/PAGE gels. Several subunit-specific IgG preparations (anti-B, anti-C and anti-D; Fig. 4) as well as one that recognized two subunits (anti-E) were chosen for further characterization.

Precipitation assays failed to demonstrate any ability of these anti-subunit IgG preparations to precipitate the native proteinase. The antibodies may recognize the native enzyme without causing precipitation, although an alternative explanation of these results would be that some denatured enzyme was present on the e.l.i.s.a. plates that were used to detect the antibody. Since some denaturation of the complex might be expected during coating of the e.l.i.s.a. plates even if not during the purification, another type of experiment was carried out to investigate binding to the active proteinase complex. Because none of the anti-subunit antibodies block any of the proteolytic activities of the complex, it is possible to use proteolytic activity as a measure of the amount of active enzyme in the following experiments. The proteinase was incubated with individual IgG preparations and then the proteinase/IgG mixtures were passed down Protein A–Sepharose columns. In control experiments the columns retained 96 % of the active proteinase incubated with anti-Dnp-MCP IgG, and the recovery of the enzyme incubated with non-immune IgG was 85 % under the conditions described. An additional 3–10 % of the active proteinase was lost repeatedly on the columns following incubation of proteinase with anti-subunit antibodies anti-B, anti-C and anti-D. These results suggest that anti-B, anti-C and anti-D do recognize the native complex, although the proportion of IgGs doing so may be very low.

Immunoblot analysis of cross-reactivity between subunits with affinity-purified antibodies against individual subunits

Immunoblots of SDS/PAGE gels with anti-B, anti-C and anti-D antibodies show only a single band (Fig. 4). For the anti-Dnp-MCP and anti-E antibodies, which recognize more than one band on blots of SDS/PAGE gels, possible relationships between immunoreactive polypeptides of different molecular masses were
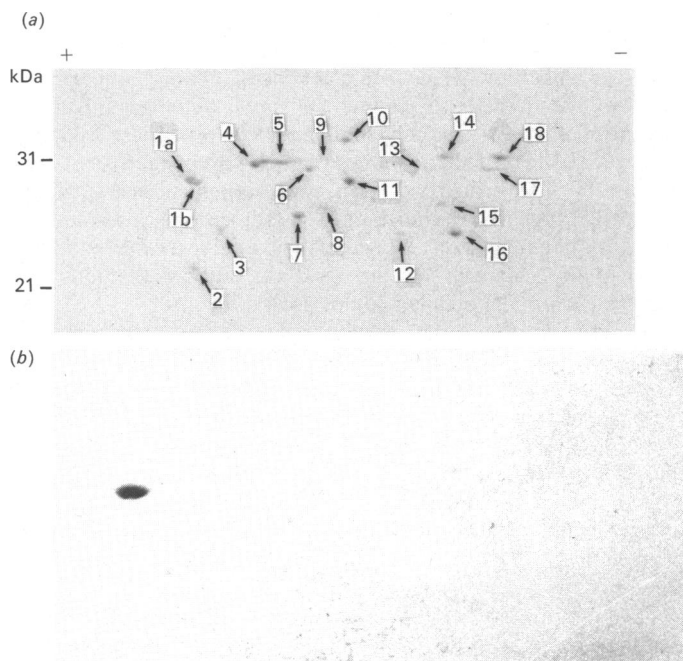
Fig. 6. Immunoblots of proteinase subunits separated by two-dimensional PAGE

(a) Polypeptides electroblotted on to nitrocellulose stained with Ponceau S and numbered for convenience. (b) Immunoblot with anti-B antibodies, which recognize polypeptides 1a and 1b. Anti-C antibodies recognize polypeptides numbered 4, 5 and 9, anti-D antibodies recognize polypeptide 7, and anti-E antibodies recognize polypeptides numbered 4, 5, 7 and 9. The two major immunoreactive species with anti-MCP antibodies were polypeptides 17 and 18.

