ELSEVIER

Molecular Brain Research 76 (2000) 103–114

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

Structural and functional characterization of 20S and 26S proteasomes from bovine brain

Marco Piccinini [a], Ornella Tazartes [a], Michael Mostert [b], Alberto Musso [b], Mario DeMarchi [c], Maria T. Rinaudo [a,*]

[a] *Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Biochimica, Via Michelangelo n. 27 / B, 10126 Torino, Italy*
[b] *Dipartimento di Scienze Pediatriche e dell'Adolescenza, Piazza Polonia n. 94, 10126 Torino, Italy*
[c] *Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Ospedale San Luigi, Regione Gonzole n. 10, 10143 Torino, Italy*

Accepted 30 November 1999

Abstract

Two proteins were isolated, in a stable form, from bovine brain by ion exchange chromatography, gel filtration and ultracentrifugation on glycerol gradient. They were identified as 20S and 26S proteasomes on the basis of molecular mass, migration velocity on non-denaturing gels, immunoreactivity, multipeptidase activity and the 26S proteasome also for dependence on ATP for the degradation of short peptides and ubiquitinylated proteins. However, the 26S proteasome has some properties not yet described for its counterpart of other tissues and from brain of this and other species. In particular, the ATP concentration required by the 26S proteasome to reach maximal peptidase activity was approximately 40-fold lower than the one required for maximal proteolytic activity on polyubiquitinylated substrates. Moreover, plots of substrate concentration vs. velocity gave a saturation curve for the 26S proteasome only, which, for the trypsin-like and post-glutamyl peptide hydrolase activities fitted the Michaelis–Menten equation, whereas for the chymotrypsin-like activity indicated multibinding site kinetics with positive cooperativity ($n = 2.32 \pm 0.38$). As concerns the 20S proteasome, its electrophoretic pattern on native gel revealed a single protein band, a feature, to our knowledge, not yet described for the brain particle of any species.

Keywords: 20S proteasome; 26S proteasome; Bovine brain

1. Introduction

The 26S proteasome is a multisubunit particle, distributed in the cytoplasmic and nuclear compartments, which constitutes the catalytic machinery of the ubiquitin/ATP dependent protein degradation, one of the two major pathways involved in the control of intracellular protein degradation. This pathway has a part in the constitutive turnover of intracellular proteins, rapid degradation of abnormal proteins and critical regulatory proteins (cell cycle proteins, transcriptional factors), modulation of cell surface receptors and ion channels, and presumably in the generation of peptides presented by MHC-I molecules [9,18,35,40,42]. To enter this pathway the protein has to be labeled, through a cascade of events involving ATP consumption, with multiple molecules of ubiquitin (a 8.5-kDa protein composed of 76 amino acids), largely conserved in the phylogenesis and widely distributed in tissues [7,8,21,45]), thereby giving rise to ubiquitin conjugates constituting the actual substrate of the 26S proteasome.

The 26S proteasome results from the ATP-dependent association of two particles, the 20S proteasome and the 19S complex, constituting the catalytic core and the regulatory component, respectively. The 19S complex confers upon 20S proteasome specificity for polyubiquitinylated proteins and dependence of the hydrolytic activity on ATP [3,8,9,45]. It is constituted by at least 18 different subunits with a molecular mass of 25–110 kDa, some with ATPase activity, others behaving as ubiquitin-recognising elements

Abbreviations: AMP-PCP: β,γ-methyleneadenosine 5′-triphosphate; ATP-γ-S: adenosine 5′-[γ-thio]triphosphate; DCI: 3,4-dichloroisocoumarin; DTT: dithiothreithol; EDTA: ethylenediaminetetraacetic acid; AMC: 7-amino-4-methylcoumarin; βNA: β-naphthylamine; NEM: *N*-ethylmaleinimide; PMSF: phenymethylsulfonyl fluoride; TRIS: Tris [hydroxymethyl]amino methane; TBS: TRIS-buffered saline

* Corresponding author. Fax: +0039-116707770; e-mail: mariateresa.rinaudo@unito.it

0169-328X/00/$ - see front matter
PII: S0169-328X(99)00337-X

and others with unknown function [3,9,13,46]. The 20S proteasome [EC 3.4.99.46] has been structurally and functionally characterized in different mammalian tissues and cells, in yeast and archaebacteria. In eukaryotes, the particle has been described to be composed of 28 subunits, with a molecular mass of 22–34 kDa, coded from 14 different genes, and staked in four heptameric rings ($\alpha_7\beta_7\beta_7\alpha_7$) giving rise to a cylindric-shaped structure. An interesting feature of the 20S proteasome is the location of the active sites which are distributed on the β subunits and face into the hollow of the cylinder, so that the side walls of the particle work as a barrier, preventing the free access of cytoplasmic proteins to the luminal space. Analysis of the different seven β-type subunits uncovered that only three of them are proteolytically active and bear the N-terminal threonine as the catalytic nucleophile [3,9,19]. The α-type subunits are, instead, all proteolytically inactive; they play a part in stabilizing the two β rings and in promoting the ATP-dependent capping of the cylinder with one or two 19S complex particles [3]. The 20S proteasome exists also as an independent particle; in this form it has a very limited proteolytic activity which can be revealed by its three major peptidase activities, all enhanced by ATP when it becomes part of the 26S particle. These peptidase activities are prevalently directed against peptide bonds at the carboxyl side of basic and hydrophobic (trypsin-like and chymotrypsin-like activities) as well as acidic amino acid (post-glutamyl peptide hydrolase or PGPH activity) residues, as defined by the degradation of short fluorogenic peptides [3,9]. For this multiple substrate specificity the 20S proteasome is also defined as multiple catalytic protease or MCP.

As concerns the distribution of the 20S and 26S proteasomes in nervous tissue, they have been described in bovine [2,28] and rat [1,36] brain. In this study we developed a purification procedure that allows to obtain the two proteasomes, and in particular the 26S one, in a stable form suitable for biochemical characterization. This is an important preliminary step for a better understanding of structural and functional properties of the brain ubiquitin-dependent pathway proteolytic machinery. This issue is of interest because the brain is a non-mitotic tissue with a very active protein turnover; further, disorders of this pathway, as well as of the catalytic activity of the two proteasomes, have been envisaged in some neurodegenerative diseases [11,15–17,25,27,29,31,32,45,47].

