Molecular interaction of the proteasome (multicatalytic proteinase)

Evidence that the proteasome is not a constituent of the '26 S' multienzyme complex

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On the basis of recent reports that suggested that proteasomes, via an ATP-dependent process, become integral components of a '26 S' complex possessing 3-carboxypropionyl-Leu-Leu-Val-Tyr 4-methylcoumarin-7-ylamide-hydrolysing activity, we have investigated the molecular interaction of proteasomes in ATP-stabilized fraction II (proteins adsorbed on DEAE-matrix and eluted with 0.5 M-KCl) of rabbit reticulocytes and mouse liver. Analysis of the various extracts by $(\text{NH}_4)_2\text{SO}_4$ fractionation, velocity-gradient centrifugation, non-denaturing PAGE and SDS/PAGE and immunoblotting with proteasome-specific antisera failed to identify the proteasome as part of a higher-molecular-mass '26 S' multienzyme complex. In all instances proteasomes are identified in their 'free' 650 kDa '20 S' form. In addition to the proteasome and independent of the presence of MgATP, we isolated a high-molecular-mass proteinase whose electrophoretic migration behaviour and sedimentation rate correspond to that of the previously described '26 S' proteinase. This '26 S' proteinase possesses a strong 3-carboxypropionyl-Leu-Leu-Val-Tyr 4-methylcoumarin-7-ylamide-hydrolysing activity and is composed of several non-identical polypeptides in the molecular-mass range 20–150 kDa. Despite its similarity to proteasomal enzyme activity, protein analysis and immunoblotting experiments demonstrate that neither the intact proteasome nor subunits thereof are components of the '26 S' proteinase complex.

INTRODUCTION

The proteasome or multicatalytic proteinase, which was first identified in bovine pituitary (Wilk & Orlowski, 1980), is a ubiquitous non-lysosomal proteinase complex that has been isolated from a large variety of eukaryotic cells (for reviews see Rivett, 1989; Dahlmann *et al.*, 1989a) as well as from the prokaryotic archaebacterium *Thermoplasma acidophilum* (Dahlmann *et al.*, 1989b). The proteasome is a reel-shaped particle (Baumeister *et al.*, 1988) composed of a characteristic set of non-identical protein subunits with molecular masses in the range 21–35 kDa (Kloetzel, 1987). As judged from immunological experiments, the subunits are conserved between species (Falkenburg *et al.*, 1988; Tanaka *et al.*, 1988), and it has been shown that proteasome subunits are encoded by an evolutionarily conserved gene family (*PROS* genes) (Haass *et al.*, 1990; Tanaka *et al.*, 1990). The ubiquitous occurrence of this enzyme and its early development in evolution indicates that it serves an important cellular function. Although there exists general agreement on the multicatalytic nature of the proteasome, little is known about its functions *in vivo*, and quite contradictory reports have been published on the ATP-dependence of its proteolytic activity and its involvement in ubiquitin-dependent proteolysis (Dahlmann *et al.*, 1983; Rivett, 1985; Hough *et al.*, 1987; Waxman *et al.*, 1987; Tanaka & Ichihara, 1988; Kuehn *et al.*, 1989; McGuire *et al.*, 1988; Matthews *et al.*, 1989).

Hough *et al.* (1987) isolated an ATP-dependent high-molecular-mass proteinase, designated '26 S' proteinase, from reticulocytes that hydrolyses ubiquitin conjugates and the peptide substrate 3-carboxypropionyl-Leu-Leu-Val-Tyr 4-methylcoumarin-7-ylamide (Suc-LLVY-NH-Mec) but not unconjugated proteins. This proteinase complex was shown to be composed of a larger number of high-molecular-mass proteins and a set of proteins in the molecular-mass range 20–35 kDa. On the

basis of experiments performed *in vitro*, Hershko and co-workers (Ganath *et al.*, 1988; Eytan *et al.*, 1989) suggested that in rabbit reticulocytes the proteasome, which they designate conjugate-degrading factor CF-3, associates in an MgATP-dependent assembly process with two other factors, CF-1 and CF-2, to form a high-molecular-mass multienzyme complex of > 1000 kDa with a designated sedimentation coefficient of 26 (i.e. '26 S' proteinase). In a more recent publication Driscoll & Goldberg (1990) arrived at a similar conclusion. They showed that after incubation of rabbit reticulocyte fraction II (proteins adsorbed on DEAE-matrix and eluted with 0.5 M-KCl) at 37 °C in the presence of MgATP the proteasomes are precipitated as part of the '26 S' complex in a 38%-saturated- $(\text{NH}_4)_2\text{SO}_4$ cut and migrate as part of a higher-molecular-mass complex when analysed by gel electrophoresis. However, detailed molecular data, e.g. from SDS/PAGE and immunological analysis, for the '26 S' complex were not presented by either of these groups of authors.

