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Different ratios in 20 S proteasomes and regulatory subunit complexes in two isoforms of the 26 S proteasome purified from rabbit skeletal muscle

Hitoshi Sawada[a,b,*], Kazuko Muto[a], Masahiro Fujimuro[a], Takahiro Akaishi[a], Michiko Takagi Sawada[b,**], Hideyoshi Yokosawa[a], Alfred L. Goldberg[b]

[a]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060, Japan*
[b]*Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115, USA*

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A ubiquitin/ATP-dependent proteinase complex (26 S proteasome) was highly purified from rabbit skeletal muscle. The purified 26 S proteasome easily dissociated into a 20 S proteasome and a regulatory subunit complex on non-denaturing PAGE. By using cleavable and non-cleavable cross-linkers, it was revealed that the 26 S proteasome exists in two isoforms: one (D complex) consists of the 20 S proteasome and the regulatory subunit complex in the ratio of one to two, while the other (C complex) exists in an equal molar ratio. Molecular masses of the former and the latter isoforms were estimated to be 1,700 kDa and 1,400 kDa, respectively, by gel filtration, and 2,400 kDa and 1,400 kDa, respectively, by Ferguson plot analysis. Furthermore, both isoforms efficiently hydrolyzed Suc-Leu-Leu-Val-Tyr-MCA and ubiquitin-conjugated [^{125}I]lysozyme. These results suggest that the D and C complexes are active proteinase complexes, most probably corresponding to the dumbbell-like and mushroom-like (or space capsule-like) molecules, respectively.

ATP-dependent; Ubiquitin; Protease; Proteasome; Muscle (rabbit)

1. INTRODUCTION

A ubiquitin(Ub)/ATP-dependent proteinase complex, 26 S proteasome, plays a key role in intracellular proteolysis of the abnormal and short-lived proteins (for review see [1–4]). In this pathway, substrate proteins are first poly-ubiquitinated by E1 (Ub activating enzyme), E2 (Ub carrier protein, Ub conjugating enzyme) and E3 (Ub ligase, N-recognin) in an ATP-dependent manner, and degraded by the 26 S proteasome in an ATP-dependent fashion again.

An ATP-dependent 26 S protease, which has recently been called 26 S proteasome [1,2], was first purified from rabbit reticulocyte lysate by Hough et al. [5]. Their purified preparation gave two bands on non-denaturing PAGE, both of which had a Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity and appeared to comprise the 20 S proteasome and the higher molecular mass subunits (34–110 kDa), the latter of which is believed to be a regulatory subunit complex including ATPase subunits [5,6]. Although recent progress in their studies of the 26 S proteasome agrees with their previous findings concerning the presence of two isoforms in the 26 S proteasome [7], molecular entities of the two isoforms and the reason for the presence of two isoforms in a purified preparation of the 26 S proteasome remain unclear.

Several ideas have been proposed concerning the constituent complexes of the 26 S proteasome: the rabbit reticulocyte 26 S complex seems to comprise three factors: CF-1 (600 kDa), CF-2 (250 kDa), and CF-3 (650 kDa) [8], among which CF-3 is identified as a 20 S proteasome, a multicatalytic proteinase complex [9,10], and CF-2 appears to be an ATP-stabilized inhibitor of the proteasome [11]. CF-1 seems likely to be an activator of the 20 S proteasome [2,11]. Alternatively, the rabbit reticulocyte 26 S proteasome is reported to be composed of the protease complex (20 S proteasome) and the regulatory subunit complex [6,7]. In rabbit skeletal muscle, the 26 S proteasome is thought to be composed of the 20 S proteasome and a novel cysteine proteinase complex, multipain (500 kDa), since both of them form a ubiquitin/ATP-dependent higher molecular mass proteinase complex in an ATP-dependent manner [1,2]. On the other hand, several investigators still suspect the presence of the 26 S proteasome which comprises the 20 S proteasome and the regulatory subunits, on the basis of the analysis of the partially purified 26 S proteasome and on the immunoblot analysis using anti-20 S proteasome antibody [12].

*Corresponding author. Fax: (81) (11) 717 3167.

