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ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells

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The role of proteasomes in ubiquitin (Ub)-dependent protein degradation was studied by analyzing lysates of human promyelocytic leukemia HL-60 cells by glycerol density gradient centrifugation. High succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide hydrolyzing activity was found in the 26S fraction, whereas the 20S fraction containing proteaomes had no activity. Addition of 0.05% sodium dodecylsulfate to the latter fraction, however, induced marked activity. The 26S, but not the 20S fraction catalyzed ATP-dependent degradation of [^{125}I]lysozyme–Ub conjugate. Depletion from the lysate of ATP caused complete shift of the active 26S complex to the latent 20S form, whereas in the lysate prepared from ATP-depleted cells, ATP converted 20S proteasomes to 26S complexes. The immunoprecipitated 26S complexes were found to consist of proteasomes and 13–15 other proteins ranging in size from 35 to 110 kDa. We conclude that in the lysate, latent proteasomes undergo reversible, ATP-dependent association with multiple protein components to form 26S complexes that catalyze ATP-dependent degradation of Ub–protein conjugates.

Proteasome; 26S Protease complex; ATP; Ubiquitin; HL-60 Cell

1. INTRODUCTION

ATP is involved in several steps of energy-dependent proteolysis. One role of ATP is in the covalent modification of substrate proteins by ubiquitin (Ub), a process involving multiple enzymatic reactions [1]. This Ub ligation to substrate proteins serves as a signal for their proteolytic degradation. There are reports that a large 26S protease complex catalyzes ATP-dependent breakdown of Ub-conjugated proteins [2,3] and that the 20S proteasome, a multicatalytic proteinase complex [4,5], is a component of this 26S complex [6,7]. However, the properties of the 26S complex are not yet well characterized.

In this work, we examined the role of proteasomes in ATP-dependent, Ub-mediated protein degradation in human promyelocytic leukemia HL-60 cells, which express very high levels of proteasomes [8]. We found that the incorporation of 20S proteasomes into 26S particles is ATP-dependent and reversible. By immunoprecipitation with anti-proteasome monoclonal antibody, we also found that 26S complexes contain multiple other protein components in addition to proteasomes.

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Abbreviations: Ub, ubiquitin; Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; mAb, monoclonal antibody; IgG, immunoglobulin G.

2. MATERIALS AND METHODS

Human promyelocytic leukemia HL-60 cells (approximately 2–5 × 10^8 cells) were lysed by sonication, the sonicate was centrifuged for 30 min at 15 000 × *g*, and the resulting supernatant was used as crude cell lysate. The lysate was incubated for 60 min at 37°C in buffer A (20 mM Tris-HCl buffer, pH 7.5, containing 2 mM ATP, 5 mM $MgCl_2$ and 1 mM dithiothreitol) with an ATP-regenerating system (10 mM creatine phosphate and 10 μg/ml of creatine kinase) or in buffer A without ATP and supplemented with an ATP-depleting system (10 mM glucose and 1 μg/ml of hexokinase). About 1.0 ml of crude lysate (0.8–5.0 mg protein) was loaded on a 16-ml linear gradient of 10–40% (v/v) glycerol in buffer A. The gradient was centrifuged at 27 000 rpm for 18 h in a Hitachi SRP28SA rotor, and then 16 fractions of 1 ml each were collected at 4°C.

Suc-LLVY-MCA-hydrolyzing activity was measured after incubation for 10 min at 37°C as described [5]. Breakdown of [^{125}I]lysozyme–Ub conjugates was assayed by measuring the acid-soluble radioactivity after incubation for 60 min at 37°C in the presence or absence of 5 mM ATP as described [3]. Lysozyme was radioiodinated and [^{125}I]lysozyme–Ub conjugates were prepared with rabbit reticulocyte extracts by the method of Hough et al. [2].

Monoclonal antibody against human liver proteasomes, named mAb 2-17, was prepared as described [8]. Immunoelectrophoretic blot analysis was carried out by the method of Towbin et al. [9]. For immunoprecipitation experiments, approximately 5.0 mg of protein of cell lysate was mixed with 150 μg of anti-proteasome mAb 2-17 for 60 min at room temperature. Then the mixture was treated for 30 min with Protein A-Sepharose CL-4B in an amount corresponding to a 2-fold excess over the binding capacity of the added IgG, or with CH-Sepharose 4B coupled with a similar amount of mAb 2-17. The resulting precipitate was washed extensively with buffer A containing 0.15 M NaCl and 0.05% Tween 20 and solubilized in 400 μl of 20 mM glycine-HCl buffer (pH 3.0). The solubilized proteins were precipitated with cold acetone and subjected to SDS-PAGE (10–20% gradient gel) [10]. Proteins were detected by silver staining.

