Purification and Characterization of the 26 S Proteasome from Spinach Leaves*

(Received for publication, February 15, 1994, and in revised form, May 31, 1994)

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The 26 S proteasome complex catalyzing ATP-dependent breakdown of ubiquitin-ligated proteins was purified from spinach leaves to near homogeneity by chromatography on DEAE-cellulose, gel filtration on Biogel A-1.5, and glycerol density gradient centrifugation. The purified enzyme was shown to degrade multi-ubiquitinated, but not unmodified, lysozymes in an ATP-dependent fashion coupled with ATPase activity supplying energy for proteolysis and isopeptidase activity to generate free ubiquitin. By nondenaturing electrophoresis, the purified enzyme was separated into two distinct forms of the 26 S complex, named 26 Sα and 26 Sβ proteasomes, with different electrophoretic mobilities. The 26 S proteasome was found to consist of multiple polypeptides with molecular masses of 23–35 and 39–115 kDa, which were thought to be those of a 20 S proteasome with multicatalytic proteinase activity and an associated regulatory part with ATPase and deubiquitinating activities, respectively. The subunit multiplicity of the spinach 26 S proteasome closely resembled that of rat liver with minor differences in certain components. No sulfhydryl bond was involved in the assembly of this multicomponent polypeptide complex. Electron microscopy showed that the 26 S proteasome complex had a "caterpillar"-like shape, consisting of four central protein layers, assumed to be the 26 S proteasome, with asymmetric V-shaped layers at each end. These structural and functional characteristics of the spinach 26 S proteasome showed marked similarity to those of the mammalian 26 S proteasomes reported recently, suggesting that the 26 S proteasome is widely distributed in eukaryotic cells and is of general importance for catalyzing the soluble energy- and ubiquitin-dependent proteolytic pathway.

Two large multisubunit proteases are abundant constituents of the cytosol and nuclear compartments of eukaryotic cells (for reviews, see Hershko and Ciechanover (1992), Goldberg (1992), Tanaka et al. (1992), and Rechsteiner et al. (1993)). The smaller of the two is most often referred to as the multicatalytic proteinase complex (Orlowski, 1990; Rivett, 1993) or the 20 S proteasome (Goldberg, 1992; Tanaka et al., 1992). This 20 S proteasome has been isolated from a variety of organisms including higher plants (Ozaki et al., 1992; Skoda and Malek, 1992). The larger of the two, discovered by its ability to degrade ubiquitin (Ub)-lysozyme conjugates in an ATP-dependent fashion, is called the 26 S protease complex (Hough et al., 1987; Waxman et al., 1987) or the 26 S proteasome (Goldberg, 1992). The 20 S proteasome is known to associate with multiple protein components ATP-dependently to form the 26 S protease complex (Eytan et al., 1989; Driscoll and Goldberg, 1990; Orino et al., 1991; Kanayama et al., 1992). The abundance and wide distribution of proteasomes and their ability to degrade protein substrates ATP dependently suggest that they play an important role in nonlysosomal pathways of protein turnover (here and Hershko and Ciechanover, 1992). Proteasomes function in both ATP-dependent and ATP-independent pathways of protein breakdown, and their relative contributions to these two pathways seem to be determined by the relative proportions of the 20 and 26 S protease complexes (Hershko and Ciechanover, 1992; Goldberg, 1992; Tanaka et al., 1992; Rechsteiner et al., 1993). The 26 S proteasome is believed to be responsible for ATP-dependent degradation of Ub-protein conjugates (Hershko and Ciechanover, 1992; Rechsteiner et al., 1993), but it also appears to be involved in the Ub-independent degradation of certain proteins, such as ornithine decarboxylase, ATP dependently (Murakami et al., 1992). The existence of the 26 S proteasome in plants was suggested by the detection of ATP-dependent activities that degrade ubiquitinated proteins in vivo (Vierstra and Sullivan, 1988; Hatfield and Vierstra, 1989), but no 26 S complex has yet been isolated from plants. In this work, we purified a 26 S proteasome complex that rapidly degraded Ub-ligated proteins in an ATP-dependent manner from spinach leaves and examined some properties of an integral ATPase and a Ub-specific isopeptidase of this complex. We also examined the subunit structure of the purified complex and obtained direct evidence by electron microscopic and biochemical techniques that the 26 S proteasome from spinach leaves is a heterogeneous multipolypeptide complex, consisting of the 20 S proteasome (23–35 kDa) and a characteristic set of other nonidentical protein components (39–115 kDa). Finally, we analyze the gross structure of the 26 S proteasome by electron microscopy and discuss the similarity of its molecular properties with those of other eukaryotic 26 S proteasomes.