***Present address:* Bioscience and Chemistry Division, Hokkaido National Industrial Research Institute, Toyohira-ku, Sapporo 062, Japan.

Abbreviations: AMC, 7-amino-4-methylcoumarin; DMA, dimethyladipimidate; DTT, dithiothreitol; DTBP, dimethyl 3,3′-dithiobispropionimidate; MCA, 4-methylcoumaryl-7-amide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Suc, succinyl; 2D, two-dimensional; Ub, ubiquitin.

In this context, it is important to clarify the entity of the two isoforms of the 26 S proteasome and also the constituent components of the 26 S complex in order to understand the molecular structure and function of the 26 S proteasome. Thus, we attempted to purify the 26 S proteasome from rabbit skeletal muscle and carefully examined the molecular structure of the constituent complexes of the 26 S proteasome by electrophoretic techniques. Our purified preparation of the 26 S proteasome was easily dissociated into the 20 S proteasome and the regulatory subunit complex during non-denaturing PAGE, which was convincingly demonstrated by using the cleavable and non-cleavable bifunctional cross-linking reagents. Furthermore, we found that the 26 S proteasome exists in two isoforms composed of the 20 S proteasome and the regulatory subunit complex. One isoform is a complex of both constituent complexes in an equal molar ratio, while the other is a complex composed of the 20 S proteasome and the regulatory subunit complex in the ratio of one to two.

2.MATERIALS AND METHODS

2.1. *Materials*

Suc-Leu-Leu-Val-Tyr-MCA and AMC were purchased from Peptide Institute (Osaka, Japan). DEAE-cellulose (DE-32) and phosphocellulose (P-11) were obtained from Whatman. FPLC system equipped with Mono Q HR 5/5 and Superose 6 HR 10/30 were from Pharmacia-LKB. Centricon 10 and PM-10 membrane were products of Amicon.

2.2. *Buffers*

The following buffers were used for extraction and purification of enzymes: Buffer A, 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM EDTA, and 1% (v/v) glycerol; Buffer B, 20 mM Tris-HCl (pH 7.2), 1 mM DTT, 0.1 mM ATP, 0.1 mM EDTA, and 10% (v/v) glycerol; Buffer C, 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 500 mM NaCl, 0.1 mM ATP, 0.1 mM EDTA, and 10% glycerol; Buffer D, 100 mM sodium phosphate (pH 6.5), 1 mM DTT, 0.1 mM ATP, 0.1 mM EDTA, and 10% glycerol; Buffer E, 20 mM Tris-HCl (pH 7.8), 1 mM DTT, 0.1 mM ATP, 0.1 mM EDTA, and 10% glycerol; Buffer F, 50 mM Tricine-NaOH (pH 7.8), 1 mM DTT, 0.1 mM ATP, 0.1 mM EDTA, 100 mM NaCl, and 10% glycerol.

2.3. *Assays*

Unless otherwise noted, the ubiquitin/ATP-dependent protease activity was measured at 37°C by using ubiquitinated [^{125}I]lysozyme as a substrate according to the method described by Tamura et al. [13] with a slight modification (Fujimuro, M. et al., in preparation). The reaction mixture (200 μl) contained 50 mM Tris-HCl (pH 7.8) U, 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 5 ng of ubiquitinated [^{125}I]lysozyme (18,000 cpm). After incubation for 1–3 h, the reaction was stopped by chilling, followed by the addition of 25 μl of 10% bovine serum albumin and 600 μl of 10% trichloroacetic acid. After standing for 10 min at 0°C, the acid soluble radioactivity was measured. Assay was also carried out by using an equal molar amount of [^{125}I]lysozyme instead of Ub-[^{125}I]-lysozyme as a substrate.

2.4. *Protein*

Protein concentration was determined by the method of Bradford [14] with bovine serum albumin as a standard.

2.5. *Electrophoreses*

SDS-PAGE (12.5% slab gel) was carried out by the method of Laemmli [15]. Non-denaturing PAGE (4% slab gel) was performed by the method of Laemmli [15] in the absence of SDS. For estimation of molecular mass, Ferguson plot analysis [16] was carried out by the above non-denaturing PAGE with polyacrylamide gels (2.5%, 3.0%, 3.5%, and 4.0%) containing 0.5% agarose. In the 2D-PAGE, the first dimensional gel was immersed in the sample buffer for 10 min at room temperature and then placed on the top of the slab polyacrylamide gel in the presence or absence of SDS and subjected to electrophoresis in the second dimension.

