Purification of an 11 S Regulator of the Multicatalytic Protease*

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We have identified and purified a protein complex from human red blood cells that activates the multicatalytic protease (MCP). The complex, which we call the regulator, sediments at 11 S and is composed of 30-kDa subunits. The regulator does not hydrolyze fluorogenic peptides, but when multicatalytic protease and regulator are combined, MCP cleaves succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin and Leu-Leu-Glu-p-nitroanilide as much as 60-fold faster. Hydrolysis of several other fluorogenic peptides is stimulated to a lesser extent, and activated MCP does not degrade ubiquitin-lysozyme conjugates, bovine serum albumin, or lysozyme. Latent and activated forms of MCP display similar sensitivity to protease inhibitors, suggesting that activation does not generate new kinds of catalytic sites. In addition, ATP suppresses peptide hydrolysis by activated and latent MCPs to the same extent.

Activation involves binding of regulator to MCP, and activated MCP migrates slower on native acrylamide gels. Dissociation of the MCP regulator complex during prolonged sedimentation on glycerol gradients releases active regulator and MCP molecules capable of being reactivated. Moreover, two-dimensional electrophoresis does not reveal changes in MCP or regulator subunits following activation. Thus, activation appears to result from reversible association of regulator subunits with MCP.

The cytosol and nuclear compartments of eucaryotic cells contain a large (~700 kDa) protease called the multicatalytic protease (MCP) or the proteosome (for review, see Refs. 1 and 2). Kinetic and inhibitor studies have provided evidence for three or more distinct proteolytic activities within the particle (3–6). Consistent with the presence of multiple active sites is the fact that the enzyme is composed of at least 12 separate subunits ranging in molecular mass from 20 to 32 kDa (7, 8). cDNAs encoding a number of these subunits have been isolated and sequenced from a variety of organisms including mammals, Xenopus, Drosophila, yeast, and archaeabacteria (9–19). The available sequences do not reveal obvious homologies to known protease families, and there has been recent debate over whether MCP is a serine or cysteine protease (20–22).

Electron microscopic analyses indicate that MCP is a cylinder measuring 11 x 16 nm in outer dimensions (23). Six or possibly seven subunits appear to form "lifesaver"-shaped rings. These in turn are stacked four deep to form a cylinder with a pore measuring ~2 nm in diameter. It is not clear that the pore is exposed to solvent (24) or that a cylinder is the only conformation adopted by the protease since raspberry-like structures have also been reported (25).

MCP subunits can be isolated as particles sedimenting at 20 S or associated with higher molecular weight polypeptides in 26 S complexes (26). The larger protease can be formed by incubating MCP in the presence of ATP/CTP and partially purified components from rabbit reticuloocyte lysozyme (27, 28), rabbit muscle (29), or human kidney (30). The 26 S protease is able to degrade ubiquitin-lysozyme conjugates in an ATP-stimulated reaction, and it exhibits enhanced degradation of succinyl-Leu-Leu-Val-Tyr-MCA (sLLVY-MCA) in the presence of nucleotides (26). Finding MCP subunits in several proteolytic complexes raises a number of questions. To what extent is each protease responsible for the breakdown of cellular proteins? How many different polypeptides associate with MCP subunits? Do the different proteolytic complexes exhibit distinct substrate specificity? In the preceding paper (28), we described electrophoretically distinct species of rabbit reticuloocyte MCP that hydrolyze sLLVY-MCA at high rates. Here, we describe the purification of a protein complex from human red blood cells that confers both slower electrophoretic mobility and increased enzymatic activity to human red blood cell MCP.

**EXPERIMENTAL PROCEDURES**

Materials—Outdated whole human blood was purchased from IHC Blood Services, LDS Hospital, Salt Lake City, UT. Calpain inhibitors I and II and DTT were purchased from Boehringer Mannheim, Glycrol was from JT Baker, TSK-Gel Toyopearl DEAE-650 S and TSK-Gel Toyopearl HW55 S chromatography resins were obtained from Supelco, Belfonte, PA. The fluorogenic peptide, succinyl-Leu-Leu-Val-Tyr-MCA and the proteolytic inhibitors leupeptin and chymostatin were supplied by Peninsula Laboratories, Inc., Belmont, CA. The fluorogenic peptides Pro-Phe-Arg-MCA and Cbz-Leu-Leu-Glu-pNA, as well as the protease inhibitors phenylmethylsulfonyl fluoride, p-hydroxymercuriphenylsulfonic acid, and N-ethylmaleimide were purchased from Sigma. The fluorogenic peptide glutaryl-Gly-Gly-MCA was from Enzyme Systems Products. The calpain inhibitor Cbz-Val-Phe-Aldehyde was a gift from S. Medhi at Merrell Dow Research Institute.

