Multiple Forms of the 20 S Multicatalytic and the 26 S Ubiquitin/ATP-dependent Proteases from Rabbit Reticulocyte Lysate*

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We have used native gel electrophoresis followed by fluorogenic peptide overlay to identify multiple forms of rabbit reticulocyte multicatalytic protease (MCP) or 20 S protease, and two forms of rabbit 26 S ubiquitin/ATP-dependent protease. An abundant, fast-migrating 20 S complex (20 S) possesses modest ability to hydrolyze the fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide. In contrast, two minor, slower migrating species cleave the peptide at high rates. A unique 30-kDa polypeptide is associated with one of the active MCPs, and a 160-kDa subunit is associated with the other.

Two electrophoretically distinct 26 S proteases can also be isolated from rabbit reticulocyte lysate. The faster migrating form, 26 S, is more resistant to inactivation by ATP depletion. Despite the differential response to nucleotides and the distinct electrophoretic mobilities of 26 S and 26 S, we have not identified any subunit differences between the two enzymes. In addition to active 26 S proteases, we have discovered and purified a proteolytically inactive particle that contains subunits characteristic of the 26 S protease (e.g., molecular masses between 30 and 110 kDa). Incubation of this protein complex with purified MCP and ATP results in the formation of the 26 S proteases.

The cytosolic and nuclear compartments of eucaryotic cells contain a variety of proteases (1). These include members from four classes of proteolytic enzymes. Cysteine proteinases are represented by the calpains (2); insulinase provides an example of a cytoplasmic metalloprotease (3). Among the cytoplasmic serine proteinases are tripeptidyl peptidase (4) and proline endopeptidase (5), and cathepsin E, an acid protease, is apparently found in red cell cytoplasm (6). The extent to which each of these enzymes contributes to overall intracellular proteolysis is presently unknown.

Eucaryotic cells also contain two large proteolytic complexes whose catalytic mechanism is a matter for some debate. The multicatalytic protease (MCP)† is a large, 20 S particle composed of 24 or so subunits ranging in molecular mass from approximately 20 to 32 kDa (for review, see Refs. 7 and 8). The subunits are stacked in four layers to produce a cylinder-shaped structure measuring 11 × 16 nm. The particle appears to have a hollow core (9).

Subunits with electrophoretic mobilities characteristic of MCP were also found associated with 10 or more polypeptides ranging from 34 to 110 kDa in size (10). This larger protease complex sediments at 26 S and is capable of degrading ubiquitin-lysozyme conjugates in an ATP-stimulated reaction. Based on similarity in subunit composition, we proposed that MCP subunits were common to both 20 S and 26 S complexes; we also suggested that the higher molecular mass chains (34–110 kDa) confer ATP dependence and ubiquitin recognition on the 26 S protease. Support for this idea has been obtained in several studies that show formation of the 26 S complex when MCP and crude protein fractions are incubated in the presence of ATP (11–13). Both large protease complexes are thought to play major roles in intracellular proteolysis, although firm evidence for this supposition is lacking.

As part of our continuing effort to characterize the 26 S protease, we conducted analyses that involve native gel electrophoresis followed by fluorogenic peptide overlays to localize protease activity. In the course of these studies, we observed that protease activity was not proportional to the distribution of protease activity. In the course of these studies, we observed that protease activity was not proportional to the distribution of MCP protein. This led to the discovery of electrophoretically distinct 26 S complexes with differing specific activities. Here we describe differences in subunit composition between the multiple forms of MCP, and we demonstrate that one highly active form of 20 S can be generated by a novel protein complex isolated from human red cells or rabbit reticulocytes. Moreover, we resolved two electrophoretically distinct species of 26 S protease and have examined the subunit composition of the two enzymes. Finally, we identify a single protein complex that associates with MCP subunits to form active 26 S proteases.

EXPERIMENTAL PROCEDURES

Materials—TSK-DEAE-650 S was obtained from Supelco (Bellefonte, PA). Centricron-30 microconcentrators were obtained from Amicon (Beverly, MA). The fluorogenic peptides Suc-Leu-Leu-Val-Tyr-MCA and Pro-Phe-Arg-MCA were obtained from Peninsula Laboratories, Inc. (Belmont, CA); the peptide Suc-Ala-Ala-Phe-MCA was obtained from Sigma. Protein silver stain reagents were obtained from Bio-Rad. Affinity-purified goat anti-rabbit IgG used as secondary antibody was obtained from Cappel Research Products (Durham, NC). Fluorescein succinimidyl ester was obtained from Molecular Probes, Inc. (Eugene, OR).