2.6. *Purification procedures*

The back skeletal muscles (about 150 g/rabbit) were dissected from 2 or 3 female white rabbits (3 kg) and homogenized in 1.5 volumes (buffer (ml)/muscle(g)) of the chilled Buffer A with Waring Blender. After centrifugation at $1,400 \times g$ for 30 min, the supernatant was further centrifuged at $100,000 \times g$ for 60 min. Glycerol was added to the resulting supernatant up to a final concentration of 10% (v/v). The crude extract thus prepared was applied to a DEAE-cellulose (DE-32) column (5×10 cm) equilibrated with Buffer B. After extensive washing with Buffer B, the adsorbed material was eluted with Buffer C (400 ml). The flow rates were 500 ml/h for adsorption and 200 ml/h for elution, respectively. To the eluate (Fraction II), an equal volume of 40% (w/v) polyethylene glycol 8,000 dissolved in 10 mM Tris-HCl (pH 8.0) was added and stirred for 30 min at 4°C. The insoluble pellet was collected by centrifugation ($6,000 \times g$, 30 min) and dissolved in 80 ml of Buffer D. After centrifugation at $6,000 \times$ g for 20 min, the supernatant was applied to a phosphocellulose P-11 column (2.7×9 cm) equilibrated with Buffer D, and was washed with the same buffer at a flow rate of 25 ml/h. The ATP-dependent proteinase-containing fractions were centrifuged ($100,000 \times$ g, 5 h), and the resulting pellet was dissolved in Buffer E containing 0.275 M NaCl. After centrifugation ($10,000 \times$ g, 30 min), the soluble materials were loaded on a Mono Q HR5/5 column and washed with the same buffer. After washing with the equilibration buffer, the adsorbed enzyme was eluted with Buffer E containing 0.375 M NaCl. One ml fractions were collected at a flow rate of 0.3 ml/min. The active fraction eluted with 0.375 M NaCl was concentrated with Centricon 10, and applied to a Superose 6 HR10/30 column (1×30 cm) equilibrated with Buffer F, which was developed at a flow rate of 0.3 ml/min collecting 0.5 ml in each tube. All steps of purification were performed at 0–4°C including FPLCs using Mono Q and Superose 6 columns.

2.7. *Cross-linking conditions*

The enzyme solutions dissolved in Buffer F were mixed with one-fifth volume of 10 mM dimethyladipimidate (DMA) or dimethyl 3,3′-dithiobispropionimidate (DTBP) dissolved in water immediately before use, and incubated for 30 min at 25°C according to the method of Uchiumi et al. [17]. After treatment of the cross-linker, the solutions were mixed with the sample buffer for electrophoresis and subjected to SDS-PAGE or non-denaturing PAGE.

3. RESULTS AND DISCUSSION

3.1. *Purification and purity of the 26 S proteasome*

As summarized in Table I, the 26 S proteasome was purified from rabbit skeletal muscle by ion-exchange chromatographies with DEAE-cellulose, phosphocellulose, and Mono Q, ultracentrifugation, and gel filtration with Superose 6. Approximately 1 mg of the 26 S proteasome was isolated with a yield of 6% from the fraction II, an adsorbed fraction on DEAE-cellulose chromatography. An ATP dependence in Ub-conjugate-degrading activity of the purified preparation was 9-fold.