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3. RESULTS

3.1. *Dissociation of 26S active complexes into latent proteasomes and multiple protein components*

Removal of proteasomes from a lysate of human promyelocytic leukemia HL-60 cells by immunoprecipitation caused parallel loss of the activities for hydrolyzing Suc-LLVY-MCA, a good substrate for proteasomes [5], and catalyzing ATP-dependent breakdown of [^{125}I]lysozyme–Ub conjugates (data not shown), indicating that proteasomes are involved in both reactions. These reactions, however, differ from each other in that ATP-Mg^{2+} is required only for lysozyme-Ub conjugate degradation. The Suc-LLVY-MCA hydrolyzing activity was lost time-dependently when the lysate was incubated in the absence, but not the presence, of ATP (data not shown). These findings suggest dual effects of ATP on proteasome-dependent proteolysis in the lysate: (a) in ATP-Mg^{2+}-dependent degradation of Ub-conjugated proteins, and (b) for protection of the peptide hydrolyzing activity from inactivation.

To characterize these proteolytic activities, we analyzed the cell lysate by glycerol density gradient centrifugation. When the lysate prepared with ATP was fractionated, Suc-LLVY-MCA hydrolyzing activity was recovered in a fraction with a sedimentation coefficient of about 26S (Fig. 1A, fraction 8), but no activity was detected in the fraction of 20S containing proteasomes (Fig. 1A, fraction 11). However, addition of 0.05% SDS, a potent artificial activator of latent proteasomes [5], markedly activated the enzyme in the 20S fraction, indicating that the proteasomes in this fraction were in a latent form. This concentration of SDS had little effect on the peptidase activity in the 26S fraction. Anti-proteasome mAb 2-17, which specifically recognizes the largest C2 subunit (M_r = 31 kDa) of proteasomes [8], reacted with the 31 kDa component in both the 26S and the 20S fraction (Fig. 1A, upper panel), confirming the presence of proteasomes in both fractions. When the ATP level in the lysate prepared without ATP was further reduced by incubation with hexokinase and glucose, the SDS-insensitive Suc-LLVY-MCA hydrolyzing activity in the 26S fraction