Purification of High Molecular Mass Proteases—Reticulocytosis was induced by phenylhydrazine injection following schedules and dosages prescribed by Lingrel (14). Blood was collected by cardiac puncture, and washed red cells (≥90% reticulocytes) were lysed in 1.6 volumes of 1 mM DTT. After centrifuging the lysate at 100,000 × g for 90 min, glycerol was added to 20% (v/v), and the lysate was loaded onto a TSK-DEAE column equilibrated in 10 mM Tris-HCl, pH 7.1
and finally developed with a 0.1-0.25 M KC1 gradient in TSDG. The diluted to 5% glycerol, and layered onto 10-40% glycerol gradients. The multicatalytic proteases elute between 175 and 195 mM KC1; the 26 S enzymes are found over the range 180-220 mM KC1 (see Fig. 2).

Appropriate fractions were combined, and the high molecular mass proteases were pelleted by centrifuging at 100,000 X g for 17 h. The pellets were resuspended in the bottom 0.5 ml of solution in the tube, diluted to 5% glycerol, and layered onto 10-40% glycerol gradients. After centrifuging for 22 h at 25,000 rpm in an SW 28 rotor at 4°C, the 28-ml gradients were fractionated into 2-ml aliquots from the bottom. Gradient-purified proteases were concentrated by spinning through Centricon-30 microconcentrators.

Preparation of Modified Fraction II—For certain experiments a modified fraction II (15) was used. This was prepared by first washing lysate-loaded TSK-DEAE with 160 ml of 0.15 M KC1 in TDI and then eluting with 0.275 M KC1 in TSDG. The modified fraction II was then taken to 38% ammonium sulfate, and the precipitated proteins were collected by cenriifugation and dialyzed against 2 liters of TSDG to produce mII38.

Protease Assays—The 20 S protease was assayed using fluorogenic peptides; the 26 S protease was measured using fluorogenic peptides as well as ubiquitin-lysosome conjugates. Spectrofluorometric assays consisted of 100 µM fluorogenic peptide in 30 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 10 mM KC1, 0.5 mM dithiotreitol, with or without 2 mM ATP or ATP-regenerating system. The reaction (100 µl of volume) was initiated by adding enzyme and incubating at 37°C for 15 min prior to quenching with 200 µl of ethanol. Fluorescence was measured on a Perkin-Elmer fluorometer using an excitation wave-length of 380 nm and an emission wavelength of 440 nm. The following fluorogenic peptides were used: Suc-Leu-Leu-Val-Tyr-7-amide, Suc-Ala-Ala-Phε-MCA, and Pro-Phe-Arg-MCA.

Electrophoresis—All samples were analyzed on mini gels using a Mini-Protean gel apparatus (Bio-Rad). Nondenaturing gels consisted of 2.5% stacking and 4.5% resolving gels cast in 90 mM Tris, pH 8.3, 5 mM EDTA, 3.75% acrylamide, and 0.08 M Tris-HCl, pH 7.25, 25 mM KCl, 10 mM MgCl2, 2 mM ATP, 200 µM fluorogenic peptide, and incubating the gels at 37°C for 30-60 min. The fluorescent gels were visualized by a UV light and photographed with a Polaroid camera.

Immunodetection of MCP Subunits—MCP was purified to homogeneity from human red blood cells using chromatography on TSK-DEAE, ammonium sulfate precipitation, gel filtration, and glycerol gradient centrifugation (10). Rabbit antibodies to human MCP were prepared by the method of Vaitukaitis (16). Human MCP (100 µg) was emulsified in Freund's adjuvant and injected subcutaneously at monthly intervals. Sera were collected after the fifth injection. Rabbit IgGs were prepared by precipitation with 40% ammonium sulfate and chromatographed on TSK-DEAE. Immunoglobulins were collected in 40% ammonium sulfate precipitation, gel filtration, and glycerol gradient centrifugation (10). Rabbit antibodies to human MCP were prepared by the method of Vaitukaitis (16). Human MCP (100 µg) was emulsified in Freund's adjuvant and injected subcutaneously at monthly intervals. Sera were collected after the fifth injection. Rabbit polyclonal antibodies were then electrophoresed for 800 V-h in a cold room. SDS-PAGE consisted of 10% resolving and 4% stacking gels in 25 mM Tris, pH 8.5, 200 mM glycine, 0.05% sodium dodecyl sulfate. Samples were run for 150 V-h at room temperature. After electrophoresis and/or substrate overlay (see below), proteins were visualized by staining in 0.2% Coomassie Brilliant Blue in 22.5% methanol, 7.5% acetic acid. Alternatively, gels were stained in the Bio-Rad silver stain method.