On Superose 6 FPLC at a final step of purification, ATP-dependent Ub-conjugate-degrading activity was

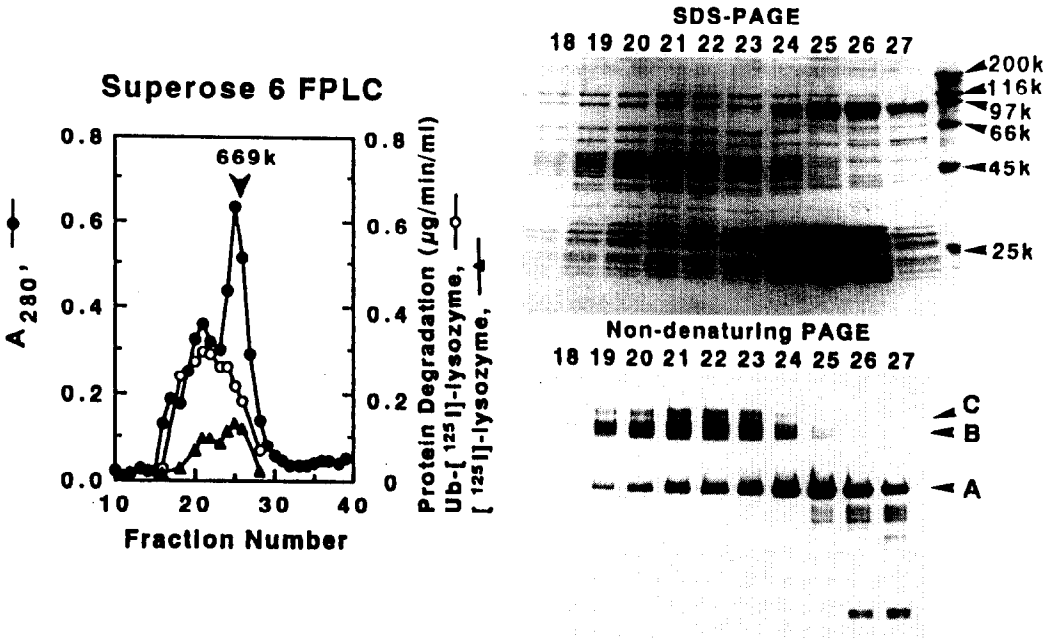

Fig. 1. Superose 6 FPLC of the 26 S proteasome from rabbit skeletal muscle. The active fractions obtained from the Mono Q column were concentrated and a part of the concentrate was applied to the Superose 6 column (1 × 30 cm) equilibrated with buffer F (left panel). Chromatography was performed at 0.25 ml/min and 0.5 ml fractions were collected. A_{280} (●); Ub-[^{125}I]lysozyme hydrolyzing activity in the presence of ATP (○); [^{125}I]lysozyme hydrolyzing activity in the presence of ATP (▲). SDS-PAGE (12.5% gel, right upper panel) and non-denaturing PAGE (4% gel, right lower panel) of the eluted fractions (20 μl each) from the Superose 6 column. After electrophoresis, protein bands were stained with Coomassie brilliant blue R-250.

eluted at around fraction 21 and coincided with the second peak of protein, which corresponds to the molecular mass of about 1,300 kDa (see Fig. 1). [^{125}I]Lysozyme-hydrolyzing activity was eluted in two peaks which correspond to the molecular masses of 1300 kDa and 700 kDa. These results indicate that the former fraction corresponds to the 26 S proteasome and the latter to the 20 S proteasome. To examine the purity and the subunit composition of the 26 S proteasome, active fractions in Superose 6 FPLC were subjected to SDS-PAGE. The peak fraction of the Ub-conjugate-degrading activity gave several bands, the pattern of which is typical to those of the 26 S proteasome of rabbit reticulocyte [5–7] (see Fig. 1). Furthermore, all of these band components eluted from the column coincidently at a peak of Ub-conjugate-degrading activity at fraction 21. These results strongly suggest that the purified 26 S proteasome preparation is highly pure.

Table I

Summary of purification of 26 S proteasome from rabbit skeletal muscle

Step	Protein (mg)	Activity* (+ATP) (units)	Specific activity (+ATP) (units/mg)	ATP stimulation (-fold)
Crude extract	16,000	590,000	37	5.5
DEAE-cellulose	470	200,000	430	0.73
PEG	330	190,000	580	8.3
Phosphocellulose	200	180,000	900	7.7
Ultra-centrifugation	30	27,000	900	3.8
Mono Q	10	22,000	2,200	4.2
Superose 6	0.98	11,000	11,000	9.0

*One unit of activity was defined as the amount of enzyme which hydrolyzes 1 ng of Ub-[^{125}I]lysozyme per min at 37°C and at pH 7.8.

