Purification and Characterization of the 26S Proteasome Complex Catalyzing ATP-Dependent Breakdown of Ubiquitin-Ligated Proteins from Rat Liver

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An ATP/ubiquitin-dependent proteasome complex with an apparent sedimentation coefficient of 26S was purified from rat liver to near homogeneity by an improved method based on procedures reported previously. Two electrophoretically distinct forms of the 26S complex, named 26Sα and 26Sβ, with very similar subunit compositions were found not only in purified preparations but also in crude extracts, indicating that the 26S proteasome is present as two isoforms. The 26S proteasome was shown to degrade multi-ubiquitinated, but not unmodified, lysozymes in an ATP-dependent fashion, to have ATPase activity supplying energy for proteolysis, and to contain isopeptidase activity to generate free ubiquitin Mg2+/ATP-dependently. The 26S proteasome also catalyzed the ATP-independent hydrolyses of three types of fluorogenic peptides with basic, neutral, and acidic amino acids at their cleavage sites, respectively. These peptides are also good substrates for the 20S proteasome, but their degradation by the free 20S proteasome and by its assembled form in the 26S complex differ markedly, suggesting a functional difference between the two forms of proteasomes. Electrophoretic and immunochemical analyses showed that the large 26S complex was composed grossly of two different structures: a core 20S proteasome with multicatalytic proteinase functions and an associated part possibly with a regulatory role. These two structures both consisted of multiple polypeptides with molecular masses of 21-31 and 35-110 kDa, respectively. The subunit multiplicity of the rat 26S proteasome closely resembled that of the human counterpart, showing only minor species-specific differences in certain components. The assembly of this multi-component complex was found not to involve a sulfhydryl bond. Electrophoretic peptide mapping with lysyl-endopeptidase indicated the non-identity of the multiple subunits of the 26S proteasome. From these structural and functional characteristics, the 26S proteasome, which is widely distributed in mammals, is suggested to be a new type of multi-molecular complex catalyzing the soluble energy- and ubiquitin-dependent proteolytic pathway.

The extralysosomal, energy-dependent proteolytic pathway consists of two distinct, sequential steps, namely, ligation of ubiquitin (Ub) to target proteins and subsequent degradation of the ubiquitinated proteins, metabolic energy being required for both steps (1, 2). This Ub pathway appears to be involved in the selective removal of unnecessary proteins with a rapid turnover, such as cyclin (3) and c-Mos (4), which are closely related with cell cycle progression, and of abnormal proteins generated in cells (5). In the first step, Ub, a highly conserved small protein, is covalently attached to various target proteins to act as a signal for proteolytic attack through a reaction catalyzed by a multi-enzymatic system that is supposed to require ATP (6). The subsequent degradation of the Ub-ligated proteins was found to be catalyzed by a novel, large, ATP-dependent intracellular protease with an apparent sedimentation coefficient of 26S named the “26S proteolytic complex” (7, 8). Subsequently, Ganoth et al. (9) reported a multi-component proteolytic system catalyzing the ATP-dependent breakdown of ubiquitinated proteins that is similar to the 26S complex, and consisting of three independent factors named CF1, CF2, and CF3. Thereafter, CF3 was suggested to be identical to the 20S proteasome (10) and then the 20S proteasome was found to be incorporated into the 26S complex ATP-dependently as a “catalytic core” (11, 12). We recently examined the role of the proteasome in the ATP-, Ub-dependent proteolytic complex in human promyelocytic leukemia HL-60 cells, and showed directly by immunological analysis that the 20S proteasome (21-31 kDa) associates ATP-dependently and reversibly with many other proteins with molecular masses of 35-110 kDa.
to form the 26S complex that degrades ubiquitinated proteins (13). Thus, ATP-energy is required for the formation of this 26S complex, although the molecular mechanism involved in its formation remains unknown. These findings are consistent with reports of possible involvement of proteasomes in the ATP-

Assaying of Peptidase Activity—For assaying of the hydrolysis of the fluorogenic substrates, Suc-LLVY-MCA, Boc-LRR-MCA, and Cbz-LLL-βNA, these compounds were incubated with the enzyme for 10-60 min at 37°C in the presence or absence of 0.05% SDS in 100 mM Tris-HCl buffer (pH 8.0), as described before (26). The reaction was stopped by adding 100 µl of 10% SDS and 2 ml of 0.1 M Tris-HCl buffer (pH 9.0), and then the fluorescence of the reaction products was measured. For detection of protease on a non-denaturing polyacrylamide gel, samples were subjected to electrophoresis at 4°C. Then the gel was overlaid with a solution of 0.1 mM Suc-LLVY-MCA and incubated for 5-10 min at room temperature. Its fluorescence then examined under ultraviolet light.

Assaying of Protease Activity—For assaying the degradation of ubiquitinated proteins, samples of about 5,000-10,000 cpm of [125I]lysozyme-Ub were incubated at 37°C for 60-120 min in a total volume of 100 µl of a reaction mixture comprising 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2 mM ATP, and an ATP-regenerating system (10 µl/m of creatine kinase and 10 mM phosphocreatine), 1 mM dithiothreitol, and a suitable amount of the purified 26S enzyme. For assaying the degradation of [125I]lysozyme-Ub conjugates, we measured the conversion of [125I]lysozyme to acid-soluble fragments, as described before (25). 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% of the activity, EDTA was added to the assay mixture.

Lysozyme was iodinated with 125I-Na by the chloramine-T method. The specific activity of the iodinated lysozyme was about 1.5-3.0 x 10⁶ cpm/µg lysozyme. [125I]Lysozyme-Ub conjugates were prepared by ligating Ub with [125I]lysozyme in the presence of Ub-protein ligation enzymes, which were partially purified from a rabbit reticulocyte extract by Ube-Sepharose affinity chromatography, as described before (27). For some experiments, [125I]Ub (about 1.0-2.0 x 10⁶ cpm/µg Ub), which was iodinated with 125I-Na as described above, was used for the preparation of lysozyme-Ub conjugates. In this case, before use, the purified lysozyme-[125I]Ub conjugates were incubated with 5 mM N-ethyl-maleimide for 30 min at 37°C to inactivate Ub-protein ligation enzymes and contaminating isopeptidases completely. This was essential to prevent re-utilization of Ub formed during the assay. The resulting conjugates were concentrated to a 1/10th volume by ultrafiltration with an Amicon PM-30 membrane and then diluted with distilled water. For removal of free [125I]Ub, this operation was repeated 4-5 times.