Substrate Overlays—Protease activity was detected in nondenaturing gels by overlaying the gels with 30 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 10 mM KC1, 0.5 mM DTT, 2 mM ATP, 200 µM fluorogenic peptide, and incubating the gels at 37°C for 30-60 min. The fluorescent gels were illuminated by a UV light and photographed with a Polaroid camera.

Fluorescein conjugation of MCP—Rabbit reticulocyte lysate was chromatographed on TSK-DEAE, and fractions enriched in 20 S or 26 S proteases were pooled separately. The proteases, collected by sedimentation at 100,000 X g for 17 h, were further purified on 10-40% glycerol gradients (see Fig. 2 and "Experimental Procedures" for details). Panel A, samples (20 µl) from gel fraction 4-9 were then electrophoresed for 800 V-h on 4.5% native polyacrylamide gels. At the left, proteolytic activity is detected by fluorogenic peptide overlay with sLLVY-MCA. The same gel is shown at the right after staining with Coomassie Brilliant Blue. The far right lane contains 5 µl of reticulocyte lysate. After peptide overlay the gel was nicked in the zone of MCP fluorescence (left), fixed and stained for protein. Note that 20 S enzyme activity is slightly offset from the major protein band. Panel B, equivalent analysis of glycerol gradient fractions from a DEAE pool (similar to fractions 168-180 in Fig. 2) containing 26 S proteases. The protein band just below the active 26 S enzymes, labeled Ball, does not exhibit proteolytic activity, but it does contain polypeptides characteristic of the 26 S protease (see "Results").

FIG. 1. Resolution of multiple 20 S and 26 S proteases on nondenaturing acrylamide gels. Rabbit reticulocyte lysate was chromatographed on TSK-DEAE, and fractions enriched in 20 S or 26 S proteases were pooled separately. The proteases, collected by sedimentation at 100,000 X g for 17 h, were further purified on 10-40% glycerol gradients (see Fig. 2 and "Experimental Procedures" for details). Panel A, samples (20 µl) from gel fraction 4-9 were then electrophoresed for 800 V-h on 4.5% native polyacrylamide gels. At the left, proteolytic activity is detected by fluorogenic peptide overlay with sLLVY-MCA. The same gel is shown at the right after staining with Coomassie Brilliant Blue. The far right lane contains 5 µl of reticulocyte lysate. After peptide overlay the gel was nicked in the zone of MCP fluorescence (left), fixed and stained for protein. Note that 20 S enzyme activity is slightly offset from the major protein band. Panel B, equivalent analysis of glycerol gradient fractions from a DEAE pool (similar to fractions 168-180 in Fig. 2) containing 26 S proteases. The protein band just below the active 26 S enzymes, labeled Ball, does not exhibit proteolytic activity, but it does contain polypeptides characteristic of the 26 S protease (see "Results").

RESULTS

Identification of Protein Complexes Containing Subunits of the 20 S and 26 S Proteases—In 1987 we showed (10) that the 20 S and 26 S protease complexes can be separated on native acrylamide gels and subsequently localized by overlaying the gel with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (sLLVY-MCA). These initial studies, in which samples were typically electrophoresed for 200 V-h on large gels, revealed two distinct 26 S activities. However, a single band of sLLVY-MCA hydrolytic activity was present in the 20 S region of the gel, and it appeared to comigrate with 26 S protease. More recently, preparations of 20 S and 26 S proteases were electrophoresed for longer periods on minigels, and as shown in Fig. 1, it became apparent that cleavage of sLLVY-MCA does not coincide with the major 20 S complex. After separating glycerol gradient-enriched 20 S protease on native gels, enzymatic activity was visualized by peptide overlay (Fig. 1, panel A). The gel was nicked at the fluorescent band in the 20 S region and then stained for protein. Whereas the 26 S activity corresponds to the major protein complex (26 SFAST), alignment of the peaks clearly demonstrates that most sLLVY-MCA hydrolysis in the 20 S region is caused by a minor, slower migrating species (20 S SLOW), not the prominent protein band. This raised several questions. Is the slower migrating protease a form of MCP or an entirely different enzyme? If a variant of MCP, what is the basis for the altered electrophoretic mobility and enhanced activity against sLLVY-MCA? Below we show that both major and minor protein complexes contain MCP subunits. We also present evidence that the active enzymes contain additional subunits.