The major conclusion of these experiments is that the proteasome as a genuine cellular component of the '26 S' complex and as identified by its Suc-LLVY-NH-Mec-hydrolysing activity represents the major proteolytic enzyme involved in the degradation of the ubiquitin conjugates within this complex. Eytan *et al.* (1989) have even considered the possibility that proteasome when isolated as 'free' '20 S' particle is an experimental artifact due to dissociation of the '26 S' complex during the isolation procedures. Since breakdown of ubiquitin conjugates appears to be a general characteristic of eukaryotic cells and in view of the amounts of proteasome present in most eukaryotic cells, the '26' complex ought to be one of the major high-molecular-mass complexes in eukaryotic tissues. The experiments described in the present paper were undertaken to assess the ubiquitous existence of the so-called '26 S' complex and to analyse the molecular organization of the proteasome within this complex.

Abbreviations used: Suc-, 3-carboxypropionyl-; -NH-Mec, 4-methylcoumarin-7-ylamide.

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MATERIALS AND METHODS

Materials

Female Chinchilla rabbits (3–3.5 kg) were purchased from Savo-Ivanovas G.m.b.H., Kisslegg, Germany.

Phenylhydrazine hydrochloride and alkaline-phosphatase-conjugated anti-(sheep IgG) antibody were from Sigma, München, Germany. Peroxidase-conjugated secondary antibody was obtained from Dianova, Hamburg, Germany. DEAE-cellulose (DEAE-Servacel), dithioerythritol and dithiothreitol were obtained from Serva Feinbiochemica, Heidelberg, Germany. DEAE-Sephacel was purchased from Pharmacia, Freiburg, Germany. ATP (disodium salt), creatine kinase (EC 2.7.3.2) and phosphocreatine (disodium salt) were obtained from Boehringer, Mannheim, Germany. Suc-LLVY-NH-Mec was from Bachem, Bubendorf, Switzerland. Other chemicals were of the highest purity available.

Induction of reticulocytosis and preparation of reticulocyte lysates

Reticulocytosis was induced in rabbits with phenylhydrazine, and reticulocytes were washed and lysates were prepared exactly as described previously (Kuehn *et al.*, 1989). After dialysis against 50 mM-Tris/HCl buffer, pH 7.8, containing 20 mM-KCl, 1 mM-dithioerythritol and 20% (v/v) glycerol, lysates were stored at -80°C . Fraction II (proteins adsorbed on DEAE-Servacel and eluted with 0.5 M-KCl) was prepared from lysates as described elsewhere (Eytan *et al.*, 1989). For treatment of reticulocyte lysate fraction II with MgATP to promote assembly of a '26 S' complex and fractionation with $(\text{NH}_4)_2\text{SO}_4$, conditions as given by Eytan *et al.* (1989) were followed. For preparation of mouse liver extracts and fraction II, 5 g of freshly isolated mouse liver was used. Buffers and procedures were as described above.

Isolation of multicatalytic proteinase from different lysate fractions

This was done by the method described (Kuehn *et al.*, 1989), except that EDTA was omitted and that buffers were supplemented to contain 1 mM-dithioerythritol, 1 mM-ATP and 20% (v/v) glycerol (Eytan *et al.*, 1989).

Assays

Proteolytic activity with Suc-LLVY-NH-Mec substrate was measured as described previously (Kuehn *et al.*, 1989; Falkenburg & Kloetzel, 1989; Haass & Kloetzel, 1989). This substrate has been shown to be split by the proteasome and the '26 S' complex (Hough *et al.*, 1987).

Sucrose-gradient centrifugation

Samples were centrifuged on 10–40% (w/v) sucrose gradients in a Beckman SW40 Ti rotor. Centrifugation conditions were 40000 rev./min, 1°C and $1 \times 10^{12} \omega^2 t \cdot \text{s}^{-1} \cdot \text{rad}^{-1}$. Sucrose gradients were prepared in 80 mM-potassium acetate buffer, pH 7.2, containing 5 mM-ATP. In one series of experiments 5% (v/v) glycerol was added to the sucrose solutions. Addition of glycerol had no effect on the results obtained. Because of the experimental procedures sucrose gradients can differ slightly in the shape of the gradients, which in turn can lead to differing sedimentation distances for the same molecule in different sucrose gradients. To be able to compare sedimentation coefficients determined for the position of enzyme activity in different sucrose gradients, gradient fractions of sucrose gradients to be compared were aligned with respect to the sucrose concentration determined for each gradient fraction. Sedimentation coefficients were calculated by the method of McEwen (1967). Since the calculated sedimentation coefficients were close to previously published

values and to avoid confusion by introducing new values 'free' proteasome was designated '20 S' proteasome and the faster-sedimenting proteinase '26 S' proteinase.