3.2. *The 26 S proteasome is composed of the 20 S proteasome and the regulatory subunit complex, which are dissociable during non-denaturing PAGE*

Regardless of the apparent high purity of the 26 S proteasome on the basis of SDS-PAGE, this preparation gave three distinct bands designated as A, B, and C under non-denaturing PAGE, among which A and B were the main bands, and band C was a minor one (see Fig. 1). Then we attempted to identify these three components by 2D-PAGE (first dimension, non-denaturing PAGE; second dimension, SDS-PAGE). The results shown in Fig. 2 indicate that the complex at band A is composed of at least 8 subunits with molecular masses between 23 kDa and 31 kDa, suggesting that this is a typical 20 S proteasome. On the other hand, the complex at band B comprised several subunits with molecular masses between 28 kDa and 110 kDa. The complex at band C appeared to be an associated form of A and B complexes. These results led us to propose the idea that the 26 S proteasome, which exists as a C complex, may be dissociated into A and B complexes during non-denaturing PAGE.

In order to prove this theory, we utilized the cleavable (DTBP), and non-cleavable (DMA) cross-linking rea-

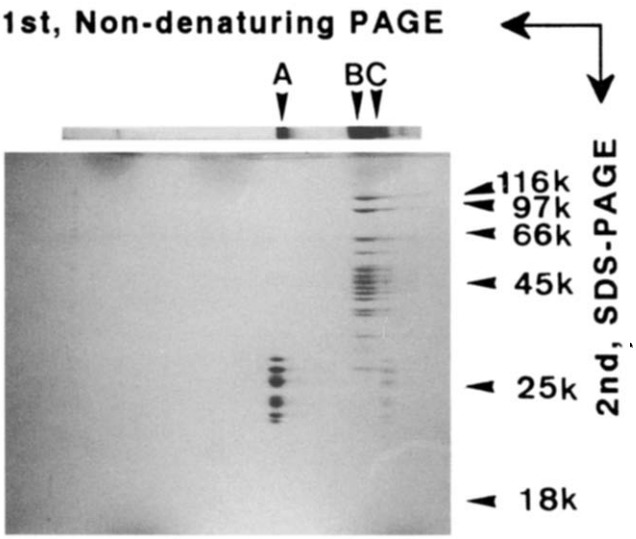

Fig. 2. 2D-PAGE of the 26 S proteasome. Approximately 3 μg of the 26 S proteasome preparation dissolved in buffer F was subjected to non-denaturing PAGE in 4% gel in the first dimension. After electrophoresis, the gel was immersed in the sample buffer and subjected to SDS-PAGE (12.5% slab gel) in the second dimension.

gents, which would block the possible dissociation of the complexes during electrophoresis. The purified 26 S proteasome was treated with these cross-linkers and subjected to non-denaturing PAGE. While the amounts of A and B complexes decreased with treatment of either of these cross-linkers, the amounts of C and D complexes increased (see Fig. 3). Bands C and D distinctly appeared as the main components after treatment of the cross-linkers, although bands C and D were also observed as minor and very faint bands, respectively, without treatment of the cross-linker (band D was detected when a large amount of the 26 S proteasome preparation was loaded on the gel; see the first dimensional PAGE pattern in Fig. 2). This result suggests that the band D complex is not an artefact or an aggregate with treatment of the cross-linkers, but one of the real isoforms of the 26 S proteasome present in a purified preparation.

In order to examine whether C and D complexes are definitely composed of A and B complexes, the 26 S proteasome preparation was treated with DTBP and subjected to 2D-PAGE (first dimension, non-denaturing PAGE under the non-reducing conditions; second dimension, SDS-PAGE or non-denaturing PAGE under the reducing conditions). As shown in Fig. 4a, the C complex was dissociated into A and B complexes in an almost equal ratio in amount, and the D complex was dissociated into B and C complexes as main components and A complex as a minor one by non-denaturing PAGE in the second dimension. Subunit compositions of C and D complexes were then examined by SDS-PAGE in the second dimension (Fig. 4b), which showed that both of them were composed of A and B complexes, and that these subunit compositions were quite similar to those of the 26 S proteasome isolated from rabbit reticulocyte [5–7]. These results also indicated that the purified 26 S proteasome exists as two isoforms (C and D complexes) in a solution, which contains 0.1 mM ATP and 10% glycerol at pH 7.8, and easily dissociates into the 20 S proteasome (band A) and the regulatory subunit complex (band B) during non-denaturing PAGE.