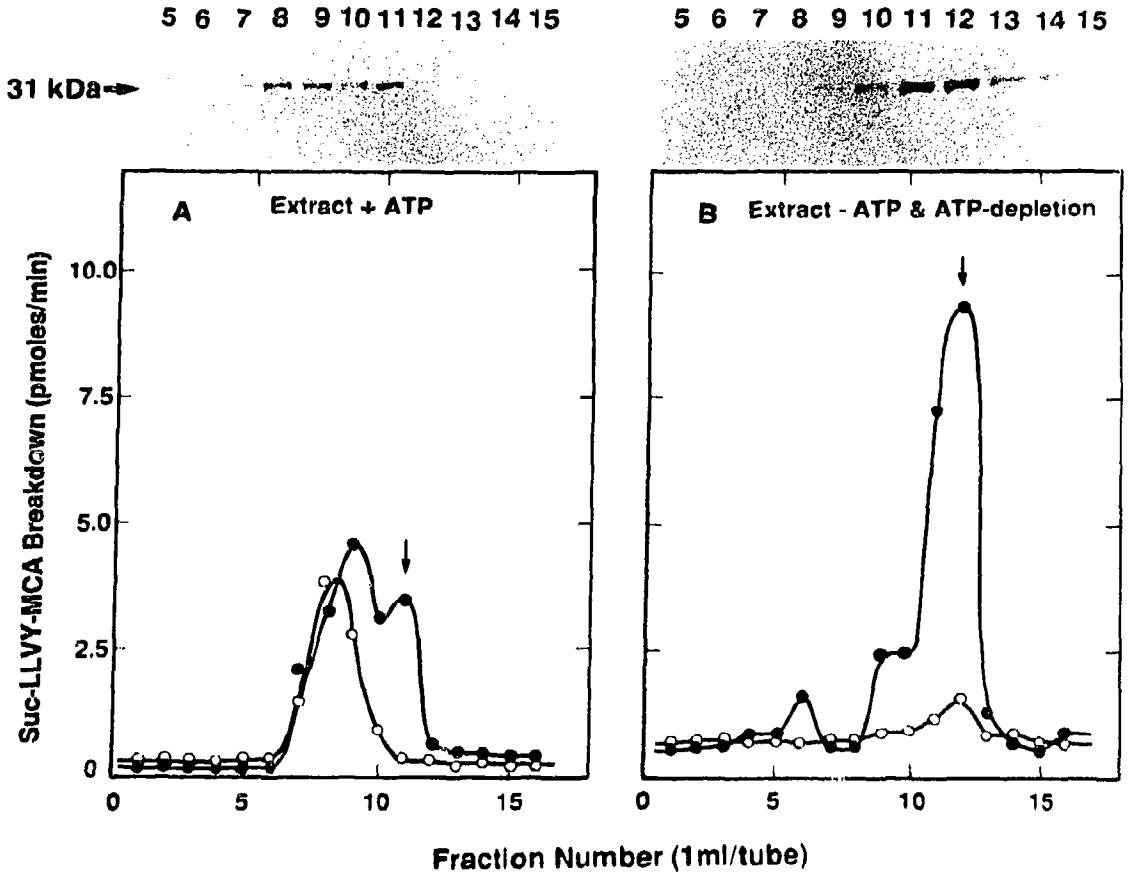

Fig. 1. Dissociation of active 26S complexes into latent 20S proteasomes in HL-60 cell lysates on depletion of ATP. Samples of about 10^8 HL-60 cells were lysed by sonication in buffer A containing 2 mM ATP (A). For B, a lysate prepared without ATP was further incubated for 60 min at 37°C with an ATP-depleting system. These crude lysates (800 μg) were fractionated by glycerol density gradient centrifugation and aliquots (20 μl) of individual fractions were used for assay of Suc-LLVY-MCA breakdown with (•) or without (○) 0.05% SDS. The upper panels show the profiles of the samples in corresponding fractions immuno-blotted with anti-proteasome mAb 2-17. The proteins in 200 μl volumes of the fractions were precipitated with acetone and subjected to immuno-blot analysis. Arrows indicate the position of elution of purified proteasomes.

was lost almost completely, and the SDS-activated activity in the 20S fraction was greatly increased (Fig. 1B). This suggests that ATP depletion caused dissociation of the 26S complexes liberating latent 20S proteasomes. In fact, immunoblot analysis showed that the C2 subunit shifted from the 26S fraction to the 20S fraction (Fig. 1B, upper panel). These findings are consistent with the apparent loss of activity for Suc-LLVY-MCA degradation upon incubation of the lysate without ATP.

As mentioned earlier, ATP-dependent degradation of Ub-protein conjugates has been shown to be catalyzed by 26S protease complexes containing proteasomes [2,3,6,7]. Actually, the 26S fraction from HL-60 cells catalyzed the degradation of [^{125}I]lysozyme-Ub conjugate in a totally ATP-dependent fashion (Table I). In contrast, the 20S fraction did not degrade the Ub-conjugated protein even in the presence of ATP-Mg^{2+}.

We next immunoprecipitated the 26S and 20S complexes with anti-proteasome mAb 2-17 from cell lysates with a high level and depleted of ATP as described in section 2. The SDS-PAGE patterns of total lysate proteins appeared identical regardless of the ATP level (Fig. 2, lanes 2 and 3), but upon silver staining of proteins immunoprecipitated from the ATP-depleted lysate only the multiple subunits of proteasomes (M_r = 21–31 kDa) and the light and heavy chains of the IgG used were detected with a few faint non-specific bands (Fig. 2, lane 5). These results confirm that the anti-C2 subunit antibody precipitated whole proteasomes as multisubunit complexes [8]. In contrast, the SDS-PAGE profile of the immunoprecipitate from the lysate with a high ATP level exhibited a number of additional protein bands ranging in size from 35 to 110 kDa (Fig. 2, lane 6). This profile was reproducible in 5 experiments. However, under these conditions some components seemed to overlap the broad band of the heavy chain of mouse IgG. Therefore, we performed another immunoprecipitation experiment using mAb 2-17 coupled to CH-Sepharose 4B and identified several more protein bands in the heavy chain region (Fig. 2, lanes 7 and 8). From these findings, we concluded that the 26S complex that degrades Ub-conjugated proteins is composed of 13–15 protein factors in addition to a proteasome. These multiple protein factors appear to be similar, but not identical in size, to those found in 26S ATP-dependent protease complexes partially purified from reticulocyte extracts [2,6].