EXPERIMENTAL PROCEDURES

Materials—The materials used were obtained as follows: Ub (Sigma); six times crystallized egg white lysozyme (Seikagaku Kogyo Co., Tokyo, Japan); NaH14CO3 (3.7 GBq/mmol) and [γ-32P]ATP (925 GBq/mmol) (Amersham Corp.); succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LYVY-MCA, Peptide Institute, Minoh, Japan); DEAE-cellulose (DE52) (Whatman); Biogel A-1.5 m (Bio-Rad); and ATP (Oriental Yeast, Osaka, Japan).

*This work was supported in part by Grants-in-aid for Scientific Research (04304004 and 06454014) from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Plant Materials—Spinach (Spinacia oleracea L.) was obtained from a local market, and cells of the leaves were cultured under continuous white light on Murashige and Skoog’s medium as described (Nakagawa et al., 1985).

Assay of Peptidase Activity—The fluorogenic substrate Suc-LLVY-MCA was incubated with a test preparation for 10 min at 30 °C in the presence or absence of 0.02% SDS in 100 mM Tris-HCl (pH 8.0), as described (Tanaka et al., 1988). The reaction was stopped by the addition of 100 μl of 10% SDS and 2 ml of 100 mM Tris-HCl (pH 9.0), and the fluorescence of the reaction products was measured.

Assay of Protease Activity—About 10,000 cpm of 125I-lysozyme-Ub was diluted at 37 °C for 60 min in a total volume of 100 μl of reaction mixture consisting of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2 mM ATP and an ATP-regenerating system (10 μg/ml of creatine kinase and 10 mM phosphocreatine), 1 mM dithiothreitol, and a suitable amount of test preparation. Then, the conversion of 125I-lysozyme to acid-soluble fragments was measured as described (Kanayama et al., 1992). ATP-dependent breakdown of 125I-lysozyme-Ub conjugates was calculated as the difference between the activities with or without Mg²⁺. For exact measurement of the activity without Mg²⁺, which was usually less than 1.5%/hour, 5 μs EDTA was added to the assay mixture. The methods for preparation of radiolabeled and Ub-lysozyme conjugates were as described (Tamura et al., 1991).

Electrophoretic Analysis—Polyacrylamide gel electrophoresis (PAGE) was carried out in 2.5% polyacrylamide gel containing 0.5% agarose under nondenaturing conditions. SDS-PAGE was carried out by the method of Laemmli (Laemmli, 1970) in 10% slab gel. Protein was detected by staining with Coomassie Brilliant Blue. Low molecular weight marker proteins (Pharmacia Biotech Inc.) were used for SDS-PAGE.

Immunological Analysis—A polyclonal antibody against the spinach 20 S proteasome was prepared as described (Ozaki et al., 1992). Immunoelectrophoretic blot analysis was carried out by the method of Towbin et al. (1979). Samples were separated by PAGE and transferred electrophoretically to Immobilon polyvinylidene difluoride membranes (Millipore Corp.). Anti-rabbit IgG conjugated with alkaline phosphatase was used as a second antibody with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as a substrate of alkaline phosphatase.