Electrophoretic techniques

Non-denaturing polyacrylamide-gel slab gels containing a continuous gradient (5–18%, w/w) of polyacrylamide were used, and proteins were electrophoresed at pH 8.3 (Davis, 1964). Non-denaturing PAGE on 5% (w/w) polyacrylamide gels was performed as described previously (Haass & Kloetzel, 1989). For SDS/PAGE on 12% (w/w) polyacrylamide gels the procedure published by Laemmli (1970) was used. Electrophoretic transfer of proteins from polyacrylamide gels was carried out in a Trans-Blot cell (Bio-Rad Laboratories, München, Germany), according to the instructions of the manufacturer. For Western blotting, nitrocellulose filters were incubated with polyclonal monospecific antibodies raised either in sheep (rabbit proteasome antibody) or in rabbits (mouse proteasome antibody). Primary antibodies were detected by using peroxidase-conjugated goat anti-(rabbit IgG) antibody or alkaline-phosphatase-conjugated donkey anti-(sheep IgG) antibody.

Other methods

Antibodies to purified rabbit proteasome were raised in sheep and isolation of IgG from whole serum was as described elsewhere (Kuehn *et al.*, 1989). Antibody to purified mouse proteasome was raised in rabbit. Protein concentrations were determined by the method of Bradford (1976), with Lab-Trol (Dade, Miami, FL, U.S.A.) as the standard. All other experimental details were as given in the legends to Figures.

RESULTS

Distribution of immunoreactive multicatalytic proteinase and Suc-LLVY-NH-Mec-hydrolysing activity in $(\text{NH}_4)_2\text{SO}_4$ precipitates of reticulocyte lysate fraction II

As summarized in Table 1, essentially all the Suc-LLVY-NH-Mec-hydrolysing activity as well as immunoreactive proteasome present in reticulocyte fraction II is precipitated in the 38–70%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction. When fraction II is pre-incubated at 37°C in the presence of MgATP, i.e. conditions under which the '26 S' complex is formed (Eytan *et al.*, 1989; Driscoll & Goldberg (1990), about 2% immunoreactive material and Suc-LLVY-NH-Mec-hydrolysing activity of that present in total lysate fraction II is precipitated at 38% $(\text{NH}_4)_2\text{SO}_4$ saturation.

The bulk of proteasome and Suc-LLVY-NH-Mec-hydrolysing activity, which remains in the supernatant during this step, is quantitatively precipitated at 70% $(\text{NH}_4)_2\text{SO}_4$ saturation (Table 1). With the very small amounts of proteolytic activity and proteasome protein in the 38%-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate, assumed to contain the '26 S' form of the proteasome, it proved difficult to purify further the proteolytic enzyme from this fraction. In an attempt to increase yields, we tested whether a further cycle of MgATP incubation might induce the formation of additional proteasome in the converted '26 S' form. Towards this end, a 38%-saturated- $(\text{NH}_4)_2\text{SO}_4$ supernatant that had previously been incubated at 37°C in the presence of MgATP, was dialysed free of $(\text{NH}_4)_2\text{SO}_4$ and of the nucleotide.

After this second round of incubation at 37°C in the presence of MgATP and subsequent $(\text{NH}_4)_2\text{SO}_4$ precipitation an additional 2% of immunoreactive proteasome and Suc-LLVY-NH-Mec-hydrolysing activity could be recovered in the 38%-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate. Analysis of this material by SDS/PAGE revealed a protein pattern that was not identical with that of lysate that had been subjected to a single 'conversion cycle' (results not shown).

Table 1. Immunological contents of multicatalytic proteinase and activity against Suc-LLVY-NH-Mec in differently treated preparations of reticulocyte lysate fraction II

Portions (5 ml) of reticulocyte fraction II, each containing 70 mg of protein, were supplemented with ATP and the regenerating system (+ATP) as described by Eytan *et al.* (1989) or with buffer only (−ATP) and were incubated for 60 min at the indicated temperature. Samples were then fractionated with solid $(\text{NH}_4)_2\text{SO}_4$. Proteins precipitated at 0–38 % and 38–70 % saturation with respect to the salt were collected by centrifugation, dissolved in 20 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithioerythritol and 20 % (v/v) glycerol and dialysed against the same buffer. Quantitative rocket immunoelectrophoresis and assay for Suc-LLVY-NH-Mec-hydrolyzing activity were as described in the Materials and methods section. Values given are averages from two separate experiments, which showed a variation of less than 10 %. Abbreviations: MCP, multicatalytic proteinase; N.D., not detected.