2. Materials and methods

2.1. Products

The fluorogenic peptides; Suc-L-L-V-Y-AMC (chymotrypsin-like activity), Z-L-L-R-AMC (trypsin-like activity) and Z-L-L-E-βNA (post-glutamyl hydrolase (PGPH) activity) were supplied by Bachem Feinchemikalien (Switzerland), DEAE Sepharose Fast Flow, Sephacryl S300 HR, High-Load Q Sepharose HP 10/16 column, Mono Q HR 5/5 column and Superose 6 HR 10/30 column were from Pharmacia Biotech, Centricon 30 were from Amicon and the other products from Sigma, Boehringer and Amersham. Ubiquitin-[^{125}I]lysozyme conjugates and part of the antisera against human 20S proteasome and 19S regulatory complex subunits were a kind gift of Dr. W. Dubiel

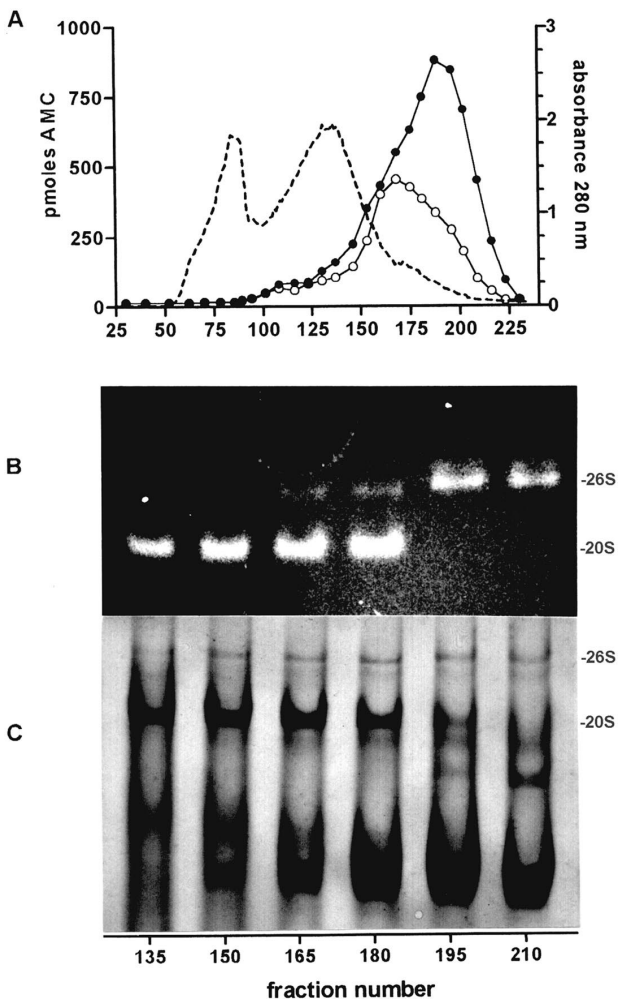

Fig. 1. Separation of the 20S and 26S proteasomes on DEAE Sepharose fast flow (fraction II). Elution profile from a 2.6×30-cm DEAE Sepharose Fast Flow column of the adsorbed proteins from the clear supernatant of bovine brain homogenate. One every seventh fraction was dialyzed against buffer C and then evaluated for peptidase activity by the fluorometric assay and analyzed through non-denaturing gel electrophoresis followed by gel overlay with the fluorogenic substrate Suc-L-L-V-Y-AMC. Gel fixation and Coomassie blue staining followed. Panel A: AMC (pmol) produced by the cleavage of the fluorogenic peptide by 10 μl of the eluate in 30 min at 37°C in the presence or absence of ATP (closed and open circles, respectively), and the absorbance of the eluate monitored at 280 nm (dashed line). Panel B: a native gel in which fractions with peptidase activity were electrophoresed and then subjected to substrate overlay. Panel C: the same gels after fixation and Coomassie blue staining. This elution profile was similar to that obtained in two other independent proteasome preparations.

(Humboldt University, Berlin, Germany). Other antisera were from Affiniti Research Products (United Kingdom).

2.2. Buffers

Buffers used for the purification of the two enzymes and for assays of their activities, were: buffer A, composed of 10 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 mM ATP, 20% glycerol (v/v) in 20 mM TRIS–HCl, pH 7.0; buffer B, composed of 10 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM ATP, 20% glycerol (v/v) in 20 mM TRIS–HCl, pH 7.0; buffer C, corresponding to buffer B with 10% glycerol (v/v); buffer D, corresponding to buffer B without glycerol; buffer E, 8 mM NaCl, 10 mM KCl, 1 mM DTT, 0.2 mM EDTA, 20% glycerol (v/v) in 51 mM TRIS–HCl, pH 7.7; buffer F, composed of 1.6 mM boric acid, 0.08 mM EDTA in 90 mM TRIS–HCl, pH 8.3.

2.3. Purification of the 20S and 26S proteasomes from bovine brain

All purification steps were performed at 4°C, according to Akaishi et al. [1] and Hoffman et al. [22], with minor modifications.

2.3.1. Tissue homogenization and fraction II preparation

Bovine brains (about 700 g), immediately after slaughter, were extensively washed with phosphate-buffered saline at pH 7.4, homogenized in two volumes of buffer A (w/v) using a Waring blendor apparatus and centrifuged at $30,000 \times g$ for 45 min. The clear supernatant was adsorbed on a 2.6×30-cm DEAE-Sepharose Fast Flow column pre-equilibrated in buffer B. The column was washed with three column volumes of buffer B followed by two volumes of buffer B supplemented with 0.1 M KCl. Proteins were desorbed at a flow rate of 0.7 ml/min with a linear gradient from 0.1 to 0.45 M KCl in seven volumes of buffer B. The eluate was monitored at 280 nm and approximately 230 fractions of 4.8 ml were collected (fraction II).

2.3.2. 26S proteasome purification

2.3.2.1. Step 1. Samples from fraction II enriched with ATP-dependent peptidase activity were pooled, dialyzed against 2×500 ml of buffer C and loaded on a 1.6×10-cm High-Load Q-Sepharose HR equilibrated with the same buffer. After extensive washing the column was developed by linearly increasing the KCl concentration in buffer C from 0 to 0.6 M in 60 min at a flow rate of 1 ml/min.