Analysis of Cultured Spinach Cell Extract by Glycerol Density Gradient Centrifugation—Cultured spinach cells (1.0 g) were homogenized with buffer A consisting of 50 mM Tris-HCl (pH 7.5) containing 2 μg ATP, 5 μg MgCl₂, and 10 mM 2-mercaptoethanol or buffer B (buffer A without ATP). The homogenates were centrifuged at 15,000 × g for 30 min, and the resulting supernatants were used as crude cell extracts. These cell extracts (2 μg of protein) were loaded onto linear gradients of 20–40% (w/v) glycerol in buffer A or buffer B and centrifuged at 25,000 rpm for 22 h in a Hitachi SRP28SS1A rotor. They were then collected in 30 fractions of 1 ml each at 4 °C.

Electron Microscopy and Image Processing—Details of the methods used for electron microscopy were described elsewhere (Yoshimura et al., 1984). Briefly, the purified enzymes were negatively stained on grids with 2% uranyl acetate and examined in a Philips EM420 electron microscope at a magnification of ×36,000. Suitable areas of the micrographs were digitized with an EIKONIX microdensitometer. Approximately 450 images of the double-ended and of the one-ended form of the 26 S proteasome complex were extracted interactively. The whole data set was subjected to an eigenvector-eigenvalue classification procedure (Van Heel and Frank, 1981; Frank and Van Heel, 1982) as implemented in the EM and SEMPER program systems (Saxton et al., 1979; Hegerl and Altbauer, 1982) to detect significant interimage variations.

Other Biochemical Analyses—ATPase activity was assayed as described by Armon et al. (1990) with γ32P]ATP. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Proteolytic Properties of Cultured Spinach Cell Extracts Analyzed by Glycerol Density Gradient Centrifugation—To determine whether the 26 S proteasome exists in a higher plant, we analyzed a crude extract of cultured spinach cells by glycerol density gradient centrifugation. Samples of cultured spinach cells were homogenized in buffer A consisting of 50 mM Tris-HCl (pH 7.5), 10 μM 2-mercaptoethanol, 5 μM MgCl₂, and 2 μM ATP (panels a and A) or in buffer B (panels b and B). Samples of the crude extracts (2 mg of protein) were fractionated by glycerol density gradient centrifugation. Suc-LLVY-MCA breakdown with (C) or without (O) 0.02% SDS and ATP-dependent breakdown of 125I-lysozyme-Ub conjugates were measured in the presence (●) or absence (□) of MgCl₂ as described under “Experimental Procedures.” The upper panels show the results of immunoblot analysis with antibodies against the purified spinach 20 S proteasome (Ozaki et al., 1992). Upper panels a and b correspond to lower panels A and B, respectively. Numbers at the top correspond to fraction numbers in the lower panels. The proteins in 200 μl of the fractions were precipitated with acetone and subjected to immunoblot analysis. The upper right panels show the immunoblots with the purified spinach 20 S proteasome (5 μg of protein).

The enzyme that sedimented more rapidly seemed to be the 26 S proteasome, because, as we reported previously, the 26 S proteasome, but not the 20 S proteasome in various mammalian cells, has activity for Suc-LLVY-MCA degradation even without SDS (Orino et al., 1991; Kanayama et al., 1992; Ugai et al., 1993). To confirm this, we examined whether the enzyme in this fraction could degrade ubiquitinated proteins ATP-dependently. As shown in Fig. 1A, activity for ATP-dependent degradation of 125I-lysozyme-Ub conjugates was observed as a single symmetrical peak, coinciding with that of activity for degrading Suc-LLVY-MCA in the absence of SDS. No significant activity for degrading 125I-lysozyme-Ub conjugates was detected in the same fractions in the absence of Mg²⁺, indicating that the degradation was energy-dependent.

