ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin

(protein breakdown/multicatalytic proteinase complex)

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ABSTRACT Previous studies have indicated that the ATP-dependent 26S protease complex that degrades proteins conjugated to ubiquitin is formed by the assembly of three factors in an ATP-requiring process. We now identify one of the factors as the 20S "multicatalytic" protease, a complex of low molecular weight subunits widely distributed in eukaryotic cells. Comparison of the subunit compositions of purified 20S and 26S complexes indicates that the former is an integral part of the latter. By the use of detergent treatment to activate latent protease activity, we show that the 20S protease becomes incorporated into the 26S complex in the ATP-dependent assembly process. It thus seems that the 20S protease is the "catalytic core" of the 26S complex of the ubiquitin proteolytic pathway.

In the ubiquitin pathway for intracellular protein breakdown, proteins are first ligated to the 76-amino acid polypeptide ubiquitin and are subsequently degraded (for reviews, see refs. 1–3). While the enzymatic reactions in the ligation of ubiquitin to proteins are relatively well characterized, much less is known about the mode of degradation of proteins conjugated to ubiquitin. Hough and Rechsteiner (4) and we (5) have initially found that ATP is required for the degradation of ubiquitin-conjugated proteins in crude extracts of reticulocytes. Subsequently, Rechsteiner and co-workers have identified (6) and extensively purified (7) a large, 26S, ATP-dependent protease complex that degrades proteins conjugated to ubiquitin but not unmodified proteins. These investigators have also purified a 20S ATP-independent protease (7). On the basis of similarities in chromatographic behavior, sensitivity to common inhibitors, and similarities in substrate specificities, Rechsteiner and co-workers (8) proposed that the 26S and 20S proteases may be related and may even share common subunits. Although this proposal was not supported by direct evidence, other investigators subsequently observed that antibodies directed against the 20S protease inhibit ATP/ubiquitin-dependent proteolysis (9–11).

The 20S protease (reviewed in ref. 8) has been observed by many workers in a wide variety of eukaryotic cells. It has been called by different names, such as high molecular weight protease (12), alkaline protease (13), multicatalytic proteinase complex (14, 15), macropain (16), ingensin (17), prosome (18, 19), and proteasome (20). It is a cylinder-shaped particle organized as a stack of four rings (21) and is composed of a characteristic set of low molecular weight subunits (22). Some of its subunits are conserved in evolution (18, 22). The role of this ATP-independent protease remains unsolved, since intracellular protein breakdown has an absolute requirement for cellular energy (23). Since protease activity is stimulated by certain denaturing treatments (14, 24, 25), it is possible that ATP-independent protease activity arises by the "uncoupling" of catalytic sites from regulation. Driscoll and Goldberg (26) have recently reported that when the 20S protease is purified rapidly in the presence of glycerol, it has an ATP-dependent (but ubiquitination-independent) proteolytic activity.

We found previously that the 26S complex that degrades ubiquitin-protein conjugates is composed of three components, designated CF-1 to CF-3 (27). The three factors combine to form the active multienzyme complex in a process that requires MgATP. The role of ATP in complex formation and the functions of the different factors remained unknown. We now identify one of the factors, CF-3, as being identical to the 20S protease. By the use of detergent treatment to uncover latent protease activity, we show that the 20S protease is incorporated into the 26S complex in the ATP-dependent assembly process.

MATERIALS AND METHODS

Materials. DEAE-cellulose (DE52) and phosphocellulose (P11) were obtained from Whatman and ammonium sulfate (enzyme grade) was from Bethesda Research Laboratories. Ubiquitin, hen egg white lysozyme, and bovine pancreatic ribonuclease oxidized with performic acid (OxRNase) were obtained from Sigma. Reductively methylated ubiquitin (MeUb) was prepared as described (28). Proteins were radiiodinated by the chloramine-T procedure (29). The following molecular mass marker proteins for SDS gel electrophoresis were obtained from Sigma: β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa.