Fig. 7. Expression of MCP subunits in various tissues

Immunoblots of crude tissue extracts with affinity-purified anti-Dnp-MCP antibodies (a) and with affinity-purified anti-D antibodies (b). Extracts were prepared from fresh tissue as described in ref. [9] but with a final centrifugation at 100000 g for 1 h. Lane 1, prestained molecular-mass markers; lane 2, purified rat liver MCP (2 μg); lanes 3–10, crude extracts (100 μg of protein) prepared from liver, lung, spleen, kidney, heart, brain, erythrocytes and psoas muscle respectively.
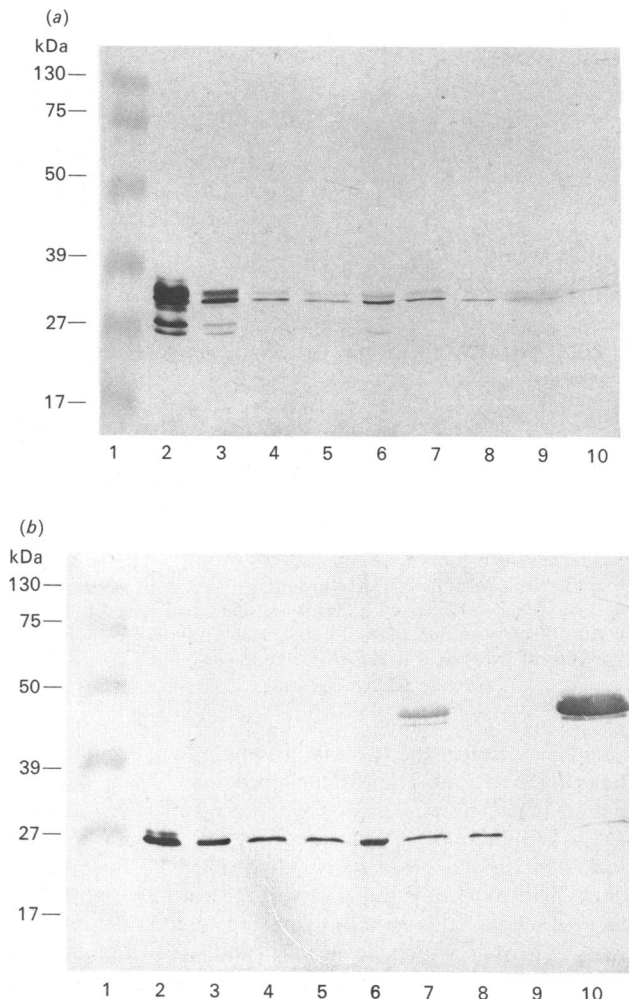
investigated by carrying out immunoblot experiments with affinity-purified IgGs directed against individual immunoreactive protein bands. The results show little evidence for cross-reactivity between three bands of different molecular masses for anti-Dnp-MCP (Fig. 5a), and two bands for anti-E (Fig. 5b).

Immunoblots of two-dimensional PAGE gels

Since SDS/PAGE does not clearly resolve all the polypeptide components of the complex, possible relationships between polypeptides were also investigated by immunoblotting of two-dimensional PAGE gels with the various IgG preparations (Fig. 6). At the acidic end of the pI range anti-B antibodies recognized two overlapping polypeptide spots (Fig. 6b) and it is likely, because of their poor separation on IEF rods, that these two polypeptides were both injected into the rabbit. Anti-C antibodies react principally with one spot with a pI value corresponding to that of the polypeptide used for immunization. However, they also react with several other spots of the same molecular mass (see legend to Fig. 6), the relative amounts of which can vary in different proteinase preparations. It is possible that these anti-C immunoreactive species are related by post-translational modifications occurring either in vivo or during purification or electrophoresis of the enzyme. Anti-D antibodies reacted with a single major spot (the polypeptide numbered 7 in Fig. 6a). It is likely that anti-E antibodies were raised as a result of co-injection of two polypeptides of the same pI value but different molecular masses.