Preincubation conditions	$(\text{NH}_4)_2\text{SO}_4$ step (% saturation)	Concn. of protein (mg/ml)	Total protein (mg)	Immunological MCP ($\mu\text{g}/\text{mg}$)	Total MCP (μg)	Suc-LLVY-NH-Mec hydrolysis	
						Sp. activity (pmol/min per mg)	Total activity (pmol/min)
−ATP/4 °C	38	16	18.4	N.D.	–	15	276
	70	10	49	25	1.230	3.912	191.7
+ATP/4 °C	38	16.7	18.4	N.D.	–	20	370
	70	8.8	40.5	29	1.180	4.425	179.2
−ATP/37 °C	38	16	19.2	N.D.	–	17	326
	70	9.7	47.5	26	1.240	3.846	182.7
+ATP/37 °C	38	16.3	18.4	1	19	220	4.010
	70	9.3	43.3	27	1.180	4.210	182.3

Fig. 1. Hydrolysis of Suc-LLVY-NH-Mec by reticulocyte fraction II proteinases separated by non-denaturing PAGE on a 5–18% linear-gradient gel

Reticulocyte fraction II proteins were incubated as described in the legend to Table 1, and proteasomes were purified from fraction II as described in the text. The Figure shows purified proteasome and reticulocyte fraction II samples that had been overlaid with 200 μM -Suc-LLVY-NH-Mec solution and incubated at 37 °C. Lane 1, purified proteasomes. Lanes 2–5, reticulocyte fraction II proteins that had been preincubated for 60 min and subsequently fractionated with $(\text{NH}_4)_2\text{SO}_4$. Precipitates obtained at 38–70 % saturation with respect to the salt had been preincubated as follows: lane 2, +ATP/4 °C; lane 3, −ATP/4 °C; lane 4, +ATP/37 °C; lane 5, −ATP/37 °C. Lanes 6–9, precipitates from reticulocyte fraction II proteins precipitated at 0–38 % $(\text{NH}_4)_2\text{SO}_4$ saturation and preincubated as follows: lane 6, +ATP/4 °C; lane 7, −ATP/4 °C; lane 8, +ATP/37 °C; lane 9, −ATP/37 °C. The amounts of sample protein applied were: lane 1, 10 μg ; lanes 2–5, 500 μg each; lanes 6–9, 800 μg each. The positions of molecular-mass standard proteins are indicated at the left: from top to bottom, thyroglobulin, ferritin, catalase, lactate dehydrogenase and BSA.

Gel-electrophoretic analysis of proteins in differently treated preparations of reticulocyte fraction II

To analyse further the effect of MgATP on the structural organization of proteasomes in reticulocyte lysate, the various fractions from the experiment shown in Table 1 were subjected

to non-denaturing gel electrophoresis and tested for peptide-splitting activity by incubation with the fluorogenic substrate Suc-LLVY-NH-Mec. Fluorescence is restricted to a single band and at the same migration distance as that of purified proteasome (Fig. 1, lane 1). Among the proteins that had been collected from the 38 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate only that which had previously been found to contain measurable amounts of immunoreactive proteasome and Suc-LLVY-NH-Mec-hydrolysing activity (lysate preincubated at 37 °C in the presence of MgATP) was also found to hydrolyse the substrate Suc-LLVY-NH-Mec in the gel (Fig. 1, lane 8). In order to detect any proteinases that might require ATP for activity, a parallel gel was incubated with substrate plus ATP. On such gels, however, the same activity pattern as that shown in Fig. 1 was obtained. Consistent with this result, immunoblots of samples containing the 'free' or the putative '26 S' form of the proteasome, separated on non-denaturing polyacrylamide gels and developed with a monospecific polyclonal antibody to rabbit proteasome, give a single band at a position corresponding to the 650 kDa form of the enzyme (results not shown).

Our failure to detect the '26 S' form of proteasome in MgATP-treated reticulocyte lysate fraction II might be due to the following. The '26 S' complexes are not as assumed by Eytan *et al.* (1989) a major component of eukaryotic cells and therefore are only detectable after further purification by velocity gradient centrifugation. To examine this possibility, fraction II proteins incubated at 1 °C or 37 °C in the absence or in the presence of MgATP were fractionated with $(\text{NH}_4)_2\text{SO}_4$. Precipitates obtained in the 0–38 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ and 38–70 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ fractions were subjected to sucrose-gradient centrifugation. Individual gradient fractions were then assayed for their Suc-LLVY-NH-Mec-hydrolysing activity, and that precipitable at 38 % $(\text{NH}_4)_2\text{SO}_4$ saturation from control lysate (incubated at 1 °C in the absence of MgATP) is at the lower level of detection, whereas close to 100 % of the activity is contained in the 70 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate. Analysis of sucrose-gradient fractions reveals a single peak of Suc-LLVY-NH-Mec-hydrolysing activity sedimenting to a position corresponding to that of 'free' '20 S' form of the proteasome.