When fractions of glycerol gradient purified 26 S protease...
were similarly analyzed on native gels, the two bands of enzyme activity roughly match the protein present in 26 S complexes (Fig. 1, panel B). However, a slightly faster migrating protein complex is also present. Although it does not cleave sLLVY-MCA, it will be seen that this component contains subunits found in the 26 S protease. For easier description of the experiments, we call the inactive complex the “ball,” based on a “ball and cylinder” model of the 26 S protease (17). Likewise, we distinguish 20 S and 26 S enzymes as fast or slow based on their electrophoretic properties.

**Chromatographic Properties of 20 S and 26 S Complexes**—To characterize further the various species described in Fig. 1, we chromatographed reticulocyte lysate on TSK-DEAE and quantitated sLLVY-MCA cleavage in the column fractions (Fig. 2, top panel). It can be seen from the peptide overlays presented in the middle panel of Fig. 2 that the distribution of 20 S protease matches sLLVY-MCA hydrolysis in the absence of ATP (top panel, fractions 145–160). The native gels also demonstrate that slow migrating forms of 20 S are responsible for virtually all peptide cleavage in fractions 145–160, but this is difficult to discern because of the figure’s extreme reduction.

ATP-stimulated hydrolysis of sLLVY-MCA extends from fractions 152 to 184, coincident with the presence of two electrophoretic forms of 26 S protease. The two enzymes overlap extensively in their elution from DEAE; 26 Sf is present in fractions 152–183, whereas the slower migrating 26 Ss is confined to fractions 165–185. The ATP-dependent sLLVY-MCA cleavage can be attributed to 26 S enzymes, but there is a discrepancy between fluorometer and native gel assays. Although the ATP-dependent protease peak in fraction 175 is evident by both assays, the large peak of ATP-dependent peptide hydrolysis centered on fraction 163 (top panel) is not paralleled by equally intense protease activity on the native gel (middle panel). This is apparently because of enhancement of 26 S activity in the presence of ATP since incubation of fractions 155–170 with 2 mM ATP prior to electrophoresis results in greater 26 S activity in gel overlays (not shown). The bottom panel in Fig. 2 reveals that a major protein complex (denoted as ball) migrates slightly faster than 26 Sf in fractions 162–184. As shown below, this protein complex, which is composed of subunits ranging in molecular mass from 25 to 110 kDa, can assemble with MCP to form the 26 S complex.

**The Subunit Compositions of Fast and Slow Forms of 20 S**—Because they contain 20 S protease virtually free of 26 S species, DEAE-gradient fractions 145–151 were combined to form pool I. The 20 S enzyme was pelleted by overnight centrifugation at 100,000 × g, redissolved in 0.5 ml, and sedimented on a 10–40% glycerol gradient. DEAE-gradient fractions 152–166 were treated similarly to produce glycerol gradient-purified 20 S protease, termed pool II. Samples from the two 20 S preparations were then analyzed on 4.5% native gels. As expected, both slow and fast electrophoretic forms were present in each pool, and sLLVY-MCA cleavage was largely confined to the slower migrating species (Fig. 3, panel A). However, when the various bands of 20 S protease were excised from the native gel and analyzed by SDS-PAGE, the slow forms in pools I and II were found to differ in subunit compositions (Fig. 3, panel B). The highly active 20 S in pool I was deficient in the highest molecular mass subunit normally found in MCP. Instead, 20 SsI contained a novel subunit with a molecular mass of ~30 kDa. By contrast, 20 SsII contained all of the subunits characteristic of the 20 Ss species present in both pools, as well as an additional 160-kDa polyepitide. This 160-kDa subunit is absent from 20 Ss and absent from gel slices taken immediately above the active protease, so we consider it to be a component of yet another form of 20 S protease. After determining that 20 SsI has an additional 30-kDa subunit and 20 SsII has an additional 160-kDa subunit, we subsequently refer to these complexes as 20 SsII and 20 SsIII. These subunit compositions have been confirmed in six separate purifications starting with fresh blood.