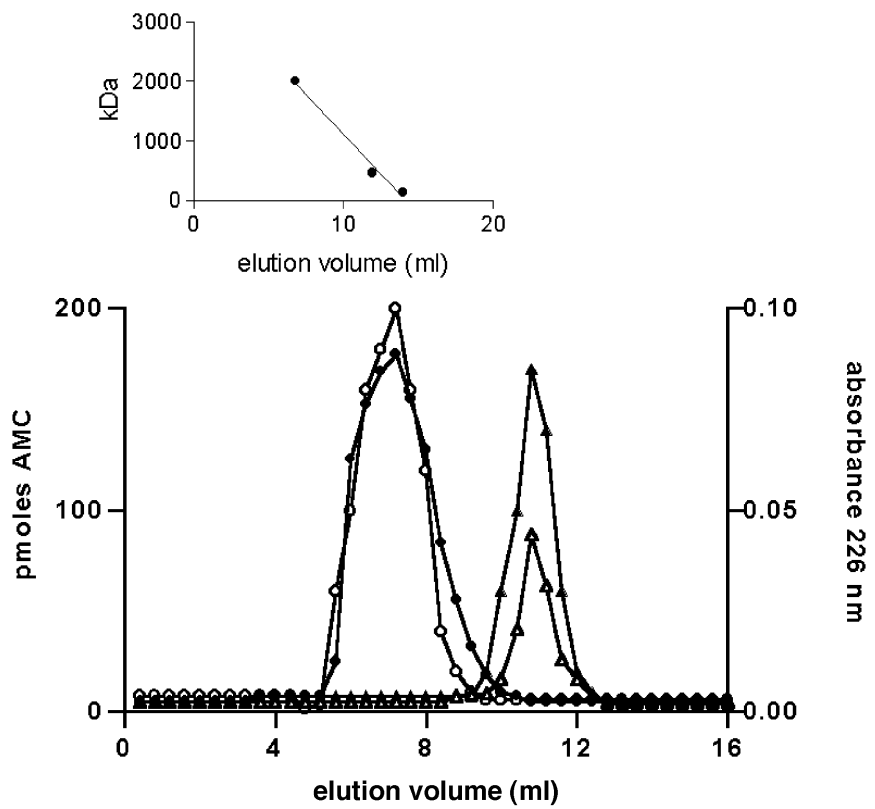

Fig. 2. Gel filtration analysis of the 20S and 26S proteasomes through Superose 6 HR column. The 20S proteasome containing fractions from the Mono Q column and the 26S proteasome containing fractions from Sephacryl S300 HR column were loaded separately on the Superose 6 HR column and eluted as detailed in Section 2. The eluate absorbance was monitored at 226 nm (open symbol). Peptidase activity (closed symbol) was assayed fluorometrically by incubating 5 μl of the eluate for 30 min at 37°C with the fluorogenic peptide Suc-L-L-V-Y-AMC in the absence or in the presence of ATP. Triangle: 20S proteasome. Circle: 26S proteasome. This elution profile was similar to that obtained in two other independent proteasome preparations.

Eluted proteins were monitored at 280 nm. Fractions of 1 ml were collected.

2.3.2.2. Step 2. Fractions with ATP-dependent peptidase activity were pooled and concentrated to 1.0 ml by centrifugal ultrafiltration on Centricon 30 and loaded on a 1.6 × 70-cm Sephacryl S300 HR column equilibrated at a flow rate of 0.5 ml/min in buffer C. The eluate was monitored at 226 nm and 0.5 ml fractions were collected.

2.3.2.3. Step 3. Active fractions were pooled, concentrated to 0.5 ml and loaded on a Superose 6 HR 10/30 column equilibrated in buffer C at a flow rate of 0.4 ml/min. The eluate was monitored at 226 nm and 0.4 ml fractions were collected.

Columns in steps 2 and 3 were calibrated using dextran blue (2000 kDa), apoferritin (443 kDa) and alcohol dehydrogenase (150 kDa) as standards.

2.3.2.4. Step 4. Fractions from step 3 were partially concentrated by centrifugal ultrafiltration and loaded on a 17–40% glycerol gradient in buffer D and centrifuged for 20 h at 25,000 rpm on a Beckman SW50.1 rotor. At the end of centrifugation 250 μl fractions were collected, searched for ATP-dependent peptidase activity, which was evaluated by the fluorometric assay, and localized on non-denaturing gels by substrate overlay and Coomassie blue staining. Active fractions were pooled, partially concentrated and used for the characterization of the protein in structural and functional terms.

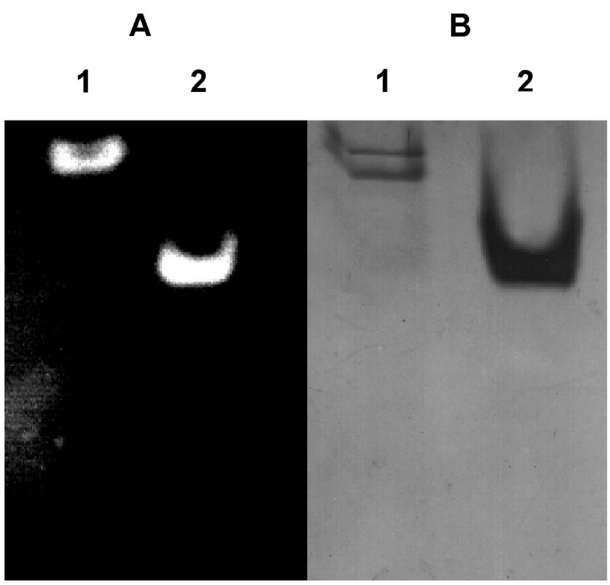

Fig. 3. Resolution of the 20S and 26S proteasomes through non-denaturing gel electrophoresis. The analysis was performed on glycerol gradient fractions (28–30% glycerol for the 20S proteasome and 32–34% glycerol for the 26S proteasome). Proteasomes were localized on the gel by substrate overlay procedures (panel A) and silver staining (panel B). This elution profile was similar to that obtained in two other independent proteasome preparations.

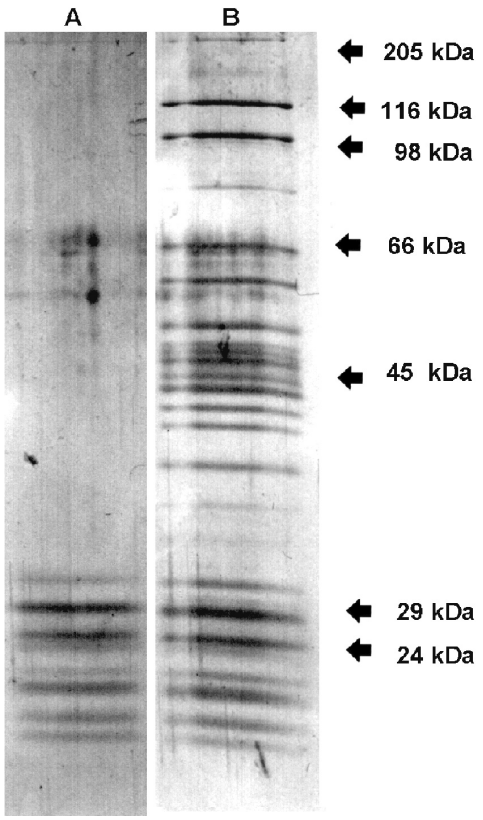

Fig. 4. Subunit composition of the purified 20S and 26S proteasomes. SDS–PAGE on 12% gel was performed on aliquots of the glycerol gradient purified proteins. Resolved proteins were detected by silver stain. Lane A: 20S proteasome. Lane B: 26S proteasome. Molecular weight markers were: rabbit muscle myosin (205,000); *Escherichia coli* β-galactosidase (116,000); rabbit muscle phosphorylase b (98,000); bovine serum albumin (66,000); ovalbumin (45,000); rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36,000); bovine carbonic anhydrase (29,000); bovine trypsinogen (24,000). This subunit composition was similar to that obtained in two other independent proteasome preparations.

2.3.3. 20S proteasome purification

The ATP-dependent peptidase activity was purified essentially through the same procedures described above with the following modifications: 1) from step 1 the used buffer were ATP-free; 2) an anion chromatography step on MONO Q HR 5/5 was introduced between step 2 and 3: the column was developed as described in step 1 except that the flow rate was 0.5 ml/min and fractions of 0.5 ml were collected.

2.3.4. Proteasome identification

At each step the two proteasomes were identified by: 1) a fluorometric assay carried out in the presence and in the absence of ATP using as a substrate the fluorogenic peptide Suc-L-L-V-Y-AMC; 2) non-denaturing gel electrophoresis followed by gel overlay with the above peptide and Coomassie blue staining; 3) SDS–PAGE performed on the dissociated fluorescent band resolved by non-dena-

Table 1
Summary of the purification steps of 20S and 26S proteasomes from bovine brain
Activity was assayed in the presence of the fluorogenic peptide Suc-L-L-V-Y-AMC (130 μM) and without (20S proteasome) or with (26S proteasome) 25 μM ATP. Data are the mean values of three different proteasome preparations (variation less than 5%).