Purification of Regulator from Human Red Blood Cells—Twenty units of outdated human blood were washed three times with phosphate-buffered saline (2.7 mM KCl, 136.9 mM NaCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 0.7 mM CaCl2, 0.5 mM MgCl2) by centrifugation at 8,000 rpm in a JA20 Beckman rotor at 4°C. Theuffy coat was removed after each spin, and the washed red cells were lysed in 4 volumes of cold 1 mM DTT followed by centrifugation at 10,000 rpm for 60 min to remove membranes. The supernatant was brought to a final volume of 18 liters by adding glyceral to 20% (v/v), mixed with
1.7 liters of TSK-DEAE resin previously equilibrated in 10 mM Tris, pH 7.0, 1 mM DTT, 20% glycerol, pH 7.0 (TDG), and rotated overnight at 4 °C. The resin was collected by filtration and washed with 4 liters of TSDG, which is 10 mM Tris, pH 7.0, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 20% glycerol, pH 7.0. The resin was then transferred to a 5 × 100-cm column of TSDG and 1 liter of TSDG containing 100 mM KCl. The column was developed with an 8-liter linear gradient from 100 to 500 mM KCl in TSDG at a flow rate of 2.5 ml/min. Fractions of 20 ml were collected (see Fig. 2).

Those fractions containing significant amounts of MCP regulator (fractions 65-95 in Fig. 2) were concentrated to 42 ml by pressure filtration and passed over a 5 × 90-cm column of TSK-HW55 at 2.5 ml/min in TSDG (see Fig. 3). Fractions 40-47 (160 ml) were pooled and used for the assays shown in all figures except 6 and 8. Partially purified regulator is stable for at least 4 months at -80 °C, and this preparation retained more than 50% of the initial activity after 6 months at 4 °C.

A portion (25 ml) of the TSK-HW55 pool was reapplied to a 1 × 20-cm TSK-DEAE column equilibrated in TSDG, and the column was developed with a gradient of KCl extending from 0.05 to 0.15 M (see Fig. 4). Regulator activity eluted in two peaks, which were pooled separately. Pool A (fractions 100-104) was diluted to 14% glycerol by the addition of TSDG and was layered onto 30-ml glycerol gradients separately. Pool A (fractions 100-104) was diluted to 14% glycerol by the addition of TSDG and was layered onto 30-ml glycerol gradients (16-40% v/v in TSD). After centrifuging for 41 h at 25,000 rpm in an SW 28 rotor, the gradient was collected in 2-ml fractions by bottom puncture. MCP regulator activity resolved as a single symmetrical peak centered at fraction 8. Similar results were obtained using pool B (fractions 108-112), although the resulting specific activity for this regulator fraction was 2-fold lower after glycerol gradient centrifugation.

**Assay of Regulator**—The multicatalytic protease and the regulator were assayed by spectrophotometry. The standard regulator assay consists of 3 μg of human red cell 20 S (1 μl of a 3 mg/ml stock in TSD (no glycerol)), 0-40 μl of regulator (in TSDG), and reticulocyte buffer (RB; 20 mM Tris, pH 7.8, 5 mM MgCl₂, 10 mM KCl, and 5 mM DTT) to 50 μl. The mixture was incubated at 37 °C for 10 min before reactions were initiated by the addition of 50 μl of 200 μM sLLVY-MCA in RB. Fluorescence was monitored continuously for 1-2 min in a Perkin-Elmer fluorometer using an excitation wavelength of 380 nm and an emission wavelength of 440 nm with MCA peptide substrates (see Fig. 1A) and excitation at 335 nm, emission at 410 nm for LLE-pNA. The 20 S activity in the absence of regulator is 5 pmol of sLLVY-MCA hydrolyzed per min/μg. One unit of regulator doubles the rate of sLLVY-MCA cleavage from 3 μg of MCP under standard conditions.

**Separation Techniques**—Native gel electrophoresis and substrate overlay gels were performed as described in the preceding paper (28). The method of O’Farrell (31) was used for two-dimensional PAGE. Proteins were stained with Coomassie Brilliant Blue or by use of the Bio-Rad silver stain kit. Glycerol gradient centrifugation was performed as described in Hough et al. (26).

**Radiolabeling of Proteins and Autoradiography**—Proteins were radioiodinated by the chloramine-T or Bolton-Hunter procedures as described (32). Ubiquitin-lysosome conjugates were prepared as described previously (33). After SDS-PAGE, radiolabeled proteins were detected by exposing gels to Kodak XAR film.

**RESULTS**

**Purification of a Regulator of MCP**—In the course of isolating the 26 S protease from outdated human blood, we surveyed fractions from the initial DEAE column for their ability to stimulate peptide hydrolysis by purified human MCP. Such an activity was found among proteins eluting at relatively low salt concentrations (see Fig. 2). The original mixing experiments were done in the presence of ATP because we were searching for the ball component described in the preceding paper (28). To our surprise, sLLVY-MCA hydrolysis by MCP was stimulated by the DEAE components in the absence of ATP. Thus, activation was not caused by the ball component of the 26 S protease; rather it was a result of a novel factor which we call regulator of MCP or simply the regulator.