MATERIALS AND METHODS

Materials—The compounds used were as follows: Ub and Cbz-LLL-βNA (Sigma Chem., St. Louis), [3,7 G Bq/m], and [2,8-³H]ATP (925 Bq/mm (Amersham), Suc-LLVY-MCA and Boc-LRR-MCA (Peptide Institute, Minoh), Biogel A-1.5 m, Biogel A-5 m, and hydroxyapatite HTP (Bio-Rad Industries), and PEI (polyethylenimine)-cellulose F-TLC plastic sheets (Merck, Darmstadt). ATP was kindly supplied by Oriental Yeast (Osaka).
mined (28). Another assay performed as described by Armon et al. (29) with \[\gamma-^{32}P\]ATP gave essentially the same results.

**Electrophoretic Analyses**—Electrophoretic analyses were carried out in 2.5% polyacrylamide (PAA) gels containing 0.5% agarose, or 3%, 4%, or 5% PAA gels under non-denaturing conditions. SDS-PAGE was carried out by the method of Laemmli in a 12.5% or 10–20% gradient slab gel (30). Peptide mapping of the purified enzyme was carried out by the two-dimensional electrophoretic method of Bordier and Crettol-Jarvinen (31). The multiple components of the enzyme were separated by SDS-PAGE in the 1st dimension. After electrophoresis, the gel was equilibrated with the stacking gel buffer and then transferred to a second slab gel (12.5% gel). The gel was overlaid with a solution of lysyl-endopeptidase (4 \(\mu\)g/ml) and the peptides produced were separated by SDS-PAGE in the 2nd dimension. Proteins were detected by staining with Coomassie Brilliant Blue or silver. Low molecular weight marker proteins (Pharmacia LKB Biotechnology) were used for SDS-PAGE. Various acetylated cytochromes c with pI values of 4.1, 4.9, 6.4, and 8.4 (Oriental Yeast, Osaka) were used as pI markers.

**Other Biochemical Analyses**—Immunoelectrophoretic blot analysis was carried out by the method of Towbin et al. (32). The method for raising antibodies against rat liver proteasomes in rabbits and their specificity were reported previously (26). Protein concentrations were measured by the method of Bradford (33) with bovine serum albumin as a standard. For glycerol density gradient centrifugation, samples of the enzyme preparation (1–5 mg protein) were loaded onto a linear gradient of 10–40% (v/v) glycerol in the standard buffer (see text). After centrifugation at 25,000 rpm for 22 h in a Hitachi SRP28SA1 rotor, the gradient was separated into 30 fractions of 1 ml each at 4°C.

**RESULTS**

**Purification of the 26S Proteasome from Rat Liver by an Improved Method**—All purification procedures were performed at 4°C. "Standard buffer," consisting of 25 mM Tris-HCl buffer (pH 7.5), 1 mM dithiothreitol, 2 mM ATP, and 20% glycerol, was used unless otherwise specified. ATP (2 mM) and glycerol (20%) were added to all solutions used, because they stabilized the 26S proteasome complex, as reported previously (25).

**Step 1: Precipitation of the 26S proteasome complex by ultracentrifugation**—Male Wistar rats, weighing 300 g, were fed ad libitum on laboratory chow. Approximately 500 g specimens of liver from these animals were perfused with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 2 mM ATP, and 0.25 M sucrose, and then homogenized in 3 volumes of the same buffer in a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 1 h at 30,000 rpm in a Hitachi RP42 rotor (average, 70,100 \(\times\) g) and the resulting supernatant containing about 3–4 g of protein was used as the starting material. Recentrifugation of the crude extract for 5 h at 70,100 \(\times\) g precipitated the 26S complex almost completely. The precipitate was dissolved in a suitable volume (approximately 50 ml) of the standard buffer and then the solubilized extract was centrifuged at 20,000 \(\times\) g for 30 min to remove insoluble material. Approximately 1.5 g of protein was recovered at this step. This preparation showed high ATP-dependent activity as to the breakdown of \([^{125}I]\)lysozyme-ligated to Ub (approximately 4.3 ng/h/mg protein). This activity was stable during storage of the crude precipitate at −70°C for Fig. 1. Separation of 26S-proteasome complexes and 20S-proteasomes by molecular sieve chromatography. A: Chromatography on a Biogel A-1.5m column. B and C: Chromatography on a Biogel A-5m column. Pooled fractions of 26S complexes from the Biogel A-1.5m column were concentrated to about 50 ml by ultrafiltration on an Amicon PM-10 membrane and then applied to the column. The bar indicates the fractions of 26S complexes pooled for further purification. The procedure was as described in the text. Aliquots (10–20 \(\mu\)l) of individual fractions were used for measuring various enzyme activities. Suc-LVY-MCA breakdown with (●) or without (○) 0.05% SDS, ATPase activity (+), protein (−), and ATP-dependent breakdown of \([^{125}I]\)lysozyme-ubiquitin conjugates (□) were measured as described under "MATERIALS AND METHODS."
Fig. 2. Fractionation of 26S-proteasomes by glycerol density gradient centrifugation. Samples of 2 mg of protein were used for this analysis. The details of the procedure are given in the text. Aliquots (20 µl) of individual fractions were used for measuring various enzyme activities. The symbols are as for Fig. 1. A: Suc-LLVY-MCA breakdown with (●) or without (○) 0.05% SDS, and protein (−). B: ATPase activity (x) and ATP-dependent breakdown of [125I]lysozyme-ubiquitin conjugates (○). The upper panel shows the electrophoretic pattern of the fractions on SDS-PAGE. The numbers above the panels are the fraction numbers. Marker proteins of known molecular weight (Pharmacia LKB Biotechnology) were run in the left lane. Proteins were detected by silver-staining. The bar indicates the fractions pooled.