Step	20S proteasome				26S proteasome			
	Total protein, mg	Total activity, nmol/min	Specific activity, nmol/min/mg protein	Yield, %	Total protein, mg	Total activity, nmol/min	Specific activity, nmol/min/mg protein	Yield, %
Homogenate	8800.0	352.0	0.04	100.0	8880.0	880	0.10	100.0
Fraction II	1450.0	72.5	0.05	20.5	1292.0	181	0.14	20.0
Q-Sepharose HP	343.0	41.2	0.12	11.7	16.5	132	8.00	15.0
Sephacryl S300 HR	34.2	14.7	0.43	4.0	3.0	63	21.00	7.2
Mono Q HR 5/5	5.6	8.2	1.47	2.3	–	–	–	–
Superose 6 HR	2.8	5.9	2.10	1.6	0.8	29.3	38.00	3.3
Glycerol gradient	1.7	4.7	2.70	1.3	0.4	18.8	47.00	2.1

turing gel electrophoresis and on aliquots of the proteins at the end of the purification procedures. A detailed description of the procedures at points 1–3 is given below.

2.4. Enzyme activity assay

ATP-dependent and ATP-independent peptidase activities were evaluated according to Hoffman et al. [22] by using a spectrofluorometric assay and/or a non-denaturing gel electrophoresis followed by a substrate overlay procedure described below. For the fluorometric assay the fluorogenic peptides Suc-L-L-V-Y-AMC, Z-L-L-R-AMC and Z-L-L-E-βNA were used. The assay was carried out at 37°C in the presence or absence of 25 μM ATP (unless otherwise stated) in samples that in a final volume of 100 μl contained: 130 μM fluorogenic peptide, 50 μl buffer E and 5 mM $MgCl_2$. The reaction was started by the addition of variable amounts (see tables) of the two proteins and quenched at defined time intervals (see tables) with 200 μl of cold absolute ethanol. In control samples, run in parallel, the proteins were added at the end of incubation immediately before ethanol. Samples were centrifuged and evaluated for ethanol soluble fluorescence on a Perkin-Elmer fluorometer using an excitation wavelength of 380 and 335 nm, and an emission wavelength of 440 and 410 nm for AMC and βNA releasing reactions, respectively. Fluorescence was quantified using AMC and βNA as a standard.

The purified ATP-dependent peptidase activity was also evaluated for the ability to cleave ubiquitinylated [^{125}I]lysozyme according to Hough and Rechsteiner [23], in samples that in a final volume of 100 μl contained 30 μl buffer E, 5 mM $MgCl_2$, variable ATP amounts (see tables), 4 μg of the purified protein and the radiolabeled substrate (25,000 cpm); the reaction was started by the addition of the purified protein and was left to go on at 37°C for 3 h. In control samples the purified protein was pre-incubated at 24°C for 30 min with apyrase (potato type VIII from Sigma), and then the assay was performed as reported above. Rate of conjugate degradation is expressed as percentage of acid soluble radioactivity formed in 60 min by 1 μg of protein vs. total radioactivity.

2.5. Kinetic measurements

Initial velocities of the hydrolytic reaction in the presence of the fluorogenic peptides Suc-L-L-V-Y-AMC, Z-L-L-R-AMC and Z-L-L-E-βNA were plotted vs. substrate concentrations and the experimental data were fitted by least squares non-linear regression analysis to the Michaelis–Menten and Hill equations.

2.6. Electrophoresis

At the different stages of purification, proteins were analysed by non-denaturing and/or denaturing polyacrylamide gel electrophoresis. Non-denaturing gel electrophoresis was performed according to Hoffman et al. [22] and buffer F was used. Resolved proteins were evidenced with either Coomassie blue or silver staining [26,33]. In some cases, Coomassie Brilliant Blue-stained proteins were excised, extensively washed in TBS, extracted in sample buffer and subjected to SDS–PAGE followed by detection of subunit composition. Denaturing gel electrophoresis (SDS–PAGE) was carried out according to Laemmli [26].

2.7. Immunoreactivity assay

Aliquots of purified proteins were subjected to SDS–PAGE followed by transfer to nitrocellulose or PVDF membranes [41,48]. Immunodecorated bands were identified using the ECL Western blotting detection system.

2.8. Protein quantification

The protein content of samples in each purification step was determined according to Bradford [4] using bovine serum albumin as a standard.

3. Results

3.1. Enzyme purification

Resolution of the 20S and 26S proteasomes on DEAE-Sepharose Fast Flow column and their identification by fluorometric assay and non-denaturing gel electrophoresis is reported in Fig. 1.

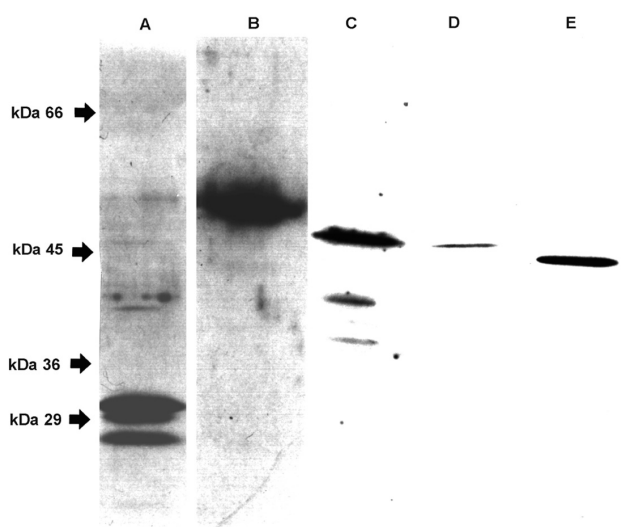

Fig. 5. Immunodecoration of the dissociated 26S proteasome. Aliquots of the purified proteasome were subjected to SDS–PAGE, electrotransferred to nitrocellulose and immunodecorated with antisera directed against human 20S proteasome (lane A) and subunits of human 19S complex, S4 (lane B), S5a (lane C), S6′ (Tbp1) (lane D) and S7 (Mss1) (lane E) [14]. Molecular weight markers are described in Fig. 4.

Table 2
Dependence of the hydrolytic reaction rate of the three fluorogenic peptides Suc-L-L-V-Y-AMC, Z-L-L-R-AMC and Z-L-L-E-βNA on the concentration of the 20S and 26S proteasomes
The experiments were carried out on purified proteasomes dialyzed against buffer E for 1 h, and then incubated, at the concentrations reported in the table, without (20S proteasome) and with (26S proteasome) 25 μM ATP and the three fluorogenic peptides (130 μM), as described in Section 2. Data are the mean values ± S.D. of experiments performed in duplicate on three independent proteasome preparations. Activity is expressed as pmol of AMC or βNA released/min.