Analysis of protein present in fractions covering the '20 S'–'26 S' region of the gradient by non-denaturing PAGE

Fig. 2. Identification of Suc-LLVY-NH-Mec-hydrolysing activity in fraction II of rabbit reticulocyte lysates and analysis of '20 S' and '26 S' hydrolysing activity by immunoblotting and enzyme assay *in situ*

Fraction II was incubated in the presence of MgATP at 37 °C and at 1 °C. The 38%-saturated-(NH₄)₂SO₄ and 70%-saturated-(NH₄)₂SO₄ precipitates were subjected to sucrose-gradient centrifugation, and individual fractions (30 µl) were analysed with respect to their Suc-LLVY-NH-Mec-hydrolysing activity. Portions (30 µl) of sucrose-gradient fractions corresponding to sedimentation coefficients of '20 S'-'26 S' were subjected to non-denaturing PAGE on 5% polyacrylamide gels and analysed with respect to their Suc-LLVY-NH-Mec-hydrolysing activity. Proteins were blotted on to nitrocellulose filters and detected by monospecific antibody against rabbit multicatalytic proteinase. (a) Enzymic activity profile of the 38%-saturated-(NH₄)₂SO₄ precipitate of fraction II (●—●) and the 70%-saturated-(NH₄)₂SO₄ precipitate of fraction II (■—■) incubated at 1 °C. (b) Enzymic activity profile of the 38%-saturated-(NH₄)₂SO₄ precipitate of fraction II (●—●) and 70%-saturated-(NH₄)₂SO₄ precipitate of fraction II (■—■) incubated at 37 °C. (c) Analysis of the 38%-saturated-(NH₄)₂SO₄ precipitate of reticulocyte lysate fraction II incubated at 1 °C and at 37 °C by non-denaturing PAGE and immunoblotting. (d) Analysis of the 70%-saturated-(NH₄)₂SO₄ precipitate of reticulocyte fraction II incubated at 1 °C and at 37 °C by non-denaturing PAGE and immunoblotting. Numbers below (c) and (d) refer to the first and the last gradient fraction analysed. (e) Analysis of the Suc-LLVY-NH-Mec-splitting activity in the 70%-saturated-(NH₄)₂SO₄ precipitate of reticulocyte lysate fraction II incubated at 1 °C (control) and at 37 °C after non-denaturing PAGE. Suc-LLVY-NH-Mec-hydrolysing activity in the 38%-saturated-(NH₄)₂SO₄ precipitates could not be detected in the gels.

and immunoblotting shows that proteasome accumulation in gradient fractions 12 and 13 (Fig. 2c) coincides with the main peak of Suc-LLVY-NH-Mec-hydrolysing activity in the '20 S' position of the gradient. Interestingly, proteasomes sedimenting in gradient fractions > '20 S' exhibit an electrophoretic migration behaviour that is identical with that observed for the 'free' '20 S' form of the proteasome. This is most probably due to streaking towards the bottom of the gradient as a result of unspecific aggregation of the enzyme complex. Such unspecific aggregation and altered sedimentation characteristics of the enzyme complex are observed even when highly purified '20 S' proteasome is re-centrifuged under identical conditions (results not shown).

After incubation of the lysate at 37 °C in the presence of

MgATP minor amounts of Suc-LLVY-NH-Mec-hydrolysing activity is contained in the precipitate forming between 38% and 70% (NH₄)₂SO₄ saturation. In contrast with control lysates, incubation at 37 °C in the presence of MgATP induces a relative increase in Suc-LLVY-NH-Mec-hydrolysing activity in the '26 S' fractions of the gradient. In the 70%-saturated-(NH₄)₂SO₄ precipitate a pronounced shoulder of enzymic activity is observed beyond the main '20 S' proteolytic-activity peak. In the 38%-saturated-(NH₄)₂SO₄ precipitate Suc-LLVY-NH-Mec-hydrolysing activity is present in sucrose-gradient fractions corresponding to the '26 S' region of the gradient (Fig. 2b). Although this latter result is similar to the data shown by Eytan *et al.* (1989), gel-electrophoretic analysis demonstrates that the enhanced proteolytic activity in gradient fractions > '20 S'

Fig. 3. Identification of Suc-LLVY-NH-Mec-hydrolysing activity in fraction II of mouse liver extracts and gel-electrophoretic analysis of '20 S' and '26 S' hydrolysing activity by immunoblotting and enzyme assay *in situ*

Fraction II was incubated at 37 °C in the presence of MgATP or in the absence of ATP at 1 °C (control). The 38 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ and 70 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitates were subjected to sucrose-gradient centrifugation, and individual gradient fractions (30 μl) were tested with respect to their Suc-LLVY-NH-Mec-hydrolysing activity. Portions (30 μl) of sucrose-gradient fractions corresponding to a sedimentation coefficient of '20 S' to '26 S' were subjected to non-denaturing PAGE, proteins were blotted on to nitrocellulose filters and proteasomes were detected by monospecific antibodies against mouse proteasomes. (a) Enzymic activity profile of the 38 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate (mouse) incubated at 1 °C (●—●) and at 37 °C (■—■). (b) Enzymic activity profile of the 70 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate (mouse) incubated at 1 °C (●—●) and at 37 °C (■—■). (c) Analysis of 38 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate of mouse liver fraction II incubated at 1 °C by non-denaturing PAGE and immunoblotting. Since only minor amounts are precipitated with 38 %-saturated $(\text{NH}_4)_2\text{SO}_4$ from mouse fraction II incubated at 37 °C no protein was detectable by immunoblotting. (d) Analysis of 70 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitates of mouse liver fraction II incubated at 1 °C and at 37 °C by non-denaturing PAGE and immunoblotting. (e) Analysis of Suc-LLVY-NH-Me-splitting activity of the 70 %-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate of mouse liver fraction II incubated at 1 °C and at 37 °C after non-denaturing PAGE. Numbers below indicate the numbers of the first and the last gradient fraction analysed.