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Fig. 3. Electrophoretic and immunological identification of two types of 26S-proteasome complexes containing the 20S proteasome. Samples (2.5 mg protein) were analyzed by velocity-gradient centrifugation as for Fig. 2. A: Suc-LLVY-MCA breakdown with (●) or without (○) 0.05% SDS. B: Peptidase activity on a 2.5% polyacrylamide gel–0.5% agarose gel. Aliquots of fractions (30 µl) were subjected to electrophoresis, and the Suc-LLVY-MCA degrading proteases in the gel were assayed as described under "MATERIALS AND METHODS." The right arrow, labeled 20S, indicates the position of the purified 20S-proteasome. The left arrows, labeled 26Sα and 26Sβ, show the positions of the isoforms of the 26S complex (see text). The numbers are the fraction numbers, corresponding to those in Fig. 2. C: Immunoblot analysis with anti-proteasomal polyclonal antibodies. The same gel as for panel A was used for immunoblotting. The arrows are the same as for panel A. The fractions analyzed are connected by lines.

at least 6 months.

Step 2: Molecular sieve chromatographies on Biogel A-1.5 m and A-5 m columns: Samples (500–600 mg protein) of the preparation obtained on ultracentrifugation were applied to a Biogel A-1.5 m column (5 x 100 cm), as described before (25). Fractions of 10 ml were collected and various enzyme activities in the fractions were assayed. As
Fig. 4. Electrophoretic analysis of the 26S\textsubscript{α} and 26S\textsubscript{β} isoforms in polyacrylamide gels of different concentrations. Gels containing 3% (A), 4% (B), and 5% (C) polyacrylamide were used for non-denaturing electrophoresis. The purified 20S-proteasome fraction (10 μg), a crude extract (CE) obtained on ultracentrifugation of a liver homogenate (200 μg), and the 26S-proteasome fraction separated by velocity-gradient centrifugation (3.5 μg) were used. Suc-LLVY-MCA degradation was measured as described under "MATERIALS AND METHODS."

TABLE I. Effect of ubiquitination on the degradation of heat-denatured [\textsuperscript{125}I]lysozyme by various proteases. The molecular weights of the proteases were as follows. 20S proteasome (750 kDa), 26S proteasome (2,000 kDa), trypsin (23.3 kDa), and α-chymotrypsin (25.3 kDa). One unit of protease activity was defined as that degrading 1 pmol of lysozyme to acid-soluble fragments per 1 h. Reaction mixtures were as described under "MATERIALS AND METHODS."

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mg\textsuperscript{2+} (5 mM)</th>
<th>Hydrolysis (units/nmol)</th>
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<tr>
<td></td>
<td></td>
<td>[\textsuperscript{125}I]Lysozyme</td>
</tr>
<tr>
<td>20S proteasome</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>26S proteasome</td>
<td>4</td>
<td>8</td>
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<td></td>
<td></td>
<td>59</td>
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<tr>
<td>Trypsin</td>
<td>-</td>
<td>273</td>
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<tr>
<td>α-chymotrypsin</td>
<td>-</td>
<td>482</td>
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degrading [\textsuperscript{125}I]lysozyme-Ub conjugates ATP-dependently (data not shown). Fractions of the 26S complex (shown by the bar) containing 90–120 mg of protein were pooled.

The 26S proteasomes were eluted close to the void volume from the Biogel A-1.5 m column and appeared to be contaminated with materials eluted in the void volume. For removal of these contaminants, the pooled material (about 300 mg of protein) from the Biogel A-1.5 m column was concentrated to approximately 50 ml by ultrafiltration with an Amicon PM-10 membrane, and then fractionated on a Biogel A-5 m column (5 × 100 cm) under the same conditions as for Biogel A-1.5 m column chromatography. As shown in Fig. 1B, most contaminating materials were recovered in the void volume and thus separated from the 26S proteasomes with high SDS-independent Suc-LLVY-MCA degrading activity. Most of the ATP-dependent activity as to the degradation of [\textsuperscript{125}I]lysozyme-Ub conjugates was consistently eluted with a SDS-insensitive peptidase, although a small amount of activity was also found in the 20S fractions (Fig. 1C). The fractions exhibiting high activity were pooled (about 100 mg of protein) for the next purification step.

We measured the ATPase activities of the fractions, because ATP hydrolysis is known to be required for the degradation of [\textsuperscript{125}I]lysozyme-Ub conjugates (7, 8), and because an ATPase was found to be associated with the 26S proteasomes from rabbit reticulocytes (29) and human kidney (25). As shown in Fig. 1A, most of the ATPase activity was found in the void volume fractions, with some activity in the 20S and 26S fractions, giving a small shoulder in the eluate from the first Biogel A-1.5 m column. The ATPase activity eluted in the void volume fractions

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may be due to contaminating membranous fragments. In the second molecular sieve chromatography on a Biogel A-5m column, significant ATPase activity was observed in fractions containing 26S proteasomes, although it still overlapped that of some unidentified, possibly contaminating ATPase(s) (Fig. 1C).

**Step 3: Flow-through fraction on hydroxyapatite chromatography:** ATP was added, at a final concentration of 5 mM, to the pooled fraction corresponding to the 26S complex from the Biogel A-5 m column (approximately 100 mg of protein), and a sample was applied directly to a hydroxyapatite column of 55 ml bed volume that had been equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM dithiothreitol, 5 mM ATP, and 20% glycerol. The 26S proteasomes were recovered in the flow-through fraction, because they did not associate with this column in the presence of 5 mM ATP. Approximately 70% of the proteins bound to the hydroxyapatite resin. Most of the free 20S proteasomes associated tightly with the hydroxyapatite resin and thus were separated from the 26S proteasomes. Approximately 30 mg of protein was obtained in this way.

**Step 4: Q-Sepharose chromatography:** The active fractions corresponding to the 26S proteasomes from the hydroxyapatite column were applied on a Q-Sepharose column (2.2 × 15 cm) that had been equilibrated with the standard buffer without ATP and washed with 1 bed volume of the same buffer containing 2 mM ATP. The column was washed with 5 bed volumes of the standard buffer, and then adsorbed materials were eluted with 300 ml of a linear gradient of 0–0.8 M NaCl in the same buffer and fractions of 3.0 ml of eluate were collected. Proteins exhibiting the ability to degrade Suc-LLVY-MCA with or without SDS were eluted as a single symmetrical peak. This chromatographic profile is not shown, because it was essentially the same as that of the activity from human kidney described previously (25). As reported for the enzyme from human kidney (5), the ATPase activity and the ATP-dependent activity as to the degradation of [125I]-lysozyme-Ub conjugates were observed at the same position as the peptidase activity and eluted as similar symmetrical peaks (data not shown), suggesting the specific association of ATPase with the 26S enzyme complex. Approximately 15 mg of protein was collected in the fractions exhibiting high activity.