Protein, μg	20S proteasome			26S proteasome		
	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity
2.0	5.5 ± 0.44	5.5 ± 0.41	4.9 ± 0.51	95.2 ± 4.7	10.1 ± 1.22	16.2 ± 0.9
1.0	2.7 ± 0.20	2.9 ± 0.32	2.5 ± 0.15	47.7 ± 2.7	4.5 ± 0.7	7.7 ± 1.0
0.5	1.3 ± 0.17	1.5 ± 0.10	1.3 ± 0.10	22.3 ± 1.3	2.4 ± 0.3	3.6 ± 0.2

Following gel filtration on Sephacryl S300 HR, the 20S proteasome eluted in a volume corresponding to a molecular mass close to 800 kDa; instead, the 26S eluted in the void column volume, suggesting a molecular mass above 1500 kDa. Gel filtration on Superose 6 confirmed the molecular mass of the former enzyme and allowed to establish that of the latter as close to 1900 kDa (Fig. 2). On glycerol gradients the 26S proteasome was recovered in fractions at 32–34% and the 20S in fractions at 28–30%.

The purified proteasomes, when subjected to non-denaturing gel electrophoresis, showed different migration velocities, the 20S proteasome being the faster migrating one. Worthy of note is the electrophoretic pattern of the 20S proteasome which was characterized by a single protein band when detected with substrate overlying, or Coomassie blue, or silver staining; on the other hand, two closely paired protein bands characterized the 26S proteasome (Fig. 3). These migration modalities were a trait of the two proteasomes throughout the entire purification procedure (Fig. 1). No other bands were observed on the gel whether by substrate overlying, or Coomassie blue, or silver staining, thus excluding the presence of a specific peptidases or proteins.

The band identifying the 20S proteasome, excised from the gel and resolved by SDS–PAGE, showed a heteromultimeric nature with subunits in the size range 22–34 kDa. The subunit composition of the two protein bands identifying the 26S proteasome included all the components of the 20S proteasome and additional ones in the size range 25 kDa up to almost 120 kDa. The subunit composition was confirmed on aliquots of those samples that were used for non-denaturing gel electrophoresis, which in this case were analysed directly by SDS–PAGE (Fig. 4).

The procedures used for the purification of the two enzymes and the enrichment of the relative peptidase activity in the various steps are summarized in Table 1.

3.2. Immunological characterization

The purified 20S and 26S proteasomes cross-reacted with a series of antisera against human 20S proteasome and 19S complex (Fig. 5).

3.3. Functional characterization

The two purified enzymes hydrolyzed, although at different rates, the three fluorogenic peptides constituting a substrate for chymotrypsin-like, trypsin-like and PGPH activities.

The hydrolytic reaction rates, when tested in function of enzyme concentration and incubation time, were linear, indicating that the two enzymes were stable in the conditions selected for the assay (Tables 2 and 3).

Table 3
Dependence of the hydrolytic reaction rate catalysed by the 20S and 26S proteasomes on time of incubation
The experiments were carried out on purified proteasomes, dialyzed against buffer E for 1 h, and then incubated, at the times reported in the table, with the three fluorogenic peptides (130 μM) and without (20S proteasome) or with (26S proteasome) 25 μM ATP as described in Section 2. Data are the mean values ± S.D. of experiments performed in duplicate on three different proteasome preparations. Activity is expressed as pmol of AMC or βNA released/μg protein.

Incubation, min	20S proteasome			26S proteasome		
	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity
5	14 ± 2	14 ± 1	11 ± 1	240 ± 12	22 ± 1	39 ± 2
10	28 ± 1	29 ± 2	25 ± 1	481 ± 10	47 ± 1	77 ± 3
15	41 ± 3	43 ± 3	38 ± 2	735 ± 15	65 ± 3	111 ± 5
20	53 ± 3	59 ± 3	50 ± 3	970 ± 23	95 ± 6	155 ± 10
40	103 ± 9	121 ± 8	98 ± 4	1924 ± 38	201 ± 8	307 ± 11

The initial velocities of the hydrolytic reaction of the chymotrypsin-like, trypsin-like and PGPH activities of the two enzymes were measured at different substrate concentrations in the range 8.12–260 μM (Fig. 6). Due to the limited substrate solubility, the reaction rates for the three activities of the 20S protesome did not reach saturation. The opposite applied to the activities of the 26S proteasome; the sigmoidal kinetics of the chymotrypsin-like activity was fitted by non-linear regression analysis with the Hill equation, whereas the hyperbolic kinetics of the trypsin-like and PGPH activities with that of Michaelis–Menten. In the first case K_m, V_{max} and Hill coefficient (N) values were 44.17 ± 3.9 μM, 46.86 ± 2.3 pmol/min per μg protein and 2.32 ± 0.38, respectively; in the case of trypsin-like and PGPH activities, K_m values were 27.71 ± 2.5 and 48.5 ± 8.1 μM, respectively, and V_{max} values were, for the former 5.08 ± 0.17, and for the latter, 10.76 ± 0.6 pmol/min per μg protein.

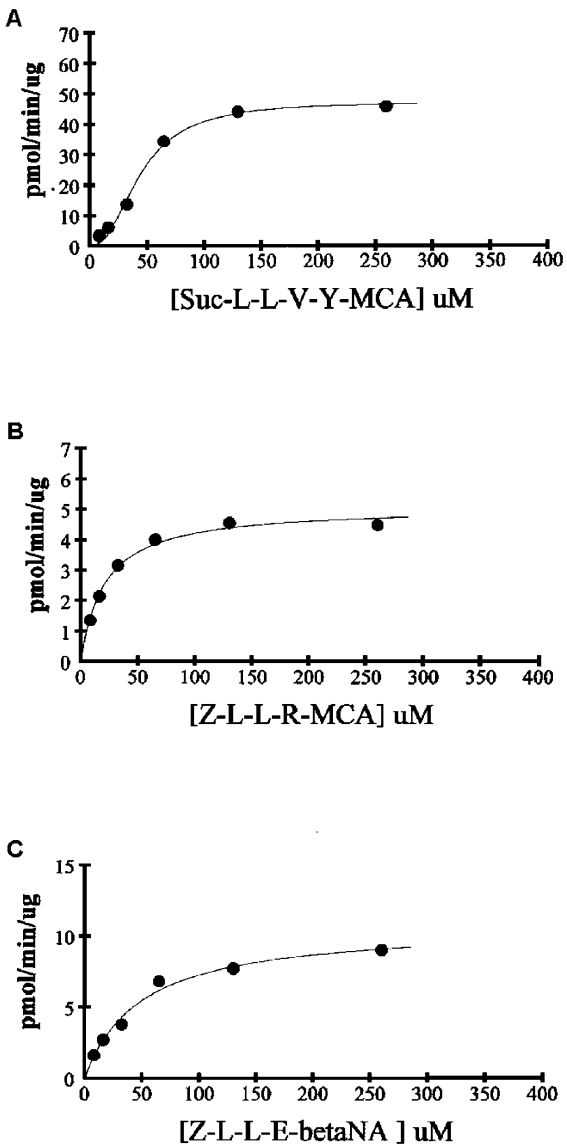

Fig. 6. Kinetic analysis of the chymotrypsin-like, trypsin-like and PGPH activities of the 26S proteasome. The experiments were carried out on purified proteasome, dialyzed against buffer E for 1 h and then incubated with the three fluorogenic peptides in presence of 25 μM ATP. For the assay of the chymotrypsin-like activity, (A) 300 ng of protein were used and the incubation lasted 15 min; for the assay of the trypsin-like (B) and PGPH (C) activities, 600 ng were used and the incubation lasted 30 min. Data are the mean values of experiments performed in duplicate from three different proteasome preparations (variation less than 5%). Activity is expressed as AMC or βNA released.