The relative activities of slow and fast 20 S proteases were

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**Fig. 2. Chromatographic properties of 20 S and 26 S proteins on TSK-DEAE.** Rabbit reticulocyte lysate (40 ml) was applied to a 1 × 20-cm column of TSK-DEAE, washed with 240 ml of TSDG and 60 ml of TSDG containing 0.1 M KCl. Proteases were then eluted with a 150-ml gradient from 0.1 to 0.25 M KCl in TSDG. One-ml fractions were collected, and 5-μl samples were assayed for protease activity. The upper panel depicts sLLVY-MCA cleavage in the presence or absence of ATP (open and closed circles, respectively). The middle panel is a composite of five nondenaturing gels in which 20-μl samples from fractions 145–184 were electrophoresed prior to overlay with sLLVY-MCA. The bottom panel shows the same gels after fixation and staining with Coomassie Brilliant Blue.

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**Fig. 3. Subunit compositions of 20 Ss and 26 Ss proteases.**

**Panel A.** Native gel separation of fast and slow 20 S complexes. Glycerol gradient-purified 20 S proteases from pool I (DEAE fractions 145–151) or pool II (fractions 152–166) were electrophoresed on native gels for 800 V·h. Enzyme activity was localized by peptide overlay (left), and protein was localized by Coomassie staining (right). Approximately 5 μg of protein was analyzed in pool I; sample II contained 5 μg of protein. **Panel B.** SDS-PAGE analyses of fast and slow forms of 20 S proteases. MCPs from pools I or II were isolated on native gels equal to those shown in panel A. After localizing the enzymes by peptide overlay, gel slices encompassing the active regions were excised, boiled in SDS-PAGE buffer, and analyzed on 10% acrylamide minigels. The silver-stained gels are shown above. I and II represent proteins in the respective glycerol gradient fractions prior to native gel electrophoresis. The lane labeled 20 SsII is the slow form of MCP present in pool I. The lane labeled 20 SsIII is the slow form of MCP present in pool II. Note the distinctly different subunit compositions. By contrast, the 20 Ss species from pools I or II exhibit identical patterns on SDS-polyacrylamide gels.
examined by the native gel overlay procedure. The native gel assays presented in panels B–D of Fig. 4 demonstrate that slow and fast forms of the 20 S protease are roughly equivalent in their ability to cleave Suc-Ala-Ala-Phe-MCA and Pro-Phe-Arg-MCA. By contrast, 20 Ss is particularly active toward sLLVY-MCA as well as toward benzoyloxycarbonyl-Leu-Leu-Glu-p-nitroanilide (data not shown). Thus, rabbit reticulocyte lysate contains at least three species of MCP. Two are highly active against sLLVY-MCA, and extra subunits of 30 or 160 kDa are associated with these slower migrating species. The third form is comprised of the traditional MCP subunits and is capable of cleaving, albeit much slower, a variety of fluorogenic peptides, including sLLVY-MCA.

Conversion of Rabbit 20 Sf to 20 S by a Protein Fraction from Human Red Cells—Inspection of Figs. 1 and 3 reveals only traces of protein in regions of the native gel that exhibit cleaving enzyme simply because, being so abundant, they trail during electrophoresis. Discovery of a regulatory factor from human red blood cells that is unable to hydrolyze peptides present in DEAE fractions 145–152. As yet, we have not been able to convert 20 Sf peptide hydrolysis (see accompanying article, Ref. 30) allowed us to eliminate this possibility. Electrophoretic analyses of 20 Sf titrated with increasing amounts of human red blood cell regulator demonstrates this activation phenomenon (Fig. 5). Comparing panels A and B, one can readily see that the enhanced ability of 20 Sf to cleave sLLVY-MCA is accompanied by a slower migration of the protease. Moreover, SDS-PAGE analysis of the activated 20 S (panel C) demonstrates that a 30-kDa subunit is now associated with the enzyme. Since we can convert 20 Sf to an activated, slower migrating protease containing an “extra” 30-kDa subunit, we conclude that the latter is equivalent to the active 20 S species present in DEAE fractions 145–152. As yet, we have not been able to convert 20 Sf to 20 Ss. Nevertheless, we consider the latter to also be a bona fide subspecies of MCP.