**Step 5. Fractionation by glycerol density gradient centrifugation:** The 26S proteasome fractions obtained on Q-Sepharose chromatography were concentrated to 2.0 mg/ml on ultrafiltration with an Amicon PM-10 membrane, and then subjected to glycerol density-gradient centrifugation. As shown in Fig. 2A, a single major peak of peptidase activity, in the absence or presence of SDS, was eluted around fraction #15, but when the activity was assayed with 0.05% SDS, another small peak was observed around
fraction #20. The latter peak corresponded to the elution position of the 20S proteasomes (data not shown). The ATPase activity was observed at the same position as the peptidase activity (Fig. 2B). Activity as to the ATP-dependent degradation of [125I]lysozyme-Ub conjugates was also observed as a single symmetrical peak, coinciding in position with the ATPase and peptidase activities in the absence of SDS. No significant [125I]lysozyme-Ub conjugate degrading activity was detected in fractions of 20S proteasomes. Pooled fractions #12-16 (about 550 μg of protein) were used for further characterization of the 26S proteasome complex.

We examined the purity of the enzyme by SDS-PAGE (Fig. 2, upper panel). After denaturation with 1% SDS, the proteins in each fraction obtained on Q-Sepharose chromatography gave multiple bands corresponding to molecular masses of 20 kDa to over 150 kDa on electrophoresis. The apparent intensities of these multiple components in fractions around #15 were consistent with the enzyme activity profile. In this work we used preparations obtained by velocity-gradient centrifugation for enzymatic and structural analyses of the 26S proteasome. In the presence of ATP and glycerol, the purified enzyme could be stored at -70°C for several months without significant loss of activity.

Identification of Two Isoforms of the 26S Proteasome Complex, 26S L and 26S S—As can be seen in Fig. 2 (upper panel), gradient SDS-PAGE revealed that the purified rat liver 26S complex contained multiple components, with a wide range of molecular masses, from 35 kDa to over 150 kDa, as well as components of 21-31 kDa with similar sizes to those of subunits of the 20S proteasome. Thus, the 26S complex from rat liver was concluded to consist of a 20S proteasome and other proteins. To distinguish the 20S proteasome more clearly from the 26S complex, we examined the electrophoretic separation of the two by non-denaturing PAGE. Figure 3A shows the profile of Suc-LLVY-MCA degrading enzyme activity on glycerol density gradient centrifugation. This profile is essentially the same as that in Fig. 2, although it was obtained with a different enzyme preparation. After electrophoresis, the polyacrylamide gel was overlaid with a solution of a fluorogenic substrate (Suc-LLVY-MCA) and then incubated for 5-10 min at room temperature. The activities as to peptide degradation were then detected under ultraviolet light. As shown in Fig. 3B, two bands with strong peptidase activity were observed, their intensities being in good agreement with the elution profiles of peptidase activities shown in Fig. 3A. The purified 20S proteasome exhibited higher electrophoretic mobility than the materials in these two bands (data not shown), migrating to the position shown by the arrow on the right in Fig. 3B, which corresponded to the positions of the weak bands observed for fractions #19-21, when the reaction proceeded for a long time. These results...
suggest that the former two bands were distinct from those of the 20S proteasome. The apparent fluorescence intensities of these two bands were similar, but the catalytic activities of the enzymes in these two bands were not examined in detail. Subsequently, we examined whether these two bands contain the 20S proteasome by immunoelectrophoretic blot analysis of the same gel. As shown in Fig. 3C, anti-proteasomal antibodies reacted with both bands. On analysis of the native gel, the fluorescence intensity, that is the peptidase activity, was apparently similar to the immunostaining intensity. Thus we concluded that these two bands represent isoforms of the 26S complex, and named them 26Sα and 26Sβ in order of their electrophoretic mobility. Importantly, on velocity-gradient centrifugation, the peaks of 26Sα and 26Sβ, but not that of the free 20S proteasome, were observed, but on SDS-PAGE analysis the proteasome components in all these fractions were detected (see, Fig. 2A), suggesting that a 20S proteasome is incorporated into the 26S complex. It is noteworthy that the proteasomes in fractions #19–21 were stained immunochemically (Fig. 3C), but that their peptidase activity was quite low (Fig. 3B). This may have been because the 20S proteasome has latent activity, as shown in Fig. 3A. In fact, the addition of 0.05% SDS resulted in slight increases in the fluorescence intensities of the proteasomal bands. The increases may have been only slight because activation of the latent proteasome in gel may be less than that in solution, as in Fig. 3A.
We compared the subunit structures of the two isoforms of the 26S complex. For this purpose, the bands of 26Sα and 26Sβ showing Suc-LLVY-MCA degrading activity were excised after non-denaturing PAGE, and the proteins extracted from the two bands detected under ultraviolet light were again subjected to SDS-PAGE. The 26Sα and 26Sβ complexes were found to consist of similar subunits of 21 to 150 kDa (data not shown). Further studies are required to clarify the exact structures of these two isoforms.

As these two bands were apparently similar in all fractions obtained on velocity-gradient centrifugation, the possibility that 26Sβ with higher electrophoretic mobility was formed through dissociation of components from the 26Sα component with lower mobility during electrophoresis cannot be ruled out. We examined whether these two bands were generated artificially during purification of the 26S enzyme complex. For this, the crude precipitate obtained on ultracentrifugation at 70,100 × g for 5 h was subjected directly to non-denaturing gel electrophoresis in 3, 4, and 5% polyacrylamide gels, and the peptidase activities in the gels were assayed as described in Fig 3B. As shown in Fig. 4, two bands with similar electrophoretic mobilities to those of the purified 26S complex were observed in the 3 and 4% gels, but the 26Sα and 26Sβ complexes did not enter the 5% gel. In contrast, the purified 20S proteosome could enter all these gels, but its electrophoretic mobility depended on the gel concentration, being less in gels with higher concentrations of polyacrylamide. Moreover, similar analysis of a crude cytoplasmic extract.