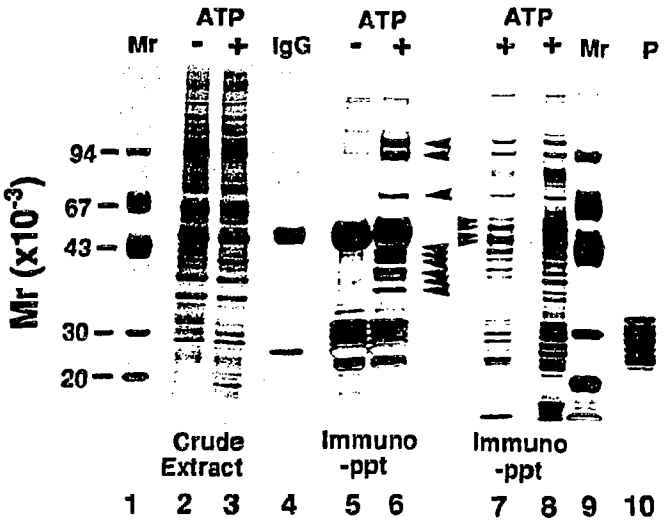

Fig. 2. Separation of the 26S complex consisting of a proteasome and multiple protein factors by immunoprecipitation in the presence of ATP. Immunoprecipitation with anti-proteasome mAb 2-17 was carried out as described in section 2. ATP (+) indicates lysates prepared in buffer A containing 2 mM ATP and then incubated for 60 min at 37°C in the same buffer supplemented with an ATP-regenerating system. ATP (−) indicates lysates prepared in buffer A without ATP and then incubated for 60 min at 37°C with an ATP-depleting system. Samples of total lysates (lanes 2 and 3, 3.0 μg), IgG of mAb 2-17 (lane 4, 1.0 μg) and P, purified human liver proteasomes [5] (lane 10, 1.0 μg) were used. Lanes 1 and 9 show M_r marker proteins. Immunoprecipitation was performed by treatment with mAb 2-17 (150 μg IgG) and protein A-Sepharose CL-4B (lanes 5 and 6) or mAb (150 μg IgG) coupled to 1 ml of CH-Sepharose 4B (lanes 7 and 8). Lysates with ATP at 1.0 mg and 5.0 mg were used for immunoprecipitation analysis in lanes 7 and 8, respectively. Arrowheads indicate various protein factors immunoprecipitated with proteasome.

Table I

Effects of ATP on breakdown of [^{125}I]lysozyme-Ub conjugates by 26S complex and 20S proteasome fractions from HL-60 cells

Fraction	Breakdown of [^{125}I]lysozyme-Ub conjugates (%/h)	
	−ATP	+ATP
Crude cell lysate	0.3	9.5
26S Protease complex	0.2	7.8
20S Proteasome	0.0	0.5

Breakdown of [^{125}I]lysozyme-Ub conjugates was assayed with samples of crude lysates (125 μg) in the presence or absence of 5 mM ATP. For assay of ATP-independent activity, an ATP-depleting system consisting of hexokinase and glucose was added to the assay mixture, because the lysates and enzyme solutions were prepared in the presence of 2 mM ATP. The fractions of 26S complex (fraction 8) and 20S proteasome (fraction 11) were prepared as for Fig. 1A except that 4.0 mg of cell lysates were fractionated. Values are means of two independent determinations.

3.2. *ATP-dependent association of the proteasome with multiple components to form the 26S complex*

Eytan et al. [6] and Driscoll and Goldberg [7] independently reported that the 20S proteasome is a component of the larger 26S complex and that it is incorporated into the complex ATP-dependently. To confirm this in our system, we incubated intact HL-60 cells with 2-deoxyglucose and dinitrophenol to deplete them of intracellular ATP, and then prepared cell lysates in buffer without ATP. These ATP-depleted lysates were completely devoid of 26S complexes, as shown by glycerol density gradient centrifugation (unpublished data). When these lysates were incubated with ATP and