The peptidase activity of the two proteasomes was tested in the presence of a series of reagents, known to affect that of the 20S proteasome from other tissues [24,39]. The three peptidase activities tested were all inhibited by NEM and DCI, and almost unaffected by iodoacetamide and PMSF; only the trypsin-like activity was inhibited by leupeptin (Table 4).

ATP maximally activated the three peptidase activities of the 26S proteasome at 12.5–25 μM, whereas at concentrations higher than 0.5 mM, it was less effective and, in the case of the chymotrypsin-like and trypsin-like activities, slightly inhibiting (Table 5). ATP 12.5–25 μM left unaffected the three activities of the 20S proteasome (unreported results).

The activity of the 26S proteasome was also evaluated in the presence of ATP analogues at 25 μM, this concentration being that at which ATP determined maximal proteasome activation (Table 6).

Ubiquitin [^{125}I]lysozyme conjugates were cleaved by the 26S proteasome, providing ATP was at least 0.1 mM; however, the reaction rate rose further as the nucleotide concentration was brought up to 1 mM (Table 7).

4. Discussion

Two heteromultimeric proteins were isolated in a stable form from the cytoplasmic compartment of bovine brain and identified as 20S and 26S proteasomes, on the basis of molecular mass [1,2,9,22,30], subunit composition [1,3,6,9,10,22,28,30,44], immunoreactivity [11], as well as migration velocity on non-denaturing gels [10,22]. Similar to the 26S proteasome from rabbit reticulocytes and rat skeletal muscle [10,22], the purified 26S proteasome migrated on non-denaturing gels as a closely paired band when visualized by substrate overlay and silver staining (Fig. 3). During the purification procedures, usually lasting 10 days, the ratio between the two fluorescent protein bands remained unvaried, indicating that in the selected conditions the bands were the expression of two stable protein conformations (Figs. 1 and 3). The yield of the 26S proteasome was instead compromised when a Mono-Q chromatography step was introduced before or after gel filtration on Superose 6. In our hands, this step caused the dissociation of a large part of 26S complex in the constituting 19S and 20S complexes (unreported results).

Table 4
Effects of group specific reagents on the chymotrypsin-like, trypsin-like and PGPH activities of the 20S and 26S proteasomes
The experiments were carried out on purified proteins, dialyzed against buffer E for 1 h and then incubated without (20S proteasome) and with 25 μM ATP (26S proteasome) and with the three fluorogenic peptides (130 μM), in the absence and presence of the various group-specific reagents. For the assay of the 26S proteasome chymotrypsin-like activity, 300 ng of protein were used and the incubation lasted 15 min; for the other two activities, 600 ng were used and the incubation lasted 30 min. For the assay of all the peptidase activities of the 20S proteasome, 500 ng of the purified protein were used and the incubation lasted 60 min. Variations are estimated with respect to the activity in the absence of specific-group reagents and considered equal to 100. Data are the mean values of experiments performed in duplicate on three different proteasome preparations (variation less than 5%).

Reagents	20S proteasome			26S proteasome		
	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity
None	100	100	100	100	100	100
DCI (1 μM)	19	37	28	2	15	3
NEM (2 mM)	216	22	86	23	32	70
PMSF (2 mM)	93	86	88	110	95	83
I-acetamide (2 mM)	117	100	83	97	106	105
Leupeptin (40 μM)	96	19	100	105	19	79

Table 5
Effect of ATP concentration on the peptidase activity of the 26S proteasome
The experiments were carried out on the purified proteasome dialysed for 1 h against buffer E and then incubated without and with ATP in the presence of the three substrates as described in Section 2. For the assay of the chymotrypsin-like activity, 300 ng of the purified complex were used and the incubation lasted 15 min; for the assay of the other two activities, 600 ng of the purified complex were used and the incubation lasted 30 min. Variations are estimated with respect to the activity tested in the absence of ATP and considered equal to 100. Data are the mean values of experiments performed in duplicate on three different proteasome preparations (variation less than 5%).

ATP, mM	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity
None	100	100	100
0.006	147	162	150
0.012	182	209	204
0.025	171	203	237
0.050	133	200	193
0.100	131	200	181
0.500	114	126	185
1.000	92	100	144
2.000	73	80	119

Table 7
Hydrolytic activity of the 26S proteasome on ubiquitin [^{125}I]lysozyme conjugates
The hydrolytic reaction was carried out at 37°C for 3 h in the presence of ubiquitin [^{125}I]lysozyme conjugates (25,000 cpm), 4 μg of purified 26S proteasome and ATP at the concentrations referred in the table. In control samples 26S proteasome was omitted or was preincubated with 1 U potato apyrase for 30 min at 24°C. Percentage difference is referred to the increase of activity in samples incubated with ATP and without apyrase, and those incubated with ATP and apyrase. Data are the mean values of an experiment performed in triplicate.

Additives	26S proteasome	
	Acid soluble/ total radioactivity, cpm	Percentage difference
Without proteasome	10.8	–
Apyrase and 1 mMATP	11.1	–
0.025 mM ATP	11.8	7
0.100 mM ATP	17.1	55
1.000 mM ATP	18.9	70

The two purified proteasomes share a number of functional properties with their counterparts from other tissues. They behaved as multicatalytic peptidases [9] and were sensitive to defined group-specific reagents with sensitivity varying in relation to the substrate used [3,9,24,39]. The 26S proteasome was highly responsive to ATP and ATP-analogues, the PGPH activity being the most sensitive one to ATPγS. AMP and CMP were instead ineffective, thus confirming that, also for 26S proteasome from brain, the hydrolysis of phosphoanhydride bonds is not essential for peptidase activity [6,10]. In this view, ATP should not be considered as a free energy donor but rather as an allosteric activator of the catalytic core of the 26S proteasome, a role that ATP shares with some of its analogues. From our data the active sites responsible for PGPH activity appear to be the most sensitive to such conformational stabilization. The 26S proteasome-mediated degradation of ubiquitin-lysozyme conjugates was instead highly responsive to increasing ATP concentrations. In this respect, it is of interest that non-hydrolyzable ATP analogues are ineffective on the 26S proteasome proteolytic activity of the 26S proteasome [20,34]. The decreasing activation or even inhibition of the 26S proteasome peptidase activity for increasing ATP concentrations, approaching those essential for proteolytic activity, further supports the different role played by the nucleotide towards peptide or protein substrates and suggests that accumulation of negatively charged compounds inside the catalytic core is detrimental for the interaction of the core with the peptide substrate, e.g., non-ubiquitinylated and unfolded substrates. Lastly, the three specific activities of the 20S proteasome were below those of the 26S proteasome, particularly the chymotrypsin-like one [3,6,10], in line with the commonly held view that the 20S proteasome activity is latent [1,5,30,37]. In addition, as concerns the purified 26S proteasome, the chymotrypsin-like to trypsin-like activity ratio is considerably greater than that of the 20S proteasome, similar to what is described for the two proteasomes from bovine red blood cells [6], but not for those from rat brain and skeletal muscle [1,10].