3.3. *Molecular entities of the two isoforms of the 26 S proteasome*

As mentioned above, the D complex cross-linked with DTBP was dissociated into the C and B complexes in an almost equal ratio on the basis of density and very small amount of A complex after reduction (Fig. 4a). On the other hand, the C complex was dissociated into A and B complexes in an almost equal ratio under the same conditions (see Fig. 4b). These results suggest that the D complex is an associated form of the B and C complexes, i.e. a complex composed of A and B complexes in a ratio of one-to-two, while the C complex is composed of the A and B complexes in a one-to-one ratio. This interpretation was well coincident with the result of SDS-PAGE in the second dimension of the 2D-PAGE: the relative amounts of the regulatory subunits to those of the 20 S proteasome subunits were much higher in the D complex than in the C complex (Fig. 4b). On the basis of the density of the protein bands, we roughly estimated the molecular masses of D and C complexes assuming that the molecular mass of the 20 S proteasome (A complex) is 700 kDa. The former (D) and the latter (C) were estimated to be 2,100 kDa and 1,600 kDa, respectively. Molecular masses of the D and C complexes were estimated to be 1,700 kDa and 1,400 kDa, respectively, by gel filtration on Super-

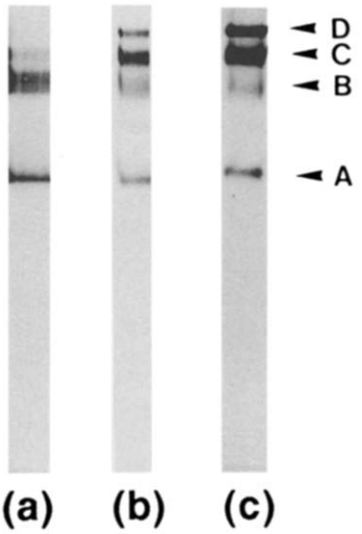

Fig. 3. Non-denaturing PAGE of the 26 S proteasome cross-linked with DMA or DTBP. The purified 26 S proteasome preparation (about 3 μg) was incubated at 25°C for 30 min in the absence (a) and presence of DMA (b) or DTBP (c) at a final concentration of 2 mM and subjected to non-denaturing PAGE.