On the other hand, when the extract prepared without ATP was fractionated in a similar way, no appreciable activity for SDS-insensitive Suc-LLVY-MCA degradation was detected in any fraction, but, on the addition of 0.02% SDS, marked activity was detected in about fraction 20 (Fig. 1B). These results suggest that ATP-depletion caused dissociation of the 26 S pro-
teosome complex liberating a latent 20 S proteasome. Interestingly, the SDS-activated putative 20 S enzyme in Fig. 1A is larger than that in Fig. 1B, which is apparently the same size as the purified 20 S proteasome, suggesting that the material with latent activity seen in Fig. 1A consisted of the 20 S proteasome with additional associated components.

Next, by immunoblot analysis with antibodies against the 20 S proteasome (Ozaki et al., 1992) we examined whether the fractions of the 26 S proteasome and SDS-activated enzyme shown in Fig. 1A actually contained the 20 S proteasome. Polyclonal antibodies against the 20 S proteasome of spinach leaves reacted with several components in fractions 12-22, showing a similar pattern on immunostaining to that of the purified spinach 20 S proteasome (Fig. 1A), and, thus, confirming the presence of the 20 S proteasome in fractions 12-22. Furthermore, in the ATP-depleted extract, anti-20 S proteasome antibodies mainly reacted with proteins recovered in about fraction 20 (upper right panels). These proteolytic properties of the spinach cell extract resembled those in human promyelocytic leukemia HL-60 cells reported previously (Orino et al., 1991). These results indicate that the 26 S proteasome, which catalyzes ATP-dependent degradation of ubiquitinated proteins, is present in cultured spinach cells.

**Purification of the 26 S Proteasome from Spinach Leaves**—To purify the plant 26 S proteasome on a large scale, we used spinach leaves as an enzyme source. All purification procedures were performed at 4 °C with standard buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 2 mM ATP, 5 mM MgCl₂, and 20% glycerol unless otherwise specified. Fresh spinach leaves (100 g) were homogenized with 200 ml of standard buffer containing 5% (w/v) polyvinylpolypyrrolidone (Sigma) in a Waring blender. The homogenate was squeezed through cheesecloth, the filtrate was centrifuged at 10,000 × g for 30 min, and the resulting supernatant was used as crude extract. The crude extract was mixed with 100 ml of DE52-cellulose equilibrated with standard buffer without ATP and transferred to a column (2.2 x 25 cm). The column was washed with 2 bed volumes of the standard buffer, and then adsorbed materials were eluted with 400 ml of a linear gradient of 0-300 mM KCl in the same buffer. Fractions of 8 ml of eluate were collected. Proteins with activity to degrade Suc-LLVY-MCA without SDS were eluted with about 0.10 × KCl as a single symmetrical peak (data not shown). The active fraction from the DEAE-cellulose column was concentrated in a Centriprep-30 concentrator (Amicon Corp.) and applied to a Bio-Gel A-1.5-m column (2.5 x 100 cm) in standard buffer. Fractions of 3 ml of eluate were collected at a flow rate of 50 ml/h. A single peak of material showing Suc-LLVY-MCA-degrading activity was obtained in about fraction 50, at close to the void volume from the column (data not shown). The active fraction from the Bio-Gel A 1.5-m gel filtration column was dialyzed against standard buffer without glycerol and concentrated to 2.0 mg/ml in a Centriprep-30 concentrator. The concentrated sample (2 mg of protein) was loaded onto a linear gradient of 10-40% (w/v) glycerol in standard buffer without 20% glycerol. After centrifugation at 25,000 rpm for 22 h, the gradient was separated into 30 fractions of 1 ml, and various enzyme activities were assayed. As shown in Fig. 2, a single major peak of peptidase activity in the absence of SDS was eluted in about fraction 11, but whenSuc-LLVY-MCA degrading activity was assayed in the presence of 0.02% SDS, another small peak was observed in about fraction 17. The latter peak corresponded to that of the latent 20 S proteasome seen in Fig. 1A. Activity for the ATP-dependent degradation of 125I-lysozyme-Ub conjugates was also observed as a single peak, coinciding with that for Suc-LLVY-MCA-degrading activity in the absence of SDS, although a small shoulder of activity was observed in heavier fractions (Fig. 2B). As reported for the enzymes from human kidney (Kanayama et al., 1992) and rat liver (Ugai et al., 1993), the ATPase activity was observed at the same position as the peptidase activity (Fig. 2B). Pooled fractions 10-12 (about 600 µg of protein) were used for further characterization of the 26 S proteasome.