There are still other structural and functional properties of the two purified proteasomes that are of note. The 20S

Table 6
Effects of ATP and ATP analogues on the chymotrypsin and trypsin-like, as well as PGPH, activities of the 26S proteasome
The experiments were carried out on the purified proteasome, dialyzed against buffer E for 1 h and then incubated with the three fluorogenic peptides (130 μM) in the absence or presence of the various nucleotides. For the assay of the chymotrypsin-like activity, 300 ng of the purified proteasome was used and the incubation lasted 15 min; for the assay of the other two activities, 600 ng of the purified complex was used and the incubation lasted 30 min. Differences are estimated with respect to the activity tested in the absence of whatever nucleotide and estimated equal to 100. Data are the mean values of experiments performed in duplicate on three different proteasome preparations (variation less than 5%).

ATP analogues, 25 μM	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity
None	100	100	100
ATP	180	203	237
ATPγS	260	207	430
AMP-PCP	127	89	113
GTP	196	123	190
CTP	129	132	227
ADP	140	96	193
AMP	109	89	93
GMP	102	87	90

proteasome migrates in non-denaturing gels, as a single band, when detected both by substrate overlay and silver staining (Fig. 3), a feature of its counterpart of other [10], but not of all [12,22], tissues, and not yet described, to our knowledge, for brain in this and other species. As concerns the 26S proteasome, plots of the degradation rate of the Suc-L-L-V-Y-AMC, Z-L-L-R-AMC, Z-L-L-E-βNA fluorogenic peptides vs. substrate concentration produced sigmoidal curves in the first case and hyperbolic ones in the latter two cases. To our knowledge, these kinetic characteristics have not yet been described for its counterpart from other tissues and from brain of this and other species. At the same substrate concentrations, a saturation curve was not obtained in the case of the 20S proteasome, in line with what is described for this complex from bovine red blood cells [5]. Therefore, at least for the 26S proteasome from bovine brain, the catalytic core should behave differently with respect to polypeptide substrates depending on the fact that it is in association with the 19S regulatory complex or a single particle. If so, the regulatory complex would also have a modulatory role on the steric conformation of the subunits, facing the cylindric hollow, once exposed to specific substrates. The sigmoidal curve obtained by the kinetic analysis of the chymotrypsin-like activity of the 26S proteasome is the expression of a positive cooperativity of the particle towards this substrate; this is an expected feature in view of the multisubunit structure of this particle; the Hill coefficient around the value of 2 suggests the participation in the catalysis of at least two β-type subunits. The absence of cooperativity towards the trypsin-like and PGPH activity substrates is, instead, surprising since both activities belong to the same multisubunit protein. At present we cannot rule out the possibility that using peptides with a different amino acid sequence [38], cooperativity can be evidenced for these two activities too. Further, the ATP concentration required by purified 26S proteasome to degrade ubiquitinylated conjugates was many times higher than that needed for optimal cleavage of small peptides (1 mM in the former case vs. 12.5–25 μM in the latter case). Thus, to cleave peptide bonds in ubiquitin conjugated proteins, brain 26S proteasome requires a 40-fold higher ATP concentration than that necessary for its activity on non-ubiquitinylated substrates. Possibly, the ATP concentration in the catalytic core is kept separate from that needed for the accomplishment of those steps preceding injection of the ubiquitinylated protein into the proteolytic compartment, and suggested to involve docking of substrate to the proteasome, its unfolding and removal of polyubiquitin chains [9,43]. Disorders of the ATPases, known to be components of the 19S complex and involved in the above mentioned early steps [3,9,13,43,46], might thus give rise to an accumulation of ubiquitin conjugates even if the catalytic core is not impaired. Interestingly, in certain neurodegenerative disorders, as well as in normal aging of brain, intraneuronal accumulation of ubiquitin conjugates has been described and in some of these disorders, alterations of the proteolytic activity of the two proteasomes can be envisaged [11,15–17,25,27,29,31,32,45,47]. In this respect, the properties described here, which appear to be unique for the two brain proteasomes, might be worth investigating in brain disorders.

Acknowledgements

The authors are indebted to Dr. W. Dubiel (Institute of Biochemistry, Humboldt University, Berlin, Germany) for antisera against human proteasomes, to Ing. G. Rossi (Istituto Elettrotecnico Nazionale Galileo Ferraris, Torino, Italy) for technical advice in image collection and to Dr. Luigi Odasso (general manager of Azienda Ospedaliera OIRM-Sant' Anna, Torino, Italy) for technical and financial support. This work was performed in part with a grant from the Ministero dell' Università e della Ricerca Scientifica e Tecnologica, Italy.

References

[1] T. Akaishi, T. Shiomi, H. Sawada, H. Yokosawa, Purification and properties of the 26S proteasome from rat brain: evidence for its degradation of myelin basic protein in a ubiquitin-dependent manner, Brain Res. 722 (1996) 139–144.
[2] A. Azaryan, M. Banay-Schwartz, A. Lajtha, The presence of ATP + ubiquitin-dependent proteinase and multicatalytic proteinase complex in bovine brain, Neurochem. Res. 14 (1989) 995–1001.
[3] W. Baumeister, J. Walz, F. Zühl, E. Seemüller, The proteasome: paradigm of a self-compartmentalizing protease, Cell 92 (1998) 367–380.
[4] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[5] M. Chu-Ping, C.A. Slaughter, G.N. DeMartino, Identification, purification and characterization of a protein activator of the 20S proteasome (Macropain), J. Biol. Chem. 267 (1992) 10515–10523.
[6] M. Chu-Ping, J.H. Vu, R.J. Proske, C.A. Slaughter, G.N. DeMartino, Identification, purification, and characterization of a high molecular weight, ATP-dependent activator (PA700) of the 20S proteasome, J. Biol. Chem. 269 (1994) 3539–3547.
[7] A. Ciechanover, The ubiquitin-proteasome pathway, Cell 79 (1994) 13–21.
[8] A. Ciechanover, The ubiquitin-mediated proteolytic pathway: mechanisms of action and cellular physiology, Biol. Chem. Hoppe-Seyler 375 (1994) 565–581.
[9] O. Coux, K. Tanaka, A.L. Goldberg, Structure and functions of the 20S and 26S proteasomes, Annu. Rev. Biochem. 65 (1996) 801–847.
[10] B. Dahlmann, L. Kuehn, H. Reinauer, Studies of the activation by ATP of the 26S proteasome complex from rat skeletal muscle, Biochem. J. 309 (1995) 195–202.
[11] D.W. Dickson, M.L. Schmidt, V.M. Lee, M.L. Zhao, S.H. Yen, J.Q. Tojanowsky, Immunoreactivity profile of hippocampal CA2/3 neurites in diffuse Lewy body disease, Acta Neuropathol. 87 (1994) 269–276.
[12] W. Dubiel, G. Pratt, K. Ferrell, M. Rechsteiner, Purification of an 11S regulator of the multicatalytic protease, J. Biol. Chem. 267 (1992) 22369–22377.