Detection of proteinase subunits in rat liver extracts with the use of affinity-purified antibodies

Anti-Dnp-MCP antibodies were affinity-purified from blots of SDS/PAGE gels containing all the subunits of the complex for

immunoblotting of crude liver extracts. Affinity-purified anti-C and anti-D antibodies were also prepared. Immunoblots of SDS/PAGE gels with each of the affinity-purified IgG preparations showed cross-reactivity with protein bands of the crude extract at the same molecular mass as for the purified proteinase (see examples in Fig. 7) and, at least under the conditions used, there was no cross-reactivity with other liver proteins. Immunoblots of extracts of cells treated immediately with SDS show bands of the expected molecular mass.

Detection of proteinase subunits in various tissues with the use of affinity-purified antibodies

The same preparations of affinity-purified antibodies used for immunoblots of crude liver extracts were also used to investigate expression of proteinase subunits in different tissues. In each case, immunoblots of crude extracts showed the highest concentrations of proteinase subunits to be present in liver. Spleen, lung and kidney also had reasonably high concentrations of MCP, but erythrocytes and skeletal muscle contained relatively little. In most of the tissue extracts there was only sufficient
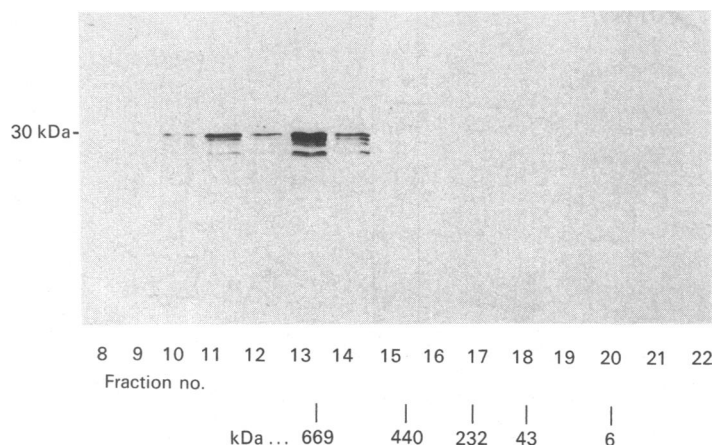
Fig. 8. Association of MCP subunits with a larger multisubunit complex

Immunoblot analysis of fractions isolated by Superose 6 gel filtration of crude extracts of rat liver. Samples of 1 ml fractions numbered 8–22 from the column were run on SDS/PAGE gel and blotted on to nitrocellulose for detection of protein with Ponceau S (not shown) and for MCP subunits with affinity-purified anti-Dnp-MCP antibodies. The positions of molecular-mass markers are indicated. Purified rat liver MCP is eluted in fractions 13 and 14.

proteinase to detect the two major immunoreactive bands with affinity-purified anti-Dnp-MCP antibodies when equivalent amounts of protein were used. There was no cross-reactivity with proteins of other molecular masses. With affinity-purified anti-D antibodies, on the other hand, cross-reactivity with a single protein band at 42 kDa was observed for heart and muscle only. It is possible that this band is actin since it could be seen to be a major protein component on Ponceau S-stained blots (results not shown). A single 30 kDa immunoreactive band was seen in all tissue extracts with the use of affinity-purified anti-C antibodies and the highest concentrations were in liver, kidney and spleen.

From the results presented in Fig. 7 and others, it is clear that immunoreactive subunits of the MCP complex are of very similar if not identical size in different tissues of the rat. However, differences both in subunit size and cross-reactivity were observed with the enzyme from widely different species (results not shown).