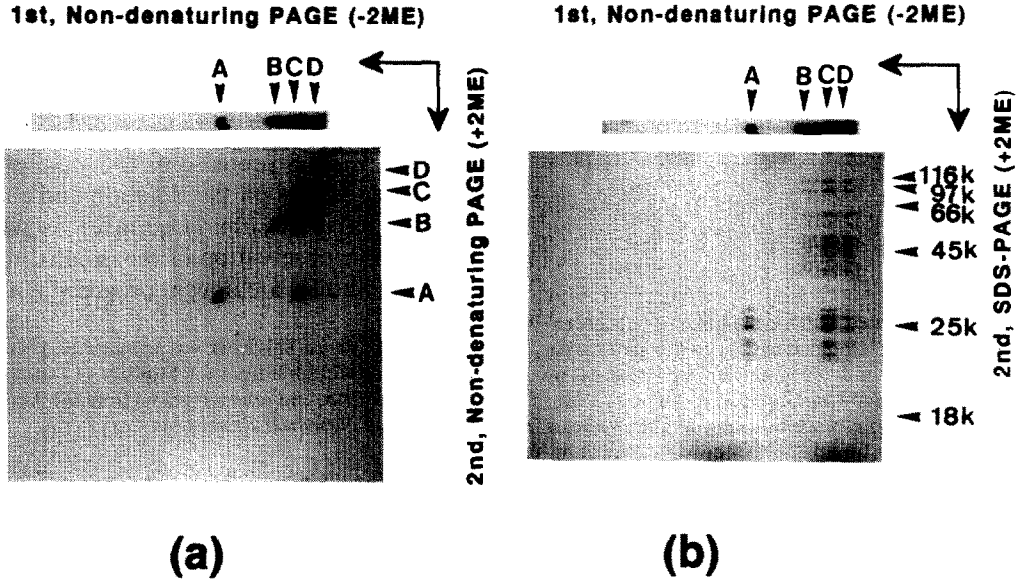

Fig. 4. 2D-PAGE of the 26 S proteasome after treatment of cross-linkers. The 26 S proteasome (about 3 μg) was incubated at 25°C for 30 min with 2 mM DTBP and subjected to 2D-PAGE. (a) Non-denaturing PAGE was performed under non-reducing conditions in the first dimension, and under reducing conditions in the second dimension. (b) The cross-linked 26 S proteasome was subjected to non-denaturing PAGE under non-reducing conditions in the first dimension followed by SDS-PAGE under reducing conditions in the second dimension.

ose 6 after cross-linking with DMA, the elution positions of which were identified by non-denaturing PAGE, while those of the D and C complexes were also estimated to be 2,400 kDa and 1,400 kDa, respectively, by Ferguson plot analysis.

All the above mentioned results indicate that there are two isoforms in the 26 S proteasome: one (D complex) consists of A and B complexes in the ratio of one to two, while the other (C complex) consists of A and B complexes in an equal ratio. Although we have not yet observed the ultrastructures of the D and C complexes by electron microscopy, the D and C complexes are most likely to be dumbbell-like and mushroom-like (or space capsule-like) structures, respectively, since both types of molecules are reported to be observed in the purified 26 S proteasome preparation [1,2,18] and our present data on the stoichiometry concerning the ratio of the constituent complexes strongly support the above consideration.

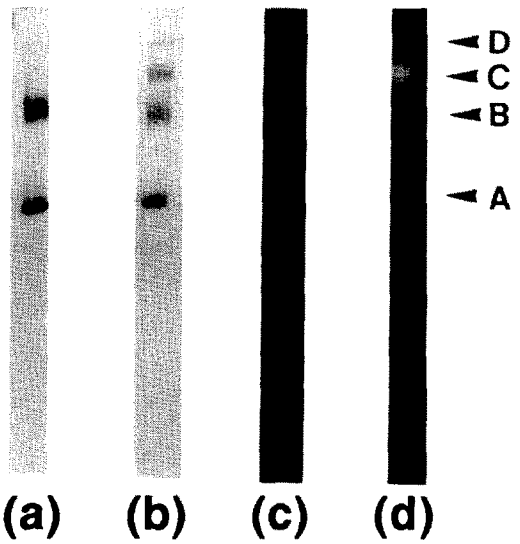

Fig. 5. Protease activities of the 26 S proteasome and its constituent complexes. The 26 S proteasome was incubated in the absence (a,c) and presence (b,d) of DTBP as described in the legend of Fig. 3, and subjected to non-denaturing PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (a, b) or subjected to active staining (c,d) with Suc-Leu-Leu-Val-Tyr-MCA as described in [5].

3.4. *Protease activities of the two isoforms of 26 S proteasome, the 20 S proteasome, and the regulatory subunit complex*

Proteolytic activities of the four complexes were tested by non-denaturing PAGE followed by active staining of the gel using Suc-Leu-Leu-Val-Tyr-MCA as a substrate. A strong fluorescence owing to the hydrolyzed product, AMC, was observed in C and D complexes, whereas very weak fluorescence was observed in the A complex, and no appreciable fluorescence was observed in the B complex (Fig. 5). This result indicates that both isoforms of the 26 S proteasome have strong proteolytic activities toward the fluorogenic peptide substrate, while the 20 S proteasome (A) and the regulatory subunit complex (B) have weak and apparently no protease activities, respectively. Ub-conjugate degrading activities of the C and D complexes were then measured after extraction with buffer F of the gel slices of the two bands which were identified by active staining. Although the total activity of the D complex appeared to be lower than that of the C complex, specific activity of the D complex was approximately 1.5-times higher than