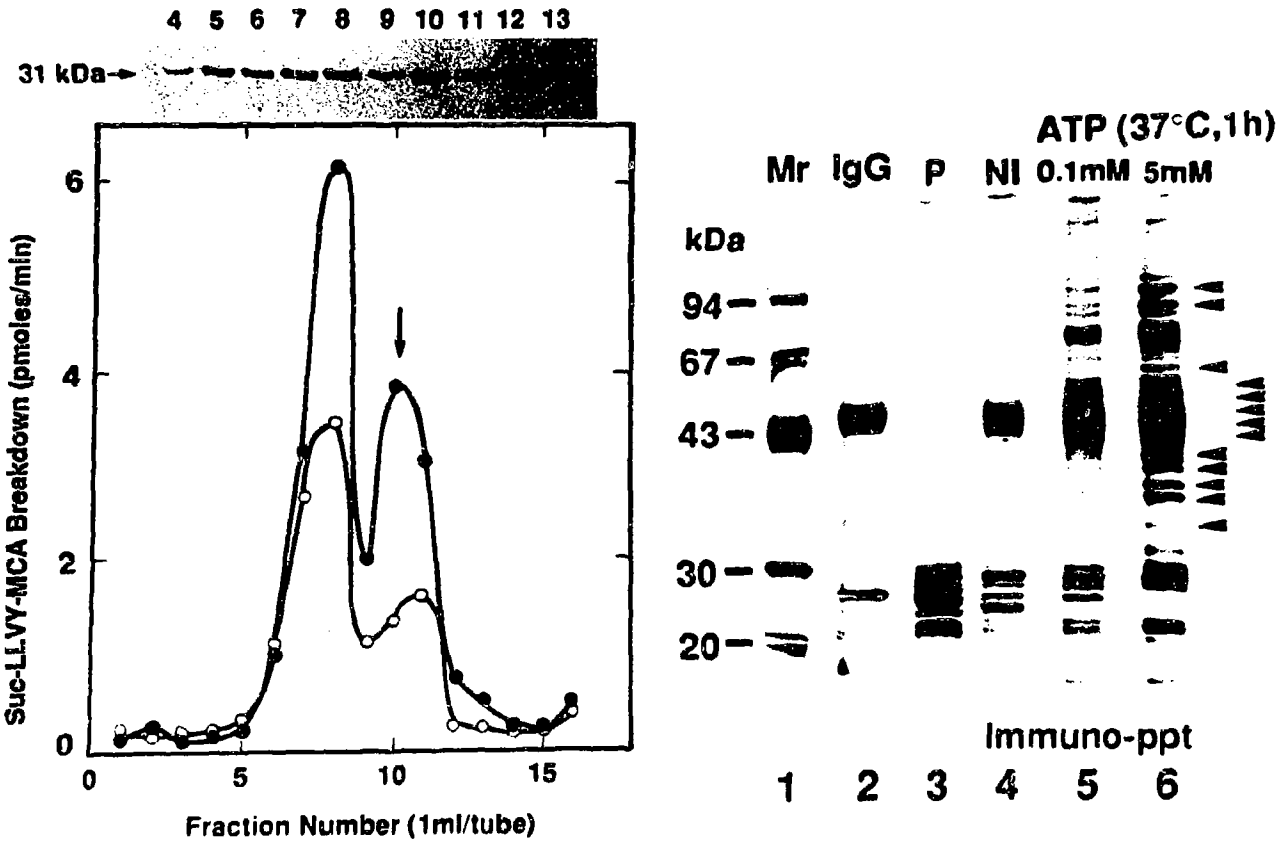

Fig. 3. ATP-dependent formation of active 26S complexes from latent proteasomes in ATP-depleted HL-60 cell lysates. For the left panel, washed intact HL-60 cells (5×10^8 cells) were resuspended in 20 mM Na-HEPES buffer (pH 7.2) containing 0.2 mM 2,4-dinitrophenol and 20 mM 2-deoxyglucose and incubated for 2 h at 37°C to deplete them of intracellular ATP. Then they were washed, resuspended in buffer A without ATP and lysed by sonication. The lysates were incubated for 60 min at 37°C in the presence of 2 mM ATP and 5 mM Mg^{2+} together with an ATP-regenerating system and analyzed by glycerol density gradient centrifugation as described in section 2. The procedures for assay of protease activity with (•) or without (○) SDS, and immuno-blot analysis were as for Fig. 2. The arrow indicates the position of elution of purified proteasomes. The right panel shows electrophoretic analysis of ATP-dependent association of proteasomes with multiple protein components to form 26S complexes. Lysates of the ATP-depleted cells described above were used. Immunoprecipitation experiments were carried out as for Fig. 2, using non-incubated lysates (NI, lane 4) or lysates after incubation with 0.1 mM (lane 5) or 5 mM (lane 6) ATP together with an ATP-regenerating system for 60 min at 37°C. Lane 1 (M_r, marker proteins), lane 2 (mAb 2-17 IgG, 1 μg), lane 3 (P, purified proteasomes, 1 μg). Arrowheads indicate various protein components co-precipitated with proteasomes by anti-proteasome mAb 2-17.