cannot be the consequence of proteasome incorporation into a larger '26 S' complex. This is most convincingly shown when proteasomes are analysed by non-denaturing PAGE and immunoblotting with proteasome-specific antisera (Figs. 2c and 2d). In all instances proteasomes reveal an electrophoretic migration behaviour that is absolutely identical with that of the 'free' '20 S' proteasomes.

Despite the observation that irrespective of sedimentation behaviour proteasomal enzyme activity is restricted to its '20 S' form, enzyme assays on non-denaturing polyacrylamide gels reveal a second proteolytically active complex in the '20 S'-'26 S' region of the sucrose gradient that, however, shows no

immunological reaction with proteasome-specific antibodies (Figs. 2c, 2d and 2e). With regard to its high Suc-LLVY-NH-Mec-hydrolysing activity, its electrophoretic migration and its sedimentation behaviour, this complex is most probably identical with the '26 S' proteinase complex previously described by Hough *et al.* (1987). The presence of this '26 S' complex is independent of MgATP and can be detected in treated as well as in control fraction II lysates. Both enzyme complexes, the proteasome and the '26 S' proteinase, show an overlapping sedimentation behaviour, whereby the '26 S' complex sediments slightly faster than the '20 S' proteasome complex. This difference in sedimentation behaviour becomes even more

pronounced in lysates that had been treated with MgATP at 37 °C (Fig. 2e). Although the absolute Suc-LLVY-NH-Mec-hydrolysing activity of the '26 S' proteinase is similar to that of the '20 S' proteasome, so far the little amount of protein present in this complex has precluded a detailed molecular analysis.

Identification and molecular characterization of the '26 S' proteinase in mouse liver fraction II extracts

In order to see whether the presence of the '26 S' proteinase complex is ubiquitous or specific for rabbit reticulocyte lysate fraction II, we prepared fraction II from mouse liver extracts. For analysis, fraction II was incubated in the presence of MgATP at 37 °C or in the absence of MgATP at 1 °C (control). The 38%-saturated-(NH₄)₂SO₄ and 78%-saturated-(NH₄)₂SO₄ precipitates of fraction II were then analysed by velocity-gradient centrifugation and the Suc-LLVY-NH-Mec-hydrolysing activity was monitored in the various gradient fractions (Figs. 3a and 3b). Irrespective of incubation conditions and the (NH₄)₂SO₄ concentration used for precipitation, no peak of proteolytic activity in sucrose-gradient fractions corresponding to '26 S' is observed. In each case the main peak of proteolytic activity is detected in the '20 S' fractions of the gradients. Similarly to the results obtained for rabbit reticulocytes, the distribution of proteolytic activity in the gradient is not restricted to the fraction corresponding to the '20 S' peak, but is also detectable in gradient fractions corresponding to sedimentation coefficients > '26 S'. Protein analysis of gradient fractions corresponding to '20 S' and '26 S' by non-denaturing gel electrophoresis shows that the '20 S' form of the proteasome is responsible for the main peak of proteolytic activity (Fig. 3c). Importantly, proteasomes sedimenting in gradient fractions > '20 S' exhibit an electrophoretic migration behaviour identical with that observed for 'free' '20 S' proteasomes. As in fraction II of rabbit reticulocyte lysates, the '20 S'-'26 S' region of sucrose gradients containing 70%-saturated-(NH₄)₂SO₄ precipitates comprises two high-molecular-mass complexes with overlapping sedimentation behaviour possessing Suc-LLVY-NH-Mec-hydrolysing activity (Figs. 3d and 3e). As demonstrated by immunoblotting, the faster-migrating band corresponds to the '20 S' form of the proteasome. However, the slower-migrating complex that hydrolyses '26 S' Suc-LLVY-NH-Mec is not recognized by the monospecific anti-proteasome antibody, which indicates that the proteasome is not a component of this proteolytically active fraction.