Table II

Ub-[^{125}I]lysozyme degrading activities of the two isoforms of the 26 S proteasome

26 S proteasome isoforms	Cross-linker	Specific activity (ng/min/mg protein)
C complex	DMA	220
C complex	DTBP	240
D complex	DMA	290
D complex	DTBP	310

The 26 S proteasome was incubated with a cross-linker (final concentration, 2 mM) for 30 min at 25°C and subjected to non-denaturing PAGE. After electrophoresis, C and D complexes were visualized by active staining of the gel using Suc-Leu-Leu-Val-Tyr-MCA as a substrate. The area with strong fluorescence was cut out and extracted with buffer F, followed by assay for Ub-[^{125}I]lysozyme degrading activity. The amount of protein in the gel extract was determined by the method of Bradford [14].

that of the C complex (Table II). Taking into account of the molecular masses of the D and C complexes, the D complex degrades the Ub-conjugate two-times more effectively than the C complex on the basis of molar ratio. These results suggest that the 26 S proteasome complex with two regulatory subunit complexes in both sides of the cylinder structure of the 20 S proteasome may more effectively degrade the Ub-conjugate than that with one regulatory subunit complex. Also, the present data strongly suggest that both mushroom-like and dumbbell-like molecules are active 26 S proteasome, and do not support the possibility that the mushroom-like complex is a defective molecule of the dumbbell-like 26 S proteasome [6,18], or the dumbbell-like complex is an artificial dimer of the mushroom-like molecule [6].

Rechsteiner and his colleagues proposed the model that the mushroom-like 26 S proteasome may comprise one regulatory subunit complex and a half (two rings among four rings) of the 20 S proteasome [6]. However, our present results do not support their model, but rather support the idea that the full size of the 20 S proteasome appears to be contained in the C complex (mushroom-like complex, see Fig. 4). If only a half of the 20 S proteasome subunits were contained in the mushroom-like 26 S proteasome (C complex) with the regulatory subunit complex, the electrophoretic mobility of the half size of the 20 S proteasome, which is contained in the C complex and dissociated during non-denaturing PAGE, would be much larger than that of the full size 20 S proteasome (A complex).

Further studies on the isolation and characterization of the regulatory subunit complex as well as electron microscopic observation of the isolated four complexes (A, B, C, and D) are now in progress in our laboratory.

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REFERENCES

[1] Goldberg, A.L. (1992) Eur. J. Biochem. 203, 9–23
[2] Goldberg, A.L. and Rock, K.L. (1992) Nature 357, 375–379
[3] Hershko, A. and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
[4] Rechsteiner, M. (1991) Cell 66, 615–618
[5] Hough, R., Pratt, G. and Rechsteiner, M. (1987) J. Biol. Chem. 262, 8303–8313
[6] Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993) J. Biol. Chem. 268, 6065–6068
[7] Hoffman, L., Pratt, G. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22362–22368
[8] Ganoth, D., Leshinsky, E., Eytan, E. and Hershko, A. (1988) J. Biol. Chem. 263, 12412–12419
[9] Eytan, E., Ganoth, D., Armon, T. and Hershko, A. (1989) Proc. Natl. Acad. Sci. USA 86, 7751–7755
[10] Driscoll, J. and Goldberg, A.L. (1990) J. Biol. Chem. 265, 4789–4792
[11] Driscoll, J., Frydman, J. and Goldberg, A.L. (1992) Proc. Natl. Acad. Sci. USA 89, 4986–4990
[12] Seelig, A., Kloetzel, P.-M., Kuehn, L. and Dahlmann, B. (1991) Biochem. J. 280, 225–232
[13] Tamura, T., Tanaka, K., Tanahashi, N. and Ichihara, A. (1991) FEBS Lett. 292, 154–158
[14] Bradford, M. (1976) Anal. Biochem. 72, 248–254
[15] Laemmli, U.K. (1970) Nature 227, 680–685
[16] Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155–164
[17] Uchiumi, T., Terao, K. and Ogata, K. (1980) J. Biochem. (Tokyo) 88, 1033–1044
[18] Ikai, A., Nishigai, M., Tanaka, K. and Ichihara, A. (1991) FEBS Lett. 292, 21–24