The purification method described above usually led to a 500-fold increase in specific Suc-LLVY-MCA-degrading activity in the absence of SDS (i.e. that of the 26 S enzyme) from the crude extract, and the overall yield was approximately 20%.

**Enzymatic Properties of the Purified 26 S Proteasome Complex**—For examination of the mechanism underlying the degradation of ubiquitinated proteins by the 26 S proteasome, changes in 125I-labeled lysozymes ligated with Ub were monitored electrophoretically. Incubation of 125I-lysozyme-Ub conjugates with the 26 S proteasome in the presence of Mg²⁺ and ATP resulted in a marked loss of material in bands of conjugates of larger sizes (Fig. 3A). The disappearance of these conjugates was not due to removal of the Ub moiety by deubiquitinating hydrolase (also called Ub isopeptidase) but to the degradation of the lysozyme by the 26 S proteasome, because no appreciable accumulation of free 125I-lysozymes was detected (Fig. 3, error). Without Mg²⁺, no significant degradation was observed (Fig. 3B). Moreover, unmodified 125I-lysozyme was not degraded appreciably when incubated with the 26 S proteasome in the presence of ATP-Mg²⁺ (data not shown). These results support the idea that the purified 26 S proteasome complex hydrolyzes Ub-ligated proteins ATP dependently.

It was of interest to examine whether Ub ligated with lysozyme was released as single molecules or as multi-Ub chains during proteolysis mediated by the 26 S proteasome. For this purpose, lysozyme ligated with 125I-Ub was treated with the 26
S proteasome; the profiles of various ubiquitinated proteins separated by SDS-PAGE were monitored by autoradiography (Fig. 4, A and B), and changes of intensity corresponding to free \(^{125}\text{I}-\text{Ub}\) migration were measured quantitatively with a Fuji BAS-2000 imaging analyzer (Fig. 4C). On incubation of lysozyme-\(^{125}\text{I}-\text{Ub}\) conjugates with the 26 S proteasome in the presence of Mg\(^{2+}\) and ATP, free \(^{125}\text{I}-\text{Ub}\) as well as intermediate bands of 25–35 kDa accumulated time dependently, indicating that the 26 S proteasome contains an enzyme with deubiquitinating activity. Moreover, in the absence of Mg\(^{2+}\), no accumulation of free \(^{125}\text{I}-\text{Ub}\) was observed (Fig. 4, B and C). These results show that the 26 S proteasome contains a Ub isopeptidase that is essential for ATP-dependent proteolysis.

Identification of Two Isoforms of the 26 S Proteasome Complex—Recently, we (Ugai et al., 1993) and Hoffman et al. (1992) reported that the 26 S proteasome in rat liver and rabbit reticulocytes is present as two isoforms, which can be separated electrophoretically. For this, we analyzed the purified spinach 26 S proteasome by nondenaturing PAGE. As shown in Fig. 5A, the final preparation of the purified 26 S proteasome gave two protein bands, both migrating slowly and being distinct from the band of the purified 20 S proteasome, which exhibited higher electrophoretic mobility. We examined whether these two bands actually contain the 20 S subunits of the purified 26 S proteasome by immunoelectrophoretic blot analysis. As shown in Fig. 5B, anti-20 S proteasomal antibodies reacted with both bands, indicating that these two slowly migrating bands contained the 20 S proteasome. We named these isoforms spinach 26 Sa and 26 Sβ proteasomes according to our nomenclature for the rat liver enzyme (Ugai et al., 1993). To determine whether these two isoforms have proteolytic function, after their separation by electrophoresis, the polyacrylamide gel was overlaid with a solution of a fluorogenic substrate (Suc-LLVTyr-MCA) and incubated for about 15 min at room temperature, and activity for peptide degradation was detected under ultraviolet light. Suc-LLVY-MCA degrading activity was detected in both these bands (Fig. 5C). Thus, spinach cells contain two active isoforms of the 26 S proteasome, 26 Sa and 26 Sβ.