Subunit Compositions of 26 Sf, 26 Ss, and the Ball—The approach used to arrive at the subunit compositions of 20 S species was also applied to the ball component and to the electrophoretic variants of the ubiquitin/ATP-dependent 26 S protease. The 26 Sf enzyme was enriched by pooling DEAE fractions 152–166 (see Fig. 2); 26 Ss and ball were obtained from fractions 167–185. After sedimenting each pool for 17 h at 100,000 × g, the pelleted protein complexes were resuspended and further purified on 10–40% glycerol gradients to yield 26 SII (mostly 26 Sf), 26 SIII (mostly 26 Ss), and a protease-free preparation of the ball component (see Fig. 6, panel A). The separated protein complexes were excised from the native gels and analyzed by SDS-PAGE. Panel B of Fig. 6 shows molecular mass distributions of the subunits present in each protein complex.

Despite close inspection of many gels, we have been unable to detect any consistent differences in the subunit patterns of the 26 S enzymes. The 26 Sf species often generates an SDS-PAGE pattern that contains more high molecular mass polypeptides (>130 kDa), but the differences between 26 Sf and 26 Ss have proved quantitative rather than qualitative. Moreover, none of the larger proteins is present at levels approaching the 110-kDa/100-kDa chains. Since the latter are thought to be present at one copy each in the 26 S protease (10), it seems unlikely that a high molecular mass component is solely responsible for the observed mobility difference. Subunit patterns for the two 26 S enzymes are also remarkably similar to that of the ball component. Aligning lanes 1–3 in Fig. 6 with the profiles from either slow or fast 26 S, one can match virtually every subunit with molecular masses greater than 30 kDa. The ball component would seem to be missing only MCP subunits, a fact confirmed by the Western blots in Fig. 8.

The Effects of Nucleotides on the Two Forms of 26 S Protease—Besides differing in electrophoretic mobility, the two 26 S species are differentially affected by nucleotides. This was shown by a series of experiments in which a mixture of the two proteases was incubated in the presence of various nucleoside triphosphates, nucleotide analogs, or apyrase.
Multiple 20 S and 26 S Proteases

Fig. 6. Subunit compositions of 26 S,, 26 S,, and ball components. Panel A, glycerol gradient-purified 26 S proteases and the ball component (see "Results") were electrophoresed on native gels and localized by peptide overlay (left) or by Coomassie staining (right). Panel B, gel lanes equivalent to those shown in panel A were overlaid with fluorogenic peptide to localize the 26 S enzymes, and the active regions of the gel were excised and boiled in SDS-PAGE sample buffer. These samples were then analyzed on 10% acrylamide gels, and the subunit patterns were obtained by silver staining. The polypeptides present in gel slices corresponding to regions of protease activity in panel A. The lanes marked slow and fast show the polypeptides present in gel slices corresponding roughly to zones 1–3 at the right side of panel A. Approximately 17, 8, and 17 μg of protein were applied in panel A to lanes III, II, and ball, respectively.

After incubation, the proteases were separated on native gels, and their activities were assayed by peptide overlay. Two representative experiments, presented in Fig. 7, demonstrate that the activity of 26 S decreases to a greater extent than 26 S when ATP is omitted or substituted by CTP, GTP or UTP, but both 26 S and 26 S remain active in the presence of ATP or the nonhydrolyzable analog AMP-PNP. EDTA appeared to have little effect on 26 S activity. The addition of apyrase results in the loss of most 26 S activity and the disappearance or altered migration of the remaining protein complexes.

Western Blot Analysis of 26 S, 20 S, and Ball Complexes Using Anti-MCP Antibodies—The ball component contains polypeptides with sizes typical of MCP subunits (e.g. 25–31 kDa), raising the possibility that inactive MCP subunits are present in the complex. This was tested by using anti-20 S antibodies to probe Western blots of the various protein assemblies. The nondenaturing gels in the upper three panels of Fig. 8 demonstrate that although immunoreactive MCP subunits are present in 26 S and 20 S proteases, they are not found associated with the ball component. The SDS-polyacrylamide gels in the lower two panels show that anti-20 S antibodies react with all Coomassie-staining subunits of human MCP. Moreover, the anti-20 S serum is shown to be highly specific for subunits of the multicatalytic protease since various other human red cell proteins, clearly evident in panel D, fail to react with the anti-20 S antibody (panel E). Thus, we interpret the absence of reactivity toward the ball (panel C) as strong evidence MCP subunits are absent from that protein complex. At the same time, the clear reactivity toward 26 S complexes confirms the presence of MCP subunits in these larger proteases.