Fig. 11. Effects of temperature on activities as to the peptide degradation of 20S- and 26S-proteasomes. Samples of the 20S-proteasomes (0.3 μg) and 26S-proteasomes (1.0 μg) were incubated for 10 min with various fluorogenic substrates at the indicated temperatures with (closed circles) or without (open circles) 0.05% SDS. Values are expressed as percentages of the maximum activity. Upper panels, 20S-proteasomes; lower panels, 26S-proteasomes.

Fig. 12. Heat stability of peptide degradation activities of 20S and 26S-proteasomes. Samples of the 20S-proteasomes (0.3 μg) and 26S-proteasomes (1.0 μg) were incubated for 30 min at the indicated temperatures, and then chilled on ice. Enzyme activities were assayed at 37°C with various fluorogenic substrates in the presence (closed circles) or absence (open circles) of 0.05% SDS. Values are expressed as percentages of the maximum activity. Upper panels, 20S-proteasomes; lower panels, 26S-proteasomes.
also gave the three bands of the free 20S proteasome, and 26Sα and 26Sβ complexes, consistent with the idea that the 26Sα and 26Sβ forms of the 26S complex are native ones. These two isoforms of the 26S complexes were also found in enzyme preparations from human kidney (data not shown) and so may be common in mammals.

**Characteristics of the Degradation of Ubiquitinated Lysozyme by the 26S Proteasome**—To clarify the enzymatic function of the purified 26S proteasome, we examined its effect on the degradation of ubiquitinated [125I]lysozyme with those of various other known proteases. As shown in Table I, the 26S proteasome degraded ubiquitinated lysozyme, but not unmodified lysozyme, and the degradation was Mg2+-dependent, suggesting the requirement of ATP hydrolysis for this activity. Treatment with the purified 20S proteasome did not cause significant breakdown of these proteins, even in the presence of Mg2+-ATP. Trypsin and α-chymotrypsin, respectively, degraded ubiquitinated and unmodified [125I]lysozymes equally well, and their activities were not dependent on Mg2+. These results suggest that the 26S proteasome specifically recognizes ubiquitinated lysozyme for degradation of the lysozyme moiety.

For examination of the mechanism underlying the degradation of ubiquitinated proteins by the 26S proteasome, changes in 125I-labeled lysozymes ligated with Ub were monitored electrophoretically. As shown in Fig. 5, on incubation of [125I]lysozyme-Ub conjugates with the respective fractions obtained on glycerol density gradient centrifugation in the presence of Mg2+-ATP, marked loss of material in bands of conjugates of larger size was observed (panel B), which apparently coincided with the protease activity monitored as to the conversion of ubiquitinated [125I]lysozyme to acid-soluble fragments (panel A). The disappearance of these conjugates was not due to removal of Ub by contaminating Ub-protein hydrolase (also called Ub isopeptidase) (34), but to the degradation of the lysozyme moiety by the 26S complex, because no accumulation of free [125I]lysozyme was detected (Fig. 5B, arrow). Moreover, without Mg2+, no significant degradation was observed, as can be seen in Fig. 5C. These results support the idea that the purified 26S proteasome complex hydrolyzes Ub-ligated proteins ATP-dependently.

It was of interest to examine whether Ub moieties ligated with lysozyme are generated singly or as multi-Ub chains during proteolysis mediated by the 26S proteasome. For this purpose, lysozymes ligated with [125I]Ub were prepared as described under "MATERIALS AND METHODS," and treated with the 26S proteasome in the fractions shown in Fig. 5. Then the profiles of various ubiquitinated proteins separated by SDS-PAGE were monitored autoradiographically (Fig. 6, A and B), and the changes of their intensities were measured quantitatively with a Fuji BAS-2000 imaging analyzer (Fig. 6, C–E). When these lysozyme-[125I]Ub conjugates were incubated with fractions containing the 26S proteasome, considerable amounts of poly-ubiquitinated lysozymes (≥70 kDa) were deubiquitinated (panel C), and conjugates of 70 to 15 kDa (panel D) and free [125I]Ub (panel E) accumulated markedly, suggesting that the purified 26S proteasome complex contains an Ub isopeptidase and that fragmented lysozymes with several Ubs accumulated. It should be noted that the lysozyme-[125I]Ub conjugates used had no isopeptidase activity, because they were pretreated with 5 mM N-ethylmaleimide to prevent deubiquitination by contaminating isopeptidases. Interestingly, in the absence of Mg2+, no changes of ubiquitinated proteins or accumulation of free [125I]Ub through deubiquitination was observed (Fig. 6B), suggesting that deubiquitination is coupled with proteolysis, and possibly that
deubiquitination promoted substantial breakdown of [125I]lysozyme ligated with Ub. As the 26S complex did not degrade Ub, free ubiquitin released during proteolysis may be reutilized in this proteolytic system. To confirm that the 26S proteasome contains the co-purified isopeptidase intrinsically, we immunoprecipitated the 26S complex with 20S proteasome-specific antibodies. As shown in Fig. 7, immunoprecipitation of the 26S proteasome caused the almost complete loss of free Ub production, which was closely related with the decrease in proteasomal peptidase activity. These results show that the 26S proteasome contains an Ub isopeptidase that is essential for ATP-dependent proteolysis.

As the ATPase was co-purified with the 26S complex (Fig. 2B), we examined the role of the intrinsic ATPase in the purified 26S proteasomes in the ATP-dependent breakdown of ubiquitinated lysozyme. The optimal pH for the degradation of both ATP and [125I]lysozyme-Ub conjugates was pH 8.0-9.0 (Fig. 8). Moreover, the Km for ATP cleavage by the purified enzymes was 0.1-0.25 mM, which is similar to the value for the ATP-dependent degradation of [125I]lysozyme-Ub conjugates. The 26S proteasomes cleaved various other nucleotides besides ATP, such as dATP, CTP, GTP, and UTP, which also supports the breakdown of ubiquitinated proteins (data not shown). Vandate and quercetin, which are known to be ATPase inhibitors, greatly inhibited not only ATP cleavage but also ATP-dependent breakdown of Ub-ligated proteins. Hemin and N-ethylmaleimide also markedly inhibited both the ATPase and the activities as to the degradation of conjugates (data not shown). These results show that the function of ATPase is closely linked to the ATP-dependent breakdown of ubiquitinated proteins.