Assay of Conjugate Breakdown. Conjugates of 125I-labeled lysozyme (125I-lysozyme) with unlabeled MeUb were prepared as described previously (28). For the determination of the activity of the 26S complex, the reaction mixture contained the following in a volume of 25 μl: 50 mM Tris-HCl (pH 7.6), 3 mM dithiothreitol (DTT), 5 mM MgCl2, 2 mM ATP, 20 mM phosphocreatine, 5 μg of creatine kinase, 125I-lysozyme–MeUb conjugates (3–6 × 102cpm), and enzyme as indicated. Following incubation at 37°C for 30 min, the release of radioactive material soluble in 15% trichloroacetic acid was determined as described (30). Parallel controls were incubated in the absence of ATP, and ATP-independent degradation was subtracted. One unit of activity is defined as that releasing 1% of acid-soluble radioactive material under these conditions in the range linear with enzyme concentration. For the assay of CF-3 activity, similar reaction conditions were used, except that CF-3 was first incubated with CF-1 (20 units) and CF-2 (90 units), in the above reaction mixture lacking 125I-lysozyme–MeUb conjugates to form the active

Abbreviations: CF-1 to CF-3, conjugate-degrading factors 1 to 3; OxRNase, bovine pancreatic ribonuclease oxidized with performic acid; MeUb, reductively methylated ubiquitin; DTT, dithiothreitol.

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26S complex. Following incubation at 37°C for 30 min, the reaction was initiated by the addition of labeled conjugates, incubation was continued for a further 30 min, and the release of acid-soluble radioactive material was monitored.

**Assay of Protease Activity.** 125I-OxRNase was the substrate of choice, since in preliminary experiments we found that it is degraded faster by the 20S protease than some other proteins, such as 125I-L-casein or 125I-hemoglobin. The reaction mixture contained the following in a volume of 25 μl: 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 3 mM DTT, 20 μg of ovalbumin, 20 mM 2-deoxyglucose, 3 μg of hexokinase, 0.1–0.2 μg of 125I-OxRNase (5–10 × 10⁶ cpm), and enzyme source as indicated. The hexokinase and deoxyglucose were used to remove ATP present in enzyme preparations. Following incubation at 37°C for 60 min, the release of radioactive material soluble in 25% trichloroacetic acid was determined. One unit of protease activity is defined as that releasing 1% of acid-soluble radioactive material under the conditions specified above in the range linear with enzyme concentration. For the determination of the effect of SDS on protease activity, a reaction mixture containing enzyme but lacking 125I-OxRNase was incubated with 0.04% SDS for 10 min at room temperature, and proteolysis was initiated by the addition of labeled substrate.

**Enzyme Preparation.** Fraction II (all proteins adsorbed to DEAE-cellulose and eluted with 0.5 M KCl) was prepared from lysates of rabbit reticulocytes and was subjected to affinity chromatography on ubiquitin-Sepharose, as described (30). The conjugate-degrading factors CF-1, CF-2, and CF-3 were prepared from the fraction not adsorbed to the affinity column, according to previously described procedures (27). Preparations of CF-1 were usually contaminated by significant amounts of CF-2 (27). When necessary, a more complete removal of CF-2 from CF-1 was attained by chromatography on phosphocellulose as follows: A preparation of CF-1 after gel filtration on Sepharose-6B (27) that contained 37,500 units of activity and 2.6 mg of protein was applied to a 3-ml column of phosphocellulose equilibrated with 20 mM potassium phosphate (pH 6.5)/1 mM DTT/1 mM ATP/20% (vol/vol) glycerol. The column was eluted successively with 15-ml portions of the above buffer containing 50, 100, or 600 mM KCl. CF-1 was eluted in the 100–600 mM KCl fraction, whereas CF-2 was eluted at lower salt concentrations. The enzyme was concentrated by ultrafiltration with Centricon CF-25 cones (Amicon) and salt was removed by two successive 10-fold dilutions with buffer A [20 mM Tris-HCl (pH 7.2)/1 mM DTT/1 mM ATP] containing 20% glycerol, followed by concentration in the same column. Preparations of CF-1 obtained by this method were virtually free of CF-2, and the recovery of activity was about 50%.

**Purification of 26S Complex.** Twenty milliliters of fraction II from reticulocyte supernatants containing 560 mg of protein, was supplemented with 2 mM ATP, 5 mM MgCl₂, 1 mM DTT, 10 mM phosphocreatine, and creatine kinase at 10 μg/ml and was incubated at 37°C for 60 min. This treatment promotes the assembly of the three factors to form the 26S complex (27). The complex was precipitated with ammonium sulfate at 38% saturation, as described previously for the preparation of fraction II A (27). This procedure separates the 26S complex from the 20S protease (31). The sediment was dissolved in 20 ml of buffer A, precipitated with ammonium sulfate at 38% saturation, and dissolved in 5 ml of buffer A containing 20% glycerol. The recovery of 26S complex activity in the 0–38% ammonium sulfate fraction was close to 100%. This preparation was applied to a column (2.5 × 40 cm) of Sepharose 6B equilibrated with buffer A containing 20% glycerol. Fractions (6 ml) were collected and conjugate-degrading activity was assayed in 1-μl samples of column fractions. Two peaks of conjugate-degrading activity were observed: a high molecular weight peak close to the void volume (fractions 11–15) and a smaller peak at a lower molecular weight region (fractions 18–24). The first peak is the 26S complex, while the second peak is presumably the overlap region between the three unassembled factors.