an ATP-regenerating system and then analyzed by glycerol density gradient centrifugation, the 26S fraction showed high Suc-LLVY-MCA hydrolyzing activity in the absence of SDS and the level of SDS-activated 20S proteasomes was decreased significantly (Fig. 3, left panel). This restoration of the level of large 26S complexes was confirmed by immuno-blot analysis (Fig. 3, left-upper panel). The sedimentation profile resembled that of a freshly prepared lysate with ATP (Fig. 1A), but a considerable amount of the 20S fraction still remained, and additional treatment with ATP did not cause any further shift of the 20S form to the 26S form, probably because of the presence of an excess amount of proteasomes over the other protein components of the 26S complex.

Finally, we confirmed that the 26S complex thus reformed by ATP treatment contained the multiple other protein components as well as proteasomes. As shown in Fig. 3 (right panel, lane 4), on SDS-PAGE, the precipitate obtained from the ATP-depleted lysate with anti-proteasome mAb 2-17 gave bands of only proteasome subunits and the added IgG. On the other hand, the immunoprecipitate from the lysate that had been incubated with ATP yielded multiple additional protein bands (Fig. 3, lane 6), the pattern being essentially similar to that seen in Fig. 2 (lane 6). We also found that larger amounts of these other protein components were immunoprecipitated after incubation with 5 mM ATP than after incubation with 0.1 mM ATP (Fig. 3, lanes 5 and 6). This difference suggests that a high ATP concentration is required for complete conversion of the 20S particle to the 26S complex or for stabilization of the active 26S complex.

4. DISCUSSION

The results reported above indicate clearly that ATP is required for the formation of a 26S protease complex

from a 20S latent proteasome and 13–15 additional proteins. This function of ATP appears to be essentially identical with its function in reticulocyte extracts proposed before [6,7]. Previous findings also showed that for this function Mg^{2+} is necessary and that ATP cannot be replaced by a non-hydrolyzable ATP analogue, indicating that ATP-hydrolysis, namely ATP-energy, is essential for assembly of this 26S complex. The reason why this energy is required is unknown. ATP also seems to be required for stabilization of the proteolytic activity, because ATP depletion causes dissociation of the 26S complexes with release of latent 20S proteasomes, and the decrease in Suc-LLVY-MCA hydrolyzing activity of the HL-60 cell lysate on aging was prevented by the nucleotide. Tsukahara et al. [11] reported a similar protective effect of ATP against the rapid inactivation of Suc-LLVY-MCA degrading activity in extracts of K562 erythroleukemia cells at alkaline pH. The 26S protease complex was precipitated with anti-proteasome mAb 2-17 only in the presence of ATP; in its absence, the multiple protein components were readily detached from the complex and the mAb prepicitated only the 20S proteasomes. As this 26S complex stabilizing effect did not require Mg^{2+}, it apparently did not require ATP-energy. A third role of ATP in proteasome-mediated proteolysis is to support the selective degradation of Ub-conjugated proteins catalyzed by the active 26S complex [2,3]. This process appears to require ATP-energy, because it requires Mg^{2+} and is not supported by ATP analogues. Recently, the purified 26S protease complex from mammalian cells has been shown to contain an ATPase essential for ATP-dependent breakdown of Ub-protein conjugates [12] and Ugai et al., in preparation. Thus the continuous requirement for ATP-hydrolysis coupled with protein breakdown in eukaryotes may be related to activation and inactivation through a resolution-association cycle of the 26S complex. This possible role of ATP is very attractive in considering the regulation of intracellular protein degradation, but further characterization of the 26S complex as an ATP-dependent protease is necessary.

We recently purified rat and human 26S complexes by conventional chromatographic techniques in the presence of ATP (manuscript in preparation) and found that the purified proteasomes contained a set of protein components closely resembling those identified by immunoprecipitation in the present study. As reported previously [4,5], a proteasome is a multi-subunit complex consisting of at least 13 non-identical components. But the 26S particle is even more complex in that it contains 13–15 additional protein components ranging in size from 35 to 110 kDa. In the 26S complex these protein components may regulate the proteolytic activity of the core-proteasome, but their precise physiological functions are still unknown.

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