Comparison of Peptidase Functions of the 20S Proteasome and Its Assembled Form in the 26S Complex—The 20S proteasome has been reported to have multifunctional catalytic sites responsible for the endoproteolytic cleavage of peptide bonds on the carboxyl side of basic, neutral and hydrophobic, and acidic amino acid residues of proteins, and these catalytic properties have been classified as trypsin-like, chymotrypsin-like, and "glutamyl-site" functions, respectively (19, 20). The 20S proteasome is normally latent and does not degrade casein or Suc-LLVY-MCA appreciably, but it is greatly activated in various ways that induce conformational changes, such as on treatment with poly-lysine, SDS or fatty acids (20, 35, 36). As no detailed studies on the breakdown of various chromogenic peptides with the 26S proteasome have been reported, we compared the proteolytic properties of synthetic peptides by the free 20S proteasome and its assembled form in the 26S proteasome using Boc-LRR-MCA, Suc-LLVY-MCA, and Cbz-LLE-βNA as typical fluorogenic substrates.

**Effect of SDS:** As shown in Fig. 9 (upper panel), the 20S proteasome had latent activity as to the degradation of Suc-LLVY-MCA and Cbz-LLE-βNA, and these latent activities were strongly enhanced by SDS. SDS caused maximal activation at about 0.05%, higher concentrations resulting in losses of these activities, probably due to denaturation of the enzyme. In contrast, the activity as to the degradation of Boc-LRR-MCA was inhibited concentration-dependently by SDS. We next examined the effect of SDS on the activities of the 26S proteasome. SDS had a complex effect on the activity as to the degradation of Suc-LLVY-MCA (Fig. 9, lower panel): the activity was high without SDS, was strongly inhibited with about 0.01% SDS, and was restored to the original level with a further increase in the SDS concentration to 0.05%. The profile of the latter activation was similar to that of the free 20S proteasome. One possible explanation for this phenomenon is that 0.01% SDS dissociated the latent 20S proteasome from the active 26S complex, and the further addition of SDS to 0.05% SDS activated the released 20S proteasome. A similar dual effect of SDS on the chymotrypsin-like activity was observed in a crude extract of rat liver (data not shown), suggesting that this effect was not due to an artifact formed during enzyme purification. We also observed a dual effect of SDS on the peptidylglutamyl-peptide (Cbz-LLE-βNA) hydrolyzing activity, but the activity with 0.05% SDS was much higher than that without SDS. Like the 20S proteasome, the 26S complex degraded a trypsin-like substrate, and SDS suppressed this activity dose-dependently, although this inhibition was stronger than that with the 20S proteasome. SDS inhibited the activities of both intrinsic ATPase and ATP-dependent breakdown of ubiquitinated lysozyme concentration-dependently (data not shown). These results suggest that the assembly of the 20S proteasome with multiple components into the 26S complex greatly affects its proteolytic properties.

**Effect of pH:** In studies on the effects of pH, we found that all the peptide degrading activities of 20S and 26S proteasomes were maximal at neutral and weakly alkaline pH values (Fig. 10). The degradation of Suc-LLVY-MCA by both types of proteasomes was maximal at about pH 8, but the mode of degradation differed. With the 26S complex, SDS somewhat increased the activity below pH 8.0, but had no significant effect above pH 8.0 (Fig. 10, lower left panel), whereas with the 20S proteasome, SDS caused strong activation in the broad pH range examined (Fig. 10, upper left panel). The pH-profiles of the two enzymes for the degradation of Cbz-LLE-βNA and Boc-LRR-MCA were similar, but 0.05% SDS caused marked activation of hydrolysis of Cbz-LLE-βNA, whereas it inhibited the degradation of Boc-LRR-MCA (Fig. 10, middle and right panels, respectively).

**Effect of temperature:** For further characterization of the differences between the catalytic functions of 20S and 26S proteasomes, we examined the effects of temperature on their activities and stabilities. As shown in Fig. 11 (upper panels), all three activities of 20S proteasomes examined were maximal at about 40°C. However, the activity of the 26S complex as to the degradation of Suc-LLVY-MCA was unstable above 40°C, but maximal at 50°C in the presence of 0.05% SDS (Fig. 11, lower left panel). In contrast, the effects of temperature on the degradations of Cbz-LLE-βNA and Boc-LRR-MCA by the 26S complex and the 20S proteasome were similar (Fig. 11, middle and right panels). These results suggest that the chymotrypsin-like activity is more stable in the 26S than in the 20S proteasome.

To clarify this phenomenon, we examined the effects of temperature on the stabilities of 20S and 26S proteasomes. Preincubation of the 20S proteasome at various temperatures revealed the similar instabilities of the three types of peptidase activities at above 30°C. Active degradation of Suc-LLVY-MCA by the 26S complex also decreased above
30°C, but this did not represent irreversible inactivation of the catalytic site, because the addition of 0.05% SDS resulted in dramatic restoration of the full activity, even at 50°C (Fig. 12, left panel). This increase was not due to dissociation of the 20S proteasome from the 26S complex, because the free 20S proteasome is unstable at high temperature. The 26S complex also exhibited high heat-stabilities as to the degradation of Cbz-LLE-βNA and Boc-LRR-MCA (Fig. 12, middle and right panels). These results indicate that the catalytic states of the proteasome in its free form and in its form assembled into the 26S complex are different. The results also suggest that the 20S proteasome interacts with other protein factors in the 26S complex. This is consistent with the results of structural analyses of the purified enzymes obtained in this study.

**Electrophoretic Analysis of the Subunit Structure of the Purified 26S Multi-Polypeptide Complex**—To determine the molecular composition of the purified 26S proteasome complex, we examined the structure of the enzyme by electrophoresis. The electrophoretic patterns of rat and human 20S proteasomes showed minor species-specific differences in subunit multiplicity (26). These differences were confirmed by analyses of their cDNA structures (21). Here, we compared the subunit structures of the 26S complexes from man and rat. As shown in Fig. 13A, on SDS-PAGE analysis, these two complexes both gave multiple bands corresponding to molecular masses of 21–110 kDa. Most of the larger sized components of both showed very similar electrophoretic mobilities, but the electrophoretic mobilities of some of their components of intermediate size differed significantly. Thus, these two enzymes were separated into polypeptides of heterogeneous sizes, showing somewhat different, species-specific patterns. Nevertheless, most of the multiple components of the 26S complex seemed to have been conserved during evolution, like the components of the 20S proteasome. Subsequently, we examined whether disulfhydryl bonds are involved in the subunit assembly. The pattern of the 26S proteasome complex was similar in the presence and absence of a sulfhydryl-reducing reagent, mercaptoethanol (ME in Fig. 13B), indicating that no disulfhydryl bonds are involved in the formation of the complex.