Association of subunits with MCP and a higher-molecular-mass complex in rat liver

In order to investigate whether MCP subunits can occur either in monomeric form or associated with other complexes in addition to MCP, fresh rat liver extracts were fractionated by f.p.l.c. (Superose 6) gel filtration before immunoblot analysis of the various fractions with affinity-purified anti-Dnp-MCP antibodies. The results of these experiments showed no evidence for free MCP subunits although subunits associated with MCP (present in fractions 13 and 14, Fig. 8) were easily detected. In addition, MCP subunits were also detected in association with a higher-molecular-mass complex (Fig. 8). The molecular mass of the larger complex was estimated to be in the range 1000–3000 kDa. The addition of ATP to the buffers as described in the Experimental section had little effect on the proportion of immunoreactive subunits associated with the larger complex.

DISCUSSION

MCP has an unusually complex subunit composition. It is composed of many different types of polypeptides with molecular

masses in the range 22–34 kDa. The components of the complex are best analysed by IEF or two-dimensional PAGE because the pI values range more widely (from pH 5 to 8.5) than the molecular masses. The pattern of about 20 spots obtained on two-dimensional gels is generally similar for the proteinase isolated from several mammalian sources (e.g. [27] and Fig. 2), but the pattern for the proteinase from some other species such as lobster [28] appears to be much simpler. However, poor resolution or the apparent loss or absence of basic polypeptides in some other studies as well as the lack of suitable methods of identification make detailed comparison difficult.

Because of the large number of different polypeptides associated with MCP, one concern is that MCP polypeptides may be proteolytic fragments of larger subunits or that some of the polypeptides may be related by proteolytic cleavage or other post-translational modifications. Comparison of the cross-reactivity of subunits in crude extracts of fresh rat liver with the pattern obtained for purified MCP shows that MCP subunits are not generated from a small number of larger polypeptides by proteolytic cleavage occurring during purification of the proteinase.

Treatment of the MCP complex with DNBS proved to be a useful method of enhancing the antigenic response, and the resulting antibodies that had a higher titre and/or affinity were more useful than the anti-(native MCP) antibodies for the studies described here as well as for subcellular localization studies [28a]. Although Wagner & Margolis [19] suggested that immunoreactive polypeptides of bovine lens MCP are related to each other, the results with affinity-purified IgGs presented here (Fig. 5) suggest that this is not the case for the rat liver enzyme. Indeed, these data, taken with the results of blotting experiments with the other subunit-specific antisera, suggest that many of the proteinase polypeptides are antigenically distinct. This lack of cross-reactivity between many of the polypeptides suggests that few, if any, of the MCP polypeptides are modified forms of other subunits.

Despite the usefulness of the various subunit-specific antibodies in analysing polypeptide components of the complex, the stoichiometry of subunits of rat liver MCP is still not clear, and the complexity of the polypeptide composition may reflect multiple forms of the proteinase, possibly from different cell types or subcellular localizations. However, MCP isolated from single cell types such as Chinese-hamster ovary cells appears to have the same complexity of subunit composition (S. T. Sweeney & A. J. Rivett, unpublished work). Falkenburg & Kloetzel [29] have separated isoforms of the Drosophila MCP by ion-exchange chromatography, and this complex has also been shown to be phosphorylated in vivo [30]. Although a covalent modification such as phosphorylation might explain the cross-reactivity of anti-C antibodies with several spots of the same molecular mass, there is no evidence as yet to show phosphorylation of the liver proteinase.

Other post-translational modifications such as deamidation and proteolytic cleavage could occur either in vivo or during purification of the complex. However, there is little evidence for proteolytic cleavage except of the 34 kDa polypeptide. Results of lectin-binding studies with the mouse MCP have suggested that the complex is glycosylated [31] and polypeptides numbered 3, 7 and 11 (Fig. 6a) of the rat liver enzyme were found to bind concanavalin A (results not shown). Other possible relationships between MCP polypeptides have been investigated and there is evidence from N-terminal amino acid sequence analysis [32,33], and now also from sequences deduced from the nucleotide sequences of cloned cDNAs [34,35], that several of the polypeptides are encoded by members of the same gene family. The weak cross-reactivity observed between some subunits when

large amounts of MCP were used for immunoblots may therefore reflect sequence similarities.