Assembly of 26 S Protease from Fraction mII38 and Fluoresceinated MCP—Hershko and his colleagues (18) have shown that in the presence of ATP, the 26 S protease can be assembled from three components termed CF1, CF2, and CF3. Although both Hershko’s group (11) and Driscoll and Goldberg (12) have provided evidence that CF3 is the multicatalytic protease, some investigators do not believe that MCP is part of the larger protease (19). Thus, we have used a different approach to confirm this assignment. Purified rabbit MCP was labeled with fluorescein and mixed with increasing amounts of protein precipitated from fraction II by 38% ammonium sulfate. It is evident from the gels in Fig. 9 that generation of 26 S activity is paralleled by association of fluoresceinated MCP subunits with the 26 S complex. We
Fig. 9. Incorporation of fluoresceinated MCP subunits into the 26 S protease. Rabbit reticulocyte MCP labeled with 4 fluoresceins (F) per particle was incubated in the presence of 2 mM ATP and varying amounts of a 38% ammonium sulfate precipitate of modified fraction II (see "Experimental Procedures"). Panel A, samples from each incubation were separated on native gels and photographed directly to localize fluorescein-labeled MCP subunits. Panel B, the same gel was then overlaid with sLLVY-MCA to detect 20 S and 26 S protease activities. The + signs above specific lanes indicate that the incubation contained fluorescein-20 S. Fraction ml138 contained 5 mg/ml protein, and the volume added to each reaction is shown. The two lanes at the right represent controls in which ml138 was boiled prior to mixing or in which ATP was omitted from the mixture.

consider this result to be a direct demonstration that MCP subunits are incorporated into the ubiquitin/ATP-dependent 26 S protease.

Assembly of the 26 S Protease from MCP and the Ball Components—When high molecular mass complexes in DEAE samples 167-185 (Fig. 2) were centrifuged on glycerol gradients, the ball component sedimented slower than the 26 S protease. The observed separation provided us with a protein complex that exhibited no protease activity and lacked MCP subunits but was comprised of polypeptides characteristic of the ubiquitin/ATP-dependent protease (see Fig. 6, panel A). Because the molecular masses of CF1 and CF2 have been estimated at 600 and 250 kDa, respectively (18), and because the ball component sediments as though it is ~700 kDa, we hypothesized that CF1 and CF2 preassemble to form the ball. If so, the multicatalytic protease and the ball component should combine to produce active 26 S. This was tested by incubating the two protein complexes in the presence of ATP followed by analysis of the reaction mixture on native gels. The peptide overlays and Coomassie patterns in Fig. 10 confirm that 26 S proteases can be assembled from two slower sedimenting particles, MCP and the ball.

DISCUSSION

The multicatalytic protease has been implicated in various reactions ranging from energy-independent degradation of oxidized proteins (20-23) to ATP-stimulated degradation of ubiquitin-lysozyme conjugates (10) and antigen presentation by major histocompatibility complex I receptors (24-29). If the enzyme participates in such diverse processes, one might expect it to interact with other cellular components that would provide specificity for substrate selection. This expectation is supported by the experiments presented above. We have identified five distinct forms of MCP; four of these (20 Ss30, 20 Ss16, and 26 Ss/26 Ss) are, in fact, associated with one or more additional polypeptides. Moreover, these extra chains can markedly affect catalytic activity or substrate selection by MCP.

The fast electrophoretic form, 20 S, is the simplest MCP, at least in terms of subunit composition. On one-dimensional SDS-PAGE, rabbit reticulocyte MCP can be resolved into 8-10 bands which vary in size from 22 to 32 kDa (see Fig. 3). We consider 20 S, to have the "traditional" MCP subunit composition reported by numerous investigators (for review, see Refs. 7 and 8). It should be noted that 20 S cleaves a variety of fluorogenic peptides, but it does so with very low apparent specific activity (Fig. 4). Of the two slower migrating forms, 20 Ss30 also contains subunits with molecular masses below 35 kDa. However, the 32-kDa subunit in 20 S is underrepresented, and a novel 30-kDa subunit is present. As shown in Fig. 5 and covered in detail in the following article (30), 20 S can be converted to 20 Ss30 by incubating the enzyme with a regulatory component that supplies the 30-kDa subunits. A second highly active species of multicatalytic protease, 20 Ss16, consists of the traditional MCP subunits and a 160-kDa chain. Like 20 Ss30, this form of the enzyme also cleaves sLLVY-MCA much better than 20 S and also migrates slower on native gels. The existence of two highly active MCPs, 20 Ss30 and 20 Ss16, raises questions concerning their interrelationships. We have considered the possibility that the 30-kDa subunit in 20 Ss30 is a proteolytic fragment of the 160-kDa polypeptide. Although we do not favor this idea, it cannot presently be eliminated.