The high molecular weight peak, which contained more than 80% of total activity, was collected. A sample of this preparation (5 ml containing 8 mg of protein and 375,000 units of conjugate-degrading activity) was diluted with 30 ml of 20 mM potassium phosphate (pH 7.0)/1 mM DTT/20% glycerol (buffer B) and was applied to a column (1.5 × 7.5 cm) of phosphocellulose equilibrated with buffer B. The column was washed further with 40 ml of buffer B and the total fraction not adsorbed to phosphocellulose was collected in the presence of 2 mg of ovalbumin and 50 μmol of ATP. Most of the conjugate-degrading activity was in the unadsorbed fraction, whereas most of the other proteins were bound to phosphocellulose, as indicated by polyacrylamide gel electrophoresis and silver staining. The extent of purification in this step could not be determined exactly due to the presence of carrier ovalbumin. The unadsorbed fraction was concentrated by ultrafiltration, diluted 10-fold with buffer A containing 20% glycerol, and concentrated again. Recovery of enzyme activity in this step varied from 30% to 50% in four different preparations. This material was subjected to glycerol density gradient centrifugation in the last step of the purification, as described under Fig. 1.

**RESULTS**

**Presence of Subunits of the 20S Protease in Purified 26S Complex.** The initial objective of this study was to characterize the processes responsible for the dissociation of the 26S complex into its components. This goal necessitated the purification of the 26S complex. We have purified the 26S complex by a modification of the procedure of Rechsteiner and co-workers (7). The main improvements in the modified procedure (see Materials and Methods) are as follows: (i) increased conversion of the three factors to the 26S complex by the incubation of reticulocyte extract with MgATP prior to purification; (ii) removal of most of the 20S protease from the 26S complex by fractionation with ammonium sulfate (31); (iii) a simple operation of passage through phosphocellulose replaced the more laborious procedures of gradient elutions on DEAE-cellulose and hydroxyapatite. The last step of purification was glycerol density gradient centrifugation under conditions similar to those of Rechsteiner and co-workers (7). Fig. 1A shows the separation of the 26S complex on such a gradient assayed by its activity in ATP-dependent conjugate breakdown. To examine the purity of the preparation, samples from the different fractions of this gradient were separated by SDS gel electrophoresis and protein bands were visualized by silver staining. As seen in Fig. 2, the 26S complex contains a set of at least 13 protein bands in the molecular mass range of approximately 35–110 kDa (marked by asterisks) that coincide with conjugate-degrading activity. This set of protein subunits is similar to that reported previously by Rechsteiner and co-workers (7). In addition, the same peak in the gradient contained another set of at least 10 protein subunits in the region of around 20–33 kDa (marked by arrows in Fig. 2). We have noted that this latter set of smaller subunits migrated on glycerol gradients in two regions: one coinciding with the 26S complex (center of peak at fraction 8, Figs. 1A and 2) and another at a lower molecular weight region (peak center at fraction 12, Fig. 2). In different preparations, there was considerable variation in the relative proportion of the low molecular weight set of subunits in the two peaks, but separation into two peaks was consistently observed in all gradients. The set of 20- to 33-kDa subunits is characteristic of the 20S protease, and the position of the second peak on the glycerol gradient
corresponds to that of the 20S protease (7). Therefore, the above observations raised the possibility that these subunits exist in two structures: as an ATP-independent 20S protease and as an integral part of the ATP/ubiquitin-dependent 26S complex. It could not be ruled out, however, that a dimeric or otherwise altered form of the 20S protease comigrates with the 26S complex on glycerol gradients (7).