[13] W. Dubiel, K. Ferrell, M. Rechsteiner, Subunits of the regulatory complex of the 26S protease, Mol. Biol. Rep. 21 (1995) 27–34.
[14] D. Finley et al., Unified nomenclature for subunits of the *Saccharomyces cerevisiae* proteasome regulatory particle, TIBS 23 (1998) 244–245.
[15] J.-M. Gallo, B.H. Anderton, Ubiquitous variations in nerves, Nature 337 (1989) 687–688.
[16] L. Gregori, C. Fuchs, M.E. Figueiredo-Pereira, W.E. Van Nostrand, D. Goldgaber, Amyloid β-protein inhibits ubiquitin-dependent protein degradation in vitro, J. Biol. Chem. 270 (1995) 19702–19708.
[17] L. Gregori, J.F. Hainfeld, M.N. Simon, D. Goldgaber, Binding of amyloid β protein to the 20S proteasome, J. Biol. Chem. 272 (1997) 58–62.
[18] M. Groettrup, A. Soza, U. Kuckelkorn, P.-M. Kloetzel, Peptide antigen production by the proteasome: complexity provides efficiency, Immunol. Today 17 (1986) 429–435.
[19] M. Groll, L. Ditzel, J. Löwe, D. Stok, M. Bochtler, H.D. Bartunik, R. Huber, Structure of 20S proteasome from yeast at 2.4 Å resolution, Nature 386 (1997) 463–471.
[20] A. Hershko, E. Leshinsky, D. Ganoth, H. Heller, ATP-dependent degradation of ubiquitin-protein conjugates, Proc. Natl. Acad. Sci. 81 (1984) 1619–1623.
[21] A. Hershko, A. Ciechanover, The ubiquitin system for protein degradation, Annu. Rev. Biochem. 61 (1992) 761–807.
[22] L. Hoffman, G. Pratt, M. Rechsteiner, Multiple forms of the 20S multicatalytic and the 26S ubiquitin/ATP-dependent proteases from rabbit reticulocytes lysate, J. Biol. Chem. 267 (1992) 22362–22368.
[23] R. Hough, M. Rechsteiner, Ubiquitin lysozyme conjugates: purification and susceptibility to proteolysis, J. Biol. Chem. 261 (1986) 2391–2399.
[24] R. Hough, G. Pratt, M. Rechsteiner, Purification of two high molecular weight proteases from rabbit reticulocytes lysate, J. Biol. Chem. 262 (1987) 8303–8313.
[25] K.S. Kosik, Alzheimer's disease: a cell biological perspective, Science 256 (1992) 780–783.
[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[27] J. Lowe, H. McDermott, M. Landon, R.J. Mayer, K.D. Wilkinson, Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinylated inclusion bodies characteristic of human neurodegenerative diseases, J. Pathol. 161 (1990) 153–160.
[28] J. Lucas, D. Lobo, E. Terry, E.L. Hogan, N.L. Banik, Susceptibility of myelin proteins to a neutral endoproteinase: the degradation of myelin basic protein (MBP) and P_2 protein by purified bovine brain multicatalytic proteinase complex (MCP), Neurochem. Res. 12 (1992) 1261–1266.
[29] R.J. Mayer, J. Arnold, L. Làszló, M. Landon, J. Lowe, Ubiquitin in health and disease, Biochim. Biophys. Acta 1089 (1991) 141–157.
[30] M.J. McGuire, M.L. McCullough, D.E. Croall, G.N. DeMartino, The high molecular weight multicatalytic proteinase, macropain, exists in a latent form in human erythrocytes, Biochim. Biophys. Acta 995 (1989) 181–186.
[31] H. Mori, J. Kondo, Y. Ihara, Ubiquitin is a component of paired helical filaments in Alzheimer disease, Science 235 (1987) 1641–1644.
[32] M. Morishima-Kawashima, M. Hasegawa, K. Takio, M. Suzuki, K. Titani, Y. Ihara, Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments, Neuron 10 (1993) 1151–1160.
[33] J. Morrissey, Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity, Anal. Biochem. 117 (1981) 307–310.
[34] Y. Murakami, S. Matsufuji, T. Kameji, S. Hayashi, K. Igarashi, T. Tamura, K. Tanaka, A. Ichihara, Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitinylation, Nature 360 (1992) 597–599.
[35] A. Murray, Cell ubiquitination: the destructive end of mitosis, Cell 81 (1995) 149–152.
[36] M. Okada, M. Ishikawa, Y. Mizushima, Identification of an ubiquitin- and ATP-dependent protein degradation pathway in rat cerebral cortex, Biochim. Biophys. Acta 1073 (1991) 514–520.
[37] M. Orlowski, C. Michaud, Pituitary multicatalytic proteinase complex. Specificity of components and aspects of proteolytic activity, Biochemistry 28 (1989) 9270–9278.
[38] M. Orlowski, C. Cardozo, M.C. Hidalgo, C. Michaud, Regulation of the peptidylglutamyl-peptide hydrolyzing activity of the pituitary multicatalytic proteinase complex, Biochemistry 30 (1991) 5999–6005.
[39] M. Orlowski, C. Cardozo, C. Michaud, Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral aminoacids, Biochemistry 32 (1993) 1563–1572.
[40] V.J. Palombella, O.J. Rando, A.L. Goldberg, T. Maniatis, The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B, Cell 78 (1994) 773–785.
[41] M. Piccinini, A. Merighi, R. Bruno, P. Cascio, M. Curto, S. Mioletti, C. Ceruti, M.T. Rinaudo, Affinity purification and characterization of protein gene product 9.5 (PGP 9.5) from retina, Biochem. J. 318 (1996) 711–716.
[42] K.L. Rock, C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, A.L. Goldberg, Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules, Cell 78 (1994) 761–771.
[43] D.M. Rubin, M.H. Glickman, C.N. Larsen, S. Dhruvakumar, D. Finley, Active site mutants in the six regulatory particles ATPases reveal multiple roles for ATP in the proteasome, EMBO J. 17 (1998) 4909–4919.
[44] H.H. Sawada, K. Muto, M. Fujimuro, T. Akaishi, M.T. Sawada, H. Yokosawa, A.L. Goldberg, Different ratios in 20S proteasomes and regulatory subunit complexes in two isoforms of the 26S proteasome purified from rabbit skeletal muscle, FEBS Lett. 335 (1993) 207–212.
[45] A.L. Schwartz, A. Ciechanover, The ubiquitin-proteasome pathway and pathogenesis of human diseases, Annu. Rev. Med. 50 (1999) 57–74.
[46] M. Seeger, K. Ferrell, M. Rechsteiner, The 26 proteasome: a dynamic structure, Mol. Biol. Rep. 24 (1997) 83–88.
[47] D.J. Selkoe, Normal and abnormal biology of the beta-amyloid precursor protein, Annu. Rev. Neurosci. 17 (1994) 489–517.
[48] H. Towbin, J. Gordon, Immunoblotting and immunobinding current status and outlook, J. Immunol. Methods 72 (1984) 313–340.