Electrophoretic Analysis of the Subunit Structure of the Purified 26 S Proteasome Complex—Next, we compared the subunit structures of spinach and rat liver 26 S proteasomes. On SDS-PAGE analysis, the spinach 26 S proteasome gave multiple bands with molecular masses of 23–115 kDa (Fig. 6). Those with molecular masses of 23–35 kDa were assumed to be components of the 20 S multicatalytic portion, and those of 39–115 kDa were assumed to be components of the regulatory part. No disulfide bonds are involved in the subunit assembly because the electrophoretic pattern of the 26 S proteasome was similar in the presence and absence of 10 mM 2-mercaptoethanol, a sulphydryl-reducing reagent (data not shown). Comparison of the subunit compositions of the spinach 26 S proteasome with those of the rat enzyme (Fig. 6) suggested that these heterogeneous subunit structures are common to plants and mammals, although they showed species-specific differences in subunit multiplicity.

Shape of the 26 S Proteasome—The gross structure of the 26 S proteasome was examined by electron microscopy and digital image analysis. Most purified 26 S proteasomes negatively stained with uranyl acetate appeared to be dumbbell-shaped complexes (Fig. 7, upper panel), like those from Xenopus oocytes (Peters et al., 1991) and rat liver (Ikai et al., 1991). Besides these double-ended complexes, some one-ended complexes, which might be partially dissociated double-ended 26 S proteasome complexes, were also observed. Images of the double- and one-ended forms were extracted, aligned with regard to translation and orientation, classified to detect interimage variations, and averaged (Fig. 7, lower panel). Side views of both the single- and double-ended forms of the 26 S proteasome showed the typical 20 S proteasome with its four striations, representing four rings of subunits collectively forming a barrel-shaped structure (Hegerl et al., 1991). The masses attached to either one or both ends of the 20 S proteasome were highly asymmetric. The native double-ended spinach 26 S proteasome had a caterpillar-like structure, like the purified enzymes from Xenopus oocytes (Peters et al., 1993) and rat liver (Yoshimura et al., 1994) analyzed similarly by the digital image analysis.
**DISCUSSION**

There is increasing genetic evidence that multiple genes encoding proteins responsible for the Ub-ligation system are present in the higher plant *Arabidopsis* (for review, see Vierstra (1993)). Nevertheless, a 26 S proteasome catalyzing the ATP/Ub-dependent pathway in plant cells has not been reported, although we have reported that spinach leaves contain the 20 S proteasome (Ozaki et al., 1992). In the present study, we demonstrated a large proteolytic complex in a spinach cell extract that was stabilized by ATP and reacted with anti-20 S proteasome antibody (Fig. 1), suggesting that the 26 S proteasome does exist in plant cells. Furthermore, we purified this complex to high homogeneity from an extract of spinach leaves by a procedure involving column chromatography and glycerol gradient centrifugation. A unique feature of 26 S proteasomes prepared by the present method is that they can be separated into two forms (26 Sα and 26 Sβ) by disc electrophoresis in nondenaturing polyacrylamide gel (Fig. 5). Detailed analyses of the differences and biological significances of the two isoforms of the 26 S proteasome require examination. The 26 S proteasome has been found in various organisms such as humans (Kanayama et al., 1992), rats (Ugai et al., 1993), rabbits (Driscoll and Goldberg, 1990; Eytan et al., 1989; Hoffman et al., 1992), *Xenopus* (Peters et al., 1991), and *Drosophila* (Udvardy, 1993). Here we discuss the structure and functions of the spinach 26 S proteasome in comparison with those of 26 S proteasomes from these other species.