The results of immunoblots with extracts of different rat tissues not only confirm the observations of Tanaka *et al.* [15] that the enzyme is present in many different tissues, but they also show that the immunoreactive subunits are of the same size in different rat tissues. There was no evidence of tissue-specific expression of subunits, nor of tissue differences in splicing. With each of the antibodies, liver was found to contain the highest concentration of proteinase subunits, whereas skeletal muscle and erythrocytes contained relatively little. The subunit composition of MCP appears to be similar in different tissues. This also seems to be the case for the bovine enzyme [16], although some differences in enzymic properties of the bovine lens and pituitary enzymes have been reported [36].

The cross-reactivity between MCP complexes from different species detected with anti-(native proteinase) antibodies [4–6,15,37] suggests that the enzymes are structurally similar. Although cross-reactivity between complexes from widely different sources (e.g. rat and yeast) can be demonstrated on immunoblots, no cross-species precipitation of the complex has been demonstrated (A. J. Rivett & D. H. Wolf, unpublished work; [37]). Moreover, IgGs may react with polypeptides of different molecular masses when immunoblots of MCPs from widely differing species are compared (S. T. Sweeney & A. J. Rivett, unpublished work; [4–6,15,37]). However, there is considerable similarity between sequences of subunits of the *Drosophila* and rat proteinase deduced from the nucleotide sequence of cDNAs [34,35] and also between *N*-terminal amino acid sequences determined for rat liver and human erythrocyte subunits [32,33].

Our observations that MCP subunits occur in association with a higher-molecular-mass complex than MCP as well as with the MCP complex itself are consistent with recent suggestions that MCP is a component of the larger 26 S ubiquitin–protein conjugate-degrading proteinase [17,12,13,38]. The amount of MCP subunits associated with the larger complex when ATP was added to the extract was investigated because of the proposed ATP-dependent incorporation of MCP into the 26 S complex [12,13,18]. Results were similar in the presence and absence of ATP. The lack of detection of lower-molecular-mass cross-reacting proteins with the anti-Dnp-MCP antibodies suggests that the immunoreactive subunits do not exist either as free subunits or in an assembly of subunits that is smaller than MCP.

Further investigations are required to understand the apparent complexity of the polypeptide composition of the MCP complex. There may be different types of polypeptides associated with the proteinase from different cellular locations, tissues or cell types, or at different stages of differentiation [39], and it is likely that the subunit-specific antibody preparations will be useful for such investigations. These antibodies will also be useful for a more detailed comparison of the components of MCP and the ubiquitin–protein-conjugate-degrading proteinase complex. The relationship between ubiquitin-dependent and ubiquitin-independent pathways of intracellular protein degradation is not clear, and the significance of common structural elements of the proteinases implicated in these pathways remains to be determined.