It was of interest to determine whether the various larger components are all essential subunits of the 26S complex or whether some of them are produced from others through limited proteolysis during purification of the enzyme complex. For this, we examined the homologies of these multiple components by means of the two-dimensional electrophoretic peptide mapping method of Bordier and Crettol-Jarvinen (31). This technique is useful for the peptide mapping of heterogeneous proteins, because proteolytic fragmentation can be performed in the stacking gel used in the second dimension of PAGE. As shown in Fig. 14, in most cases when the multiple components separated by SDS-PAGE in the first dimension were digested with lysyl-endopeptidase, they gave distinct hydrolytic patterns, suggesting differences in their primary structures. However, the component of 85 kDa gave a very similar pattern to that of the largest component of 110 kDa (asterisks), suggesting that it was produced through limited proteolysis of the latter. It is unknown whether this autolysis is necessary for the function of the complex. In this analysis, our conditions were suitable for specific digestion of larger components, because 20S proteasomal components are probably resistant to degradation by lysyl-endopeptidase. Our results support the idea that most of the multiple components of the 26S complex are not products of limited proteolysis. But before any definite conclusion can be reached, cDNA cloning and structural analyses of these components are necessary.

**DISCUSSION**

Accumulating evidence indicates that the large 26S proteolytic complex catalyzes the ATP-dependent breakdown of ubiquitinated proteins (for recent reviews, see Refs. 1 and 2), but details of the degradative process remain unknown. One reason why little is known about the degradative pathway is that a method for obtaining a sufficient amount of the purified complex for studies on its biochemical and genetic nature has not yet been developed, because this complex is very unstable and has a very complex structure. There have been several reports of the chromatographic purification of the 26S complex from rabbit reticulocytes (7, 9, 12) and muscle (8). Recently, we reported a new method for the purification of the 26S proteasome from human kidney (25). This method differed from reported procedures, as follows: i) High concentrations of ATP and glyceral were used to stabilize the complex. ii) Ultracentrifugation, instead of fractionation with ammonium sulfate and polyethylene glycol 5000, was used for separating the 26S complex, because we found that the latter procedures caused rapid destruction of the complex. However, even with this method there is still considerable dissociation of the 26S complex, probably because hydroxyapatite chromatography causes its dissociation. Therefore, in the present purification procedure we collected the enzyme in flow-through fractions from a hydroxyapatite adsorbent in the presence of 5 mM ATP. This step was mild and effective, not causing significant dissociation of the 26S complex. We increased the purity of the enzyme by molecular sieve chromatographies on Biogel A-1.5 m and A-5 m columns. The present procedures for isolation of the enzyme, such as ultra-centrifugation, molecular sieve chromatography, and velocity-gradient centrifugation, are based on differences in molecular size, and seem to be milder than reported procedures. It is difficult to judge the purity of an enzyme complex consisting of many subunits and regulatory proteins. However, the symmetrical profile of the protein exhibiting enzyme activity on glycerol density gradient centrifugation (Fig. 2) and the similar profiles of subunits on electrophoresis after each purification step (data not shown) suggest that the final preparation contained all the components, without appreciable contaminants. This simple purification procedure could be used to isolate 26S complexes from various sources, and should be useful for studying the biochemical mechanism(s) underlying energy-dependent proteolysis in vitro.

During purification of the 26S complex, we found that two types of the 26S complex, 26Sα and 26Sβ, could be separated electrophoretically (Figs. 3 and 4). We identified these two forms in crude cell extracts as well as in purified materials. This finding is consistent with a recent report of two 26S proteolytic complexes in a rabbit reticulocyte (12). These 26S complexes in rabbit reticulocytes (36) and rat
liver (this study) have similar properties, such as elec-
trophoretic mobilities and activity staining intensities, but
details of their differences are unknown. We have found by
electron microscopy that the 26S complex from rat liver
purified by essentially the same procedures as used in this
study is dumbbell-shaped, comprising two rectangular
domains attached to a thinner central structure with four
protein layers, which may be the 20S proteasome (37). A
similar ultrastructure was reported for the 26S complex
from Xenopus oocytes by Peters et al. (38). We found that
one or both of the terminal rectangular domains were
dissociated from the 26S entire complex in the absence of
ATP or during drastic chromatographic manipulations.
Therefore, we initially thought that the 26Sα form is the
native dumbbell particle comprising a 20S proteasome and
two rectangular domains, whereas the 26Sβ form is a
molecule lacking a single terminal domain from the intact
26Sα complex, which may be generated during purification
or electrophoresis. However, this is unlikely, because these
two isoforms were found to have similar subunit composi-
tions, both containing a 20S proteasome and multiple other
components of 35–150 kDa (data not shown). Therefore,
these two isoforms may differ in charge, not size, and may
be generated through some post-translational modification,
such as phosphorylation. It remains unknown whether
these two forms have the same functions in cells.