**Activation by SDS of Protease Activity in 20S and 26S Complexes.** It was previously observed by several workers that ATP-independent protease activity in the 20S complex is latent and that it can be activated by denaturing treatments such as heating, freeze-thawing, or treatment with low concentrations of detergents such as SDS (14, 24, 25). It seems reasonable to assume that partial denaturation exposes active site(s) of the protease by the disruption of an ordered, and possibly regulated, structure. To examine whether protease subunits of similar activities are present in the 20S and 26S complexes, we have tested the effect of SDS on ATP-independent protease activity in different regions of the glycerol gradient. As shown in Fig. 1B, without SDS treatment there was low protease activity in the 20S and 26S regions. Upon treatment with 0.04% SDS, there was a dramatic increase in ATP-independent protease activity, which appeared in two peaks: one coinciding exactly with the 26S complex, and another with the 20S complex. Higher concentrations of SDS inhibited protease activity in both peaks (data not shown).

**Identification of CF-3 as the 20S Protease.** Since the 20S protease apparently exists both in a free form and as a part of the 26S complex, our next question was whether it is identical to one of the three factors that assemble to form the 26S complex (27). Of the three conjugate-degrading factors, the size of CF-3 (650 kDa, ref. 27) is closest to that reported for the 20S protease (670–700 kDa; refs. 7 and 31). We have previously noted that partially purified preparations of CF-3 contained ATP-independent protease, but protease activity (without detergent treatment) was low relative to CF-3 activity and was thought to be a contaminant (27). In view of the observations described above, the possible identity of CF-3 with the 20S protease was examined. As shown in Fig. 1A, CF-3 activity (assayed by the ATP-independent breakdown of conjugates in the presence of CF-1 and CF-2) migrated in the glycerol gradient in coincidence with the second (20S) peak of SDS-activated protease activity (Fig. 1B) and of the 20- to
33-kDa subunits (Fig. 2). Similarly, SDS-activated protease activity coincided with CF-3 in chromatographic separations, such as gel filtration on Sepharose 6B, salt gradient elution on DEAE-cellulose, and chromatography on hydroxylapatite (data not shown).

These findings establish that CF-3 is a protease. They do not indicate, however, that all 20S proteases described by other authors are identical to CF-3. In different preparations of CF-3, considerable variations were observed in ATP-independent protease activity (without SDS treatment). This was presumably due to variable degrees of denaturation, caused by handling during purification. Stimulation by SDS varied between 3- and 10-fold in different preparations of CF-3.

Incorporation of the 20S Protease into the 26S Complex by an ATP-Dependent Process. If the 20S protease is identical to CF-3, it would be expected to be incorporated into the 26S complex in the ATP-dependent assembly of the three conjugate-degrading factors. This indeed was found to be the case. In the experiment shown in Fig. 3, the three conjugate-degrading factors were incubated with different additions and the mixtures were subjected to glycerol density gradient centrifugation. When either ATP or Mg$^{2+}$ was omitted, SDS-activated protease activity remained in the 20S region along with CF-3 activity (Fig. 3 A and B). Following incubation with MgATP, most SDS-activated protease activity shifted from the 20S region to the 26S peak. This was accompanied by the disappearance of free CF-3 and the formation of the active 26S multienzyme complex (Fig. 3C).

In additional experiments summarized in Table 1 we found that the presence of both CF-1 and CF-2 during incubation with MgATP was required for the shift of SDS-activated protease activity from 20S to the 26S complex. Since the

Fig. 3. MgATP-dependent incorporation of 20S protease into the 26S complex. CF-1 (3100 units), CF-2 (11,700 units), and CF-3 (2000 units) were incubated with 20 mM Tris-HCl (pH 7.2) and 1 mM DTT in a volume of 200 μl under the following conditions: (A) with 2 mM ATP and 0.5 mM EDTA, no Mg$^{2+}$ added; (B) with 5 mM MgCl$_2$, 20 mM 2-deoxyglucose, and 25 μg of hexokinase, no ATP added; (C) with 2 mM ATP and 5 mM MgCl$_2$. After incubation at 37°C for 30 min, the samples were loaded on 10-40% (vol/vol) glycerol gradients (4.3 ml) that contained buffer A supplemented with 1 mM EDTA and reduced and carbamylated bovine serum albumin at 1 mg/ml. After centrifugation at 28,000 rpm for 18 hr in a Beckman SW-65 rotor, 13 330-μl fractions were collected. SDS-activated protease activity (●) and CF-3 activity (○) in conjugate breakdown were determined in 10-μl samples of gradient fractions. All results are expressed in units of activity.