Although we used a polyclonal antibody that recognizes several of the mouse proteasome subunits, it is conceivable that proteasomes, potentially a component of the native '26 S' complex, have their epitopes masked and are therefore not detectable on immunoblots of undenatured '26 S' complex. In addition, the protein content of the fraction revealing '26 S' Suc-LLVY-NH-Mec-hydrolysing activity is very low and thus putative proteasomal protein contents may be below immunological detectability. To overcome these problems we increased the amount of mouse liver fraction II material loaded on to non-denaturing polyacrylamide gel and analysed the protein moiety of the designated '20 S' and '26 S' high-molecular-mass enzyme complexes by SDS/PAGE and immunoblotting. As shown in Fig. 4 the faster-migrating '20 S' complex represents 'free' proteasome as identified by its characteristic protein pattern in the molecular-mass range 21–32 kDa and the recognition of these proteins by a proteasome-specific antibody. Analysis by the same method of the slower-migrating '26 S' activity shows that this is a complex with a set of proteins in the molecular-mass range 20–150 kDa that closely resembles the protein pattern reported by Hough *et al.* (1987) for the '26 S' proteinase.

Fig. 4. Protein analysis of the '20 S' and '26 S' high-molecular-mass proteinase complexes of mouse liver fraction II extracts

Mouse liver fraction II extracts were subjected to sucrose-gradient centrifugation. Gradient fractions corresponding to the '20 S'-'26 S' region of the gradients were pooled and concentrated, the '20 S' and '26 S' proteinase complexes were separated by non-denaturing PAGE and the enzymically active bands were analysed for their protein moiety. (a) Analysis of the Suc-LLVY-NH-Mec-hydrolysing activity in non-denaturing polyacrylamide gels; 150 µg of protein was loaded on to the gel. (b) To analyse further the protein bands revealing Suc-LLVY-NH-Mec-splitting activity the bands were excised from the polyacrylamide gel and the gel slices were then subjected to SDS/PAGE. Lane 1, Coomassie Blue stain of the proteins contained in the '20 S' proteasome; lane 2, silver stain of the proteins contained in the '26 S' proteinase complex. (c) Immunoblotting analysis of the protein moiety in the '20 S' and '26 S' proteinase complexes. To ensure that even minor amounts of proteasomes may be detected in the '26 S' proteinase the corresponding lane was loaded with approx. 5 times as much protein as the lane containing the proteasomes, i.e. 25 µg versus 5 µg. For this experiment the '26 S' proteinase was isolated from a preparative PAGE gel. Also, in order to obtain higher sensitivity we used an alkaline-phosphatase-conjugated secondary antibody. As an additional control that the samples contained sufficient quantities of protein for immunological detection, the upper half was treated with a rabbit anti-hsp90 antibody and the lower half was incubated with a mouse proteasome-specific antibody. Whereas both samples contained hsp90, only the '20 S' proteinase sample contained proteasome subunits. Lane 1, '20 S' proteinase; lane 2, '26 S' proteinase.

Although for immunoblotting the '26 S' lane contains about 4 times as much protein as the '20 S' proteasome lane none of these proteins immuno-react with an antibody specific to mouse proteasomes.

These experiments demonstrate for the first time that the proteins in the molecular-mass range 21–32 kDa of the '26 S' complex, previously thought to represent proteasome subunits, are not components of the '20 S' multicatalytic proteinase. Thus the '26 S' proteinase complex represents a high-molecular-mass proteinase complex that possesses Suc-LLVY-NH-Mec-hydrolysing activity but whose enzymic activity is not the result of the incorporation of the proteasome itself or subunits thereof into a higher-molecular-mass proteinase complex.

DISCUSSION

In the present investigation we have analysed the molecular interaction of proteasomes in so-called fraction II of mouse liver and rabbit reticulocyte lysates. As pointed out in the Introduction, experimental data obtained with fraction II of rabbit reticulocyte lysates had suggested that upon incubation of such

lysates at 37 °C in the presence of MgATP the 'free' '20 S' form of the proteasome is converted into the '26 S' form to become the 'core' Suc-LLVY-NH-Mec-hydrolysing enzyme of the designated '26 S' multienzyme complex involved in the breakdown of ubiquitin conjugates. However, the conclusion that the '20 S' form of the proteasome becomes a component of a higher-molecular-mass '26 S' complex had been by inference from the observation that the '26 S' complex possesses enzymic properties similar to that of the proteasome and that the '26 S' complex when analysed by SDS/PAGE was found to contain in addition to proteins in the molecular-mass range 35–150 kDa a set of proteins that are similar in size to the proteasomal proteins. However, the limited amounts of reconstituted or purified '26 S' Suc-LLVY-NH-Mec-hydrolysing activity so far have precluded a detailed molecular and immunological analysis of the '26 S' complex (Eytan *et al.*, 1989).