Two MCPs identified in this study, 20 Ss30 and 20 Ss16, can be considered active, whereas 20 S probably qualifies as a latent form of the enzyme. Although there is an extensive literature on activators of MCP, most demonstrations of MCP activation have questionable biological relevance. For example, Mykles (31), who has shown that lobster MCP can be activated by heating at 60 °C, points out that lobsters reach that temperature only once in their lives. Similarly, enhanced peptide cleavage following exposure of MCPs to SDS (32-41), high levels of fatty acids (33), or supraphysiological concentrations of Ca2+ (42), demonstrates the importance of the particle's structure on its activity. But such findings do not suggest plausible physiological mechanisms. By contrast, the fact that a cellular protein complex can increase sLLVY-MCA cleavage, and do so reversibly (see 30), suggests that this activation reaction may prove to be an important regulatory step.

It is attractive to speculate that increased peptide hydrolysis by 20 Ss30 and 20 Ss16 is attended by their altered ability to
recognize and degrade cellular proteins. Although this hypothesis remains to be tested, it is clear that some forms of MCP do alter substrate specificity. The two 26 S enzymes can degrade ubiquitin-lysosome conjugates, whereas 20 S and 20 S_{26} cannot (10). Thus, the association of MCP subunits with ball components confers important substrate selection characteristics on the smaller protease.

Our assertion that MCP combines with the ball component to form the 26 S enzyme conflicts directly with a recent paper by Kloetzel and his colleagues (19). Based on their inability to detect MCP subunits in the larger complex, they concluded that the 26 S enzyme and MCP are unrelated proteases. They admit that their negative result might be explained by insufficient sensitive reagents. In Fig. 8 we show that antibodies to human MCP do, indeed, react with 26 S proteases separated on native gels. Moreover, we provide direct evidence that fluorescein-labeled MCP subunits are incorporated into the 26 S enzyme in an ATP-dependent reaction (Fig. 9). When these results are coupled with those of Herskho's group (11), Driscoll and Goldberg (12) and Orino et al. (13), the evidence that MCP and ball combine to produce 26 S proteases would seem overwhelming. Thus, we attribute the contrary view to insufficiently sensitive analyses, and we consider the ball and cylinder or “mushroom” model for the 26 S protease strengthened significantly by the results presented here.

We have shown two mechanisms for activating sLLVY-MCA cleavage by MCP. In the first, 20 S is activated by 30-kDa subunits present in the human red cell regulator fraction (Fig. 5). In the second, sLLVY-MCA hydrolysis is markedly enhanced when MCP is mixed with the ball component and assembled into 26 S proteases (Fig. 10). Interestingly, 30-kDa subunits are also present in the ball component. Although we do not yet know if the same 30-kDa proteins exist in both ball and regulator, this is clearly an important question for future study.

The present paper has focused mainly on structural features of the 20 S and 26 S proteases, such as subunit composition, electrophoretic mobility, and sedimentation characteristics. However, a functional aspect of MCP deserves some comment. Based on differential sensitivity of Leu-Leu-Glu-p-nitroanilide hydrolysis to detergents or protease inhibitors, Orlovski et al. (40) and Djaballah and Rivett (43) have described two classes of peptidylglutamyl peptide hydrolyzing sites in the multicatalytic protease. The two active sites are apparently thought to reside within the same particle. Although our identification of closely related, yet distinct, MCPs does not speak directly to the issue of the multicatalytic nature of individual complexes, they suggest an additional possibility for “multiple” sites that hydrolyze a particular peptide. Namely, Leu-Leu-Glu-p-nitroanilide active sites with distinct catalytic properties are present in separate protease complexes. We hypothesize that within cells relatively inactive 20 S_{20} particles may be activated by assembly with various other components to produce distinct proteolytic complexes. Future kinetic studies on the known species of MCP (e.g. 20 S_{26}, 20 S_{26}, and 20 S_{26}) should extend our understanding of intracellular proteolysis.

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Multiple forms of the 20 S multicatalytic and the 26 S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate.
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