Table 1. Incorporation of 20S protease into the 26S complex requires CF-1 and CF-2

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>20S</th>
<th>26S</th>
</tr>
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<tbody>
<tr>
<td>Complete system</td>
<td>57.3</td>
<td>42.7</td>
</tr>
<tr>
<td>Minus CF-1</td>
<td>94.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Minus CF-2</td>
<td>92.0</td>
<td>8.0</td>
</tr>
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</table>

Experimental conditions were as described under Fig. 3, with the following changes: CF-1 was freed from residual CF-2 by chromatography on phosphocellulose as described in Materials and Methods. The complete system contained 2300 units of CF-1, 11,700 units of CF-2, and 3000 conjugate-degrading units of CF-3. All mixtures were incubated with 2 mM ATP and 5 mM MgCl$_2$ at 37°C for 30 min and were subjected to glycerol density gradient centrifugation as in Fig. 3. Fifteen 290-μl fractions were collected and SDS-activated protease activity was estimated in 10-μl samples of gradient fractions. Protease activities in the 20S and 26S regions were calculated as the percentage of total protease activities recovered in all gradient fractions. These were as follows: complete system, 876 units; minus CF-1, 902 units; minus CF-2, 896 units. The amount of residual 20S protease in the complete system is higher than that in Fig. 3C due to the greater amount of CF-3 used.
three factors had been only partially purified (27), it was not possible in these experiments to follow the incorporation of protein subunits into the 26S complex by SDS/polyacrylamide gel electrophoresis of gradient fractions.

**DISCUSSION**

We have shown that the 20S protease becomes an integral part of the 26S complex in an ATP-dependent assembly process. We thus provide direct evidence for the suggestion of Rechtsteiner and co-workers (8) and other workers (9–11) that the two complexes share common subunits. It appears reasonable to assume that the 20S protease is the "catalytic core" of the 26S complex. It also seems plausible that the association of the protease core with subunits of the other two factors confers on the 26S complex the specificity to degrade proteins conjugated to ubiquitin. For example, some subunits may bind the ubiquitin moiety or a polyubiquitin chain (28, 32) of ubiquitin-protein conjugates and may bring the substrate to sufficient proximity to the active site(s) of the catalytic core. It was shown previously by Wilk and Orlowski (14) that the 20S protease contains at least three types of different catalytic sites, specific for the cleavage of peptide bonds at the carboxyl side of hydrophobic, basic, or acidic amino acid residues. It is possible that the structural organization of the different catalytic sites facilitates the rapid and complete endoproteolytic degradation of proteins ligated to ubiquitin.

Since the 20S complex is a part of the 26S complex, the question arises whether the free 20S complex is an artefact that results from the dissociation of the 26S complex during isolation procedures. This may well be so in at least some cases, and it may explain the presence of variable amounts of the 20S complex in purified preparations of the 26S complex (Fig. 2). It is possible, however, that the free 20S complex serves as a source of protease core for assembly into different structures with different functions. It has been shown by several workers that, when isolated from certain tissues or under certain conditions, the 20S complex has an ATP-dependent (ubiquitinylation-independent) proteolytic activity (26, 33). Though the size of this complex is similar to that of the ATP-independent 20S protease, it is possible that ATP dependence is due to a subtle alteration in subunit composition or arrangement. In addition, it has been reported that a eukaryotic pre-tRNA 5'-processing endonuclease copurifies with a particle that resembles very much the 20S complex by electron microscopy and subunit composition (34). If the particles are indeed similar, an intriguing possibility is that the 20S complex is a "degradosome" containing protease and nuclease catalytic sites.

The roles of ATP in the assembly of the 26S complex and in its function remain enigmatic. It has been pointed out that, after complex formation, the breakdown of ubiquitin-protein conjugates continues to require ATP (27). This suggests two separate roles of ATP: in the assembly and in the action of the protease complex. It is possible, for example, that assembly is due to an ATP-requiring covalent modification of one of the factors, while ATP hydrolysis may be required for the action of the protease complex. The present identification of the "catalytic core" of the 26S complex as the 20S protease suggests that ATP may not be directly involved in amide bond cleavage, as is the case in the mechanism of action of S'-oxoprolinase (35). Rather, the energy of ATP hydrolysis may drive the translocation of ubiquitin-protein conjugates to the active site or may be involved in the release of products from the catalytic center.

Note. Following the submission of this manuscript, it has come to our attention that Drs. J. Driscoll and A. L. Goldberg (Harvard Medical School) have similarly observed incorporation of the proteasome into the 26S complex (manuscript submitted). We thank Dr. Michael Fry for helpful comments on the manuscript and Atara Katznelson for expert typing. This work was supported by U.S. Public Health Service Grant AM-25614 and a grant from the United States–Israel Binational Science Foundation.