On SDS-PAGE analysis, the multiple subunits with molecular masses of 23–115 kDa of the 26 S complex from spinach leaves (Fig. 6) were found to closely resemble those of the complexes isolated from various other sources including mammals, amphibia, and insects. There is genetic evidence that all of the
genes involved in encoding 20 S proteasomes have been highly conserved during evolution (Tanaka et al., 1992). Moreover, most of the multiple distinct polypeptides ranging from 39 to over 115 kDa that are associated with the 20 S proteasome to form the 26 S proteasome (Ugai et al., 1993) are also similar in size to 26 S proteasomes from various sources (Fig. 6), indicating that the 26 S proteasome is a ubiquitous complex of multiple, nonidentical components. However the mechanism of assembly of the multiple proteasome subunits is unknown.

We found by electron microscopy in conjunction with digital image analysis that the "26 S" proteasome complex purified from spinach leaves is caterpillar-shaped with two large V- or U-like terminal complexes in opposite orientation attached to a smaller, four-layered central structure (Fig. 7). Similar ultrastructures of 26 S proteasome complexes from Xenopus and rat liver were reported by Peters et al. (1993) and Yoshimura et al. (1994), respectively. In previous studies, we showed that the 26 S proteasome from a higher plant (Ozaki et al., 1992) was similar in shape to various mammalian 20 S proteasomes (Tanaka et al., 1988). Thus, the gross structures of 20 and 26 S proteasomes have been conserved in eukaryotes during evolution, and the conserved configuration may indicate its critical role in the functions of this complex protease.

The purified 26 S proteasome complex showed similar enzymatic properties to the enzyme complexes from other sources. For instance, it rapidly degraded [35S]lysozyme-Ub conjugates in an ATP-dependent fashion (Figs. 2 and 3), suggested that covalent modification of substrate proteins through ubiquitination is essential for their proteolysis by the 26 S proteasome. Thus, the plant 26 S proteasome may recognize multiubiquitin chains bound to proteins for their specific breakdown. However, details of the mechanisms underlying these processes are unknown. In addition, it had intrinsic ATPase activity. This newly identified ATPase seems to be an essential component of the 26 S complex for catalyzing the ATP-dependent breakdown of Ub-conjugated proteins. Presumably the role of the ATPase is to supply energy continuously for the selective degradation of ubiquitinated proteins by the active 26 S complex. Moreover, recently multiple ATPase components were found to be associated with the 26 S proteasome, and these putative ATPases are suggested to operate as regulatory subunits by specifically interacting with different substrates (Rechsteiner et al., 1993). However, further characterization of the 26 S proteasome complex as an ATP-dependent protease is necessary to clarify the role of energy requirement at the molecular level. In the present work, we also found that the purified 26 S proteasome complex contains an isopeptidase whose activity is closely associated with the proteolytic reaction, as judged by the Mg<sup>2+</sup> requirement for its action (Fig. 4). Recently Ub-isopeptidase was reported to be associated with 26 S proteasomes from rabbit reticulocytes (Eytan et al., 1993) and rat liver (Ugai et al., 1993). Thus, this activity may also be common to all 26 S proteasomes.

A remarkable finding in this work was that the structure and properties of 26 S proteasomes are very similar in a plant to those in other organisms ranging from Drosophila to man. These facts indicate that the 26 S proteasome must have been conserved evolutionally and play important roles in living organisms, such as in cell cycle progression. Studies on the amino acid sequences of the subunits of plant proteasomes would provide information on the evolutionary tree of this complex.

REFERENCES