REFERENCES

1. Rivett, A. J. (1989) Arch. Biochem. Biophys. **268**, 1–8
2. Orlowski, M. (1990) Biochemistry **29**, 10289–10297
3. Baumeister, W., Dahlmann, B., Hegerl, R., Kopp, F., Kuehn, L. & Pfeifer, G. (1988) FEBS Lett **241**, 239–245
4. Falkenburg, P. E., Haass, C., Kloetzel, P. M., Niedel, B., Kopp, F., Kuehn, L. & Dahlmann, B. (1988) Nature (London) **331**, 190–192
5. Arrigo, A.-P., Tanaka, K., Goldberg, A. L. & Welch, W. J. (1988) Nature (London) **331**, 190–192
6. Kleinschmidt, J. A., Escher, C. & Wolf, D. H. (1988) FEBS Lett. **239**, 35–40
7. Skilton, H. S., Eperon, I. C. & Rivett, A. J. (1991) FEBS Lett. **279**, 351–355
8. Rivett, A. J. (1985) J. Biol. Chem. **260**, 12600–12606
9. Orlowski, M. & Michaud, C. (1989) Biochemistry **28**, 9270–9278
10. Rivett, A. J. (1989) J. Biol. Chem. **264**, 12215–12219
11. McGuire, M. J., Croall, D. E. & De Martino, G. N. (1988) Arch. Biochem. Biophys. **262**, 273–285
12. Eytan, E., Ganoth, D., Armon, T. & Hershko, A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7751–7755
13. Driscoll, J. & Goldberg, A. L. (1990) J. Biol. Chem. **265**, 4789–4792
14. 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
15. Tanaka, K., Ii, K., Ichihara, A., Waxman, L. & Goldberg, A. L. (1986) J. Biol. Chem. **261**, 15197–15203
16. Ray, K. & Harris, H. (1987) Biochem. J. **248**, 643–648
17. Hough, R., Pratt, G. & Rechsteiner, M. (1987) J. Biol. Chem. **262**, 8303–8313
18. Sweeney, S. T. & Rivett, A. J. (1989) Biochem. Soc. Trans. **17**, 1126–1127
19. Wagner, B. J. & Margolis, J. W. (1989) Biochem. J. **257**, 265–269
20. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
21. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
22. O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021
23. Duncan, R. & Hershey, J. W. B. (1984) Anal. Biochem. **138**, 144–155
24. Harlow, E. & Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor
25. Bos, E. S., Van der Dolen, A. A., Van Rooy, N. & Schuurs, A. W. H. M. (1981) J. Immunoassay **2**, 187–204
26. Towbin, H., Stachelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
27. Martins de Sa, C., Grossi de Sa, M. F., Akhayat, O., Broders, F., Scherrer, K., Horsch, A. & Schmid, H. P. (1986) J. Mol. Biol. **187**, 479–493
28. Mykles, D. L. (1989) Arch. Biochem. Biophys. **274**, 216–228
28a. Knecht, E., Palmer, A., Sweeney, S. T. & Rivett, A. J. (1991) Biochem. Soc. Trans. **19**, 2935
29. Falkenburg, P. E. & Kloetzel, P. M. (1989) J. Biol. Chem. **264**, 6660–6666
30. Haass, C. & Kloetzel, P. M. (1989) Exp. Cell Res. **180**, 243–252
31. Tomek, W., Adam, G. & Schmidt, H.-P. (1988) FEBS Lett. **239**, 155–158
32. Lilley, K. S., Davison, M. D. & Rivett, A. J. (1990) FEBS Lett. **262**, 327–329
33. Lee, L. W., Moomaw, C. R., Orth, K., McGuire, M. J., De Martino, G. N. & Slaughter, C. A. (1990) Biochim. Biophys. Acta **1037**, 178–185
34. Haass, C., Pesold-Hurt, B., Multhaup, G., Beyreuther, K. & Kloetzel, P.-M. (1990) Gene **90**, 235–241
35. Kumatori, A., Tanaka, K., Tamura, T., Fujiwara, T., Ichihara, A., Tokunaga, F., Onikura, A. & Iwanaga, S. (1990) FEBS Lett. **264**, 279–282
36. Ray, K. & Harris, H. (1986) FEBS Lett. **194**, 91–95
37. Tanaka, K., Yoshimura, T., Kumatori, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K. & Takagi, T. (1988) J. Biol. Chem. **263**, 16209–16217
38. Ishiura, S., Nomura, Y., Tsukahara, T. & Sugita, H. (1989) FEBS Lett. **257**, 123–126
39. Grossi de Sa, M. F., Martins de Sa, C., Harper, F., Coux, O., Akhayat, O., Pal, J. K., Floretin, Y. & Scherrer, K. (1988) J. Cell. Sci. **89**, 151–165