The purified 26S proteasome complex rapidly degraded
[125I]lysozyme-Ub conjugates in an ATP-dependent fash-
ion, but not unmodified [125I]lysozyme (Table I), suggesting
that covalent modification of substrate proteins through
ubiquitination is essential for their proteolysis by the 26S
proteasome. Interestingly, certain typical proteases, such as
trypsin and α-chymotrypsin, degrade both ubiquitinated
and native lysozymes similarly, suggesting that ubiquitina-
tion does not cause an increase in susceptibility to pro-
teases, in general, unlike the denaturation of various
proteins. Thus, the 26S proteasome may have the ability to
recognize multubiquitin chains bound to proteins for their
specific breakdown. Two important processes are involved
in the degradative pathway mediated by the 26S pro-
teasome: One is the energy-requiring process in the assem-
ably of the 26S complex and subsequent degradative reac-
tions, and the other is the process by which the 26S
proteasome selectively recognizes multi-ubiquitinated
proteins and degrades them. Details of the mechanisms
underlying these processes are unknown. Interestingly, an
ATPase was found to be directly associated with the 26S
proteasome, being co-purified with it and immunoprecipi-
tated almost completely by anti-proteasome antibodies
(data not shown). ATPase has also been found in 26S
complexes from rabbit reticulocytes (29) and human
kidney (25). This newly identified ATPase seems to be an
essential component of the 26S complex for catalyzing the
ATP-dependent breakdown of Ub-conjugated proteins,
judging from similarities in pH-optima, and $K_{s}$ for the
degradation of ATP and ubiquitinated lysozyme (Fig. 8),
and inhibition by various compounds, such as vanadate,
quercetin, hemin, and N-ethylmaleimide, of not only ATP
 cleavage but also the ATP-dependent breakdown of Ub-
ligated proteins (data not shown). Presumably the role of
the ATPase is to supply energy continuously for the
selective degradation of ubiquitinated proteins by the
active 26S complex. However, further characterization of

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advantageous for the rapid and selective breakdown of various types of cellular proteins. The three known endo-
proteolytic activities of proteasomes are all maximal at neutral and weakly alkaline pH values (Fig. 10), suggesting
their possible involvement in the soluble proteolytic sys-
tem. Interestingly, certain proteasome-related genes,
named RING10 (= LMP-7) and RING12 (= LMP-2), have
been reported to be located between two putative peptide
transporter loci in the class II region of the human MHC
gene cluster, suggesting the possible involvement of pro-
easomes in the antigen processing pathway (48, 49). Very
recently, we demonstrated that a cloned rat proteasome
subunit, RC1, is a homologue of human RING10 (50). Thus,
it is now clear that certain genes encoding proteasome
subunits are actually located in the MHC class II region.
Accordingly, intracellular antigens could be processed by
the proteasome and the peptides generated could be trans-
ported into the endoplasmic reticulum through two puta-
tive transporters to be assembled with class I MHC
molecules for presentation on the cell surface (51). As
the proteasome has multi-catalytic proteolytic functions, it
could have the potential to generate antigenic peptides
from various types of proteins. In this connection, an
interesting speculation is that such complexes of many
components forming 20S and 26S proteasomes may be
necessary to regulate the formation of a variety of antigenic
peptides. In fact there is evidence that the compositions of
various components in the 20S proteasome change on
treatment of various mammalian cells with interferon-γ
(unpublished data), which is known to regulate antigen
presentation. Therefore, it will also be interesting to
examine whether proteasome subunits encoded in the MHC
region, such as RING10 (= RC1) and RING12, are assem-
bled into the 20S proteasome or ATP-dependent 26S
proteasome complex, and whether the Ub-mediated path-
way is actually responsible for the processing of intra-
cellular antigens.

In the present study, we examined the biochemical
properties of the 26S proteasome. We found that the 26S
complex purified from rat liver consists of a 20S pro-
easome containing multiple subunits of 21-31 kDa and a
characteristic set of larger sized components of 35-110 kDa
(Fig. 13). Moreover, we showed the direct association of the
20S proteasome with these larger components immuno-
chemically and electrophoretically (Figs. 2, 3, and 13). We
recently reported similar findings for 26S complexes from
human promyelocytic leukemia HL-60 cells (13) and
human kidney (25). Similar, but not identical, subunit
compositions of the 26S complexes have been observed in
preparations from various eukaryotic sources, as mention-
ed in the "Introduction." However, their detailed structural
relationships with the multiple components demonstrated in
this study have not yet been examined. On the other
hand, based on the finding that anti-proteasomal antibodies
did not react with their 26S complexes, Seelig et al. (23)
and Kuehn et al. (24) concluded that the proteasome
(MCP) is not an integral component of the "26S" complex,
and that the "26S" enzymes identified by them are distinct
from the 20S proteasome. Their conclusion is not consistent
with our results, for some unknown reason. Possibly their
enzyme is different from ours, because they isolated the
26S complex in 5-18% polyacrylamide gradient gel, but we
found that the intact 26S complex cannot enter polyacryl-

amide gel at concentrations of over 5% (Fig. 4). We
found that anti-proteasomal antibodies precipitated
the activities as to both the ATPase and ATP-dependent
degradation of ubiquitinated lysozyme in addition to the
proteasomal peptidase activity (data not shown, 25), but
they did not examine the Ub-dependency of the proteolytic
function of their enzyme. Further genetic studies seem to
be required to explain these contradictory findings.

At present, the exact molecular weight of the 26S
complex is not known, but from the sum of those of its
components it is calculated to be over 2,000 kDa. This value
seems consistent with our value determined by physical
measurements of approximately 2,000 kDa (manuscript in
preparation). On SDS-PAGE analysis, the multiple sub-
units of the 26S complex in rat liver (Fig. 13A) closely
resembled those of the complex in human kidney (25),
suggesting that these species may have been conserved
during evolution. Moreover, most of the multiple associat-
ed components, ranging from 35 kDa to over 110 kDa, are
distinct polypeptides, judging from the results of electro-
phoretic peptide mapping with lysyl-endopeptidase (Fig. 14),
indicating that the 26S enzyme is a complex of multiple,
non-identical components. No sulphhydryl bond is
involved in this 26S complex (Fig. 13B), but the mechanism
of assembly of the proteasome subunits and other multiple
components is unknown. For determination of the functions
of the 26S proteasome complex at a molecular level, more
detailed structural analyses of the associated components
by genetic means are necessary.

REFERENCES
761-807
349, 132-138
4. Nishizawa, M., Okazaki, K., Furuno, N., Watanabe, N., &
Sagata, N. (1992) EMBO J. 11, 2433-2446
262, 8303-8313
Chem. 262, 2451-2457
Biol. Chem. 263, 12412-12419
Natl. Acad. Sci. USA 86, 7751-7755
4792
267, 22362-22368
13. Orino, E., Tanaka, K., Tamura, T., Sone, S., Ogura, T., &
15. Matthews, W., Tanaka, K., Driscoll, J., Ichihara, A., & Goldberg,
Res. Commun. 160, 911-916
Lett. 302, 192-196
New Biol. 4, 173-187