In agreement with previous reports (Eytan *et al.*, 1989; Driscoll & Goldberg, 1990), our data show that in fraction II of reticulocyte lysate and mouse liver extract, after incubation with MgATP at 37 °C, a small fraction of proteasome can be precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 38 % saturation. In addition, velocity-gradient centrifugation reveals that the amount of faster-sedimenting proteasomes is increased after incubation of the extracts at 37 °C in the presence of MgATP. Although the '26 S' form of the proteasome is thought to be precipitated at 38 % saturation with $(\text{NH}_4)_2\text{SO}_4$, proteasomes that are precipitated at 70 % saturation with $(\text{NH}_4)_2\text{SO}_4$ reveal a similar sedimentation behaviour. Nevertheless, this observation raises the possibility that proteasomes indeed are converted into the '26 S' form by interacting stably with other cellular components. Electrophoretic analysis, however, shows that irrespective of their sedimentation behaviour in sucrose or glycerol gradients proteasomes possess an electrophoretic migration that is identical with that of the 'free' '20 S' form. Furthermore, protein analysis of the immunologically reactive protein bands gave no evidence that proteasomes, as identified by their characteristic protein moiety, interact with multiple proteins in the molecular-mass range 35–150 kDa that appear to be typical constituents of the '26 S' proteinase (Hough *et al.*, 1987). Our data obtained by velocity-gradient analysis reveal that proteasomes, like other macromolecules, have the tendency to form aggregates and that this aggregation is enhanced after incubation of the extracts at 37 °C. One reason for this may be that incubation of cellular extracts at 37 °C in the presence of MgATP provokes extensive non-specific phosphorylation of proteins, which in turn can result in non-specific molecular interactions, in the formation of larger complexes and in alterations of enzymic activities.

Assay of Suc-LLVY-NH-Mec-splitting activity after non-denaturing gel electrophoresis identifies a second high-molecular-mass proteinase, which possesses an electrophoretic mobility that is slower than that of the 'free' '20 S' proteasome. This second high-molecular-mass proteinase complex, which we have designated '26 S' proteinase, is not separated from the proteasome, and both enzyme complexes overlap when applied to velocity-gradient centrifugation, whereby the larger complex possesses a slightly higher sedimentation rate than the proteasome. The experiments show that when fraction II extracts are analysed by velocity-gradient centrifugation the Suc-LLVY-NH-Mec-hydrolysing activity that is detected in the '20 S'–'26 S' fractions of the gradients is the result of the enzymic activity of two independent high-molecular-mass proteinase complexes. In this context it is noteworthy that the identification of the '26 S' proteinase in fraction II extracts of rabbit reticulocyte lysates and mouse liver extracts does not require the presence of MgATP, nor does incubation at 37 °C in the presence of MgATP affect the yield of '26 S' complexes. In non-denaturing gels the '26 S'

proteinase can readily be identified by its Suc-LLVY-NH-Mec-hydrolysing activity. However, this enzymic activity is the result of high specific proteolytic activity and is not correlated with large amounts of the enzyme complex. When compared with proteasomal hydrolysing activity it appears that the specific proteolytic activity of the '26 S' proteinase exceeds that of the '20 S' proteasome by approx. 8–10-fold as judged by the fluorescence signal obtained on the gels incubated with Suc-LLVY-NH-Mec detected in polyacrylamide gels. A similar result is obtained when Suc-Val-Gly-Arg-NH-Mec is used as a peptide substrate (results not shown). Protein analysis of the '26 S' complex isolated from fraction II of mouse liver shows that the '26 S' proteinase is predominantly composed of proteins with molecular masses in the range 35–150 kDa, plus some minor proteins with molecular masses between 20 kDa and 32 kDa. Thus, judged from its protein moiety, enzymic properties and electrophoretic properties as well as its sedimentation behaviour this enzyme complex seems to be identical with the '26 S' proteinase previously described by Hough *et al.* (1987). In addition to the data presented by those authors, our immunological experiments clearly demonstrate that the '20 S' proteasome or components thereof do not contribute to the protein moiety and the enzymic properties of the '26 S' proteinase. Our analyses of fraction II from rabbit reticulocytes and mouse liver provide no evidence that the 'free' '20 S' form of the proteasome is converted into a '26 S' form in an ATP-dependent process.

Although our data contrast with the conclusions drawn by Eytan *et al.* (1989) and Driscoll & Goldberg (1990), it must be emphasized that they cannot exclude a role of the proteasome in ubiquitin-dependent proteolysis (McGuire *et al.*, 1988). In fact it is well conceivable that proteasomes are involved in proteolytic step(s) following the initial proteolytic cleavage of ubiquitinated proteins that is performed by the '26 S' proteinase or are responsible for the activation of other factors that are involved in this process. On the other hand it also has to be considered that proteasome expression and accumulation depends on the developmental state of a given tissue or organism (Klein *et al.*, 1990) and that certain cell types apparently do not contain any proteasomes at all (Scherrer *et al.*, 1988), which suggest that an exclusive function of proteasomes in the breakdown of ubiquitin conjugates *in vivo* is unlikely.

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