The upper panels in Fig. 1 show that peptide hydrolysis is proportional to added regulator, and panel C in Fig. 1 demonstrates that increased proteolysis is a property of the 20 S protease which, when activated, migrates slower after gel electrophoresis of activated MCP. The upper portion of panel C shows MCA fluorescence overlaying a native gel in which MCP/regulator mixtures were separated for 3 h at 150 volts. The same gel, fixed and stained with Coomassie Brilliant Blue, is shown in the bottom half of panel C. The numbers below each lane denote the μl of regulator added to 3 μg of MCP prior to electrophoresis. The gel regions excised for SDS-PAGE buffer. The samples were then separated on 10% PAGE gels, and protein subunits were identified by silver staining. Panel C in Fig. 1 demonstrates that increased proteolysis is a property of the 20 S protease which, when activated, migrates slower after gel electrophoresis of activated MCP. The upper portion of panel C shows MCA fluorescence overlaying a native gel in which MCP/regulator mixtures were separated for 3 h at 150 volts. The same gel, fixed and stained with Coomassie Brilliant Blue, is shown in the bottom half of panel C. The numbers below each lane denote the μl of regulator added to 3 μg of MCP prior to electrophoresis. The gel regions excised for SDS-PAGE, radiolabeled proteins were detected by exposing gels to Kodak XAR film.

**FIG. 1. Fluorometric and electrophoretic assay of the MCP regulator.** Panel A, fluorometric assay of regulator. The tracings show hydrolysis of sLLVY-MCA by a fixed amount of MCP in the presence of increasing amounts of regulator. The number associated with each solid trace indicates the μl of regulator fraction added. The dotted tracing at the very bottom represents fluorescence observed from 25 μl of regulator in the absence of MCP. Conditions for the assay are described under “Experimental Procedures.” Panel B, quantitation of regulator. Rates of peptide hydrolysis at the left are plotted against volume of regulator added to demonstrate linearity between sLLVY-MCA cleavage and regulator. Panel C, native gel electrophoresis of activated MCP. The upper portion of panel C shows MCA fluorescence overlaying a native gel in which MCP/regulator mixtures were separated for 3 h at 150 volts. The same gel, fixed and stained with Coomassie Brilliant Blue, is shown in the bottom half of panel C. The numbers below each lane denote the μl of regulator added to 3 μg of MCP prior to electrophoresis. The gel regions excised for SDS-PAGE, radiolabeled proteins were detected by exposing gels to Kodak XAR film.
points between pH 5.2 and 5.5. The lower member of the doublet also resolves into three to five species that are slightly more basic. Although the basis for this isoelectric heterogeneity is unknown, phosphorylation is a likely possibility.

A major protein with a molecular mass of ~35 kDa and a pI of 5.7 is also present in Fig. 6. This is a subunit of human lactate dehydrogenase as demonstrated by direct amino acid sequencing and Western blot analyses (data not shown). Native gel detection of regulator (see below, Fig. 7) reveals that the 35-kDa subunit (lactate dehydrogenase) does not stimulate sLLVY-MCA cleavage by MCP.

Size and Subunit Composition of the MCP Regulator—The native regulator was judged to be about 150–300 kDa based on its elution from TSK-HW55 (Fig. 3). We also analyzed a crude sample of regulator by native gel electrophoresis. Upon overlaying native gels with peptide solutions containing low levels of MCP, the regulator could be localized because it enhances sLLVY-MCA cleavage by MCP molecules in the buffer above the gel (Fig. 7, upper panel). Regions of regulator activity were excised from the gel and analyzed by SDS-PAGE, revealing the two ~30-kDa subunits (lower panel in Fig. 7). When native gel electrophoresis of regulator was performed with proteins of known size, the regulator migrated between catalase (240 kDa) and aldehyde dehydrogenase (150 kDa) indicating a native molecular mass of ~200 kDa (data not shown). A final estimate of its size was obtained by comparing its sedimentation on 16–40% glycerol gradients with that of known standards. As shown by the activity profile in Fig. 8, the regulator co-sediments with catalase. Hence, its sedimentation coefficient is about 11 S.

Association of Regulator and MCP—The substrate overlay and protein stained gels in panel C of Fig. 1 demonstrate that after being mixed with regulator, the multicatalytic protease migrates more slowly on native gels. The fact that MCA fluorescence appeared directly over the new position of MCP does not, however, speak to the mechanism underlying activation. It is possible that the regulator covalently modifies subunits within the multicatalytic protease. Alternatively, the regulator might remove an inhibitor from MCP or might simply bind to the protease and alter the catalytic efficiency of MCP subunits. It is even possible that, upon forming a complex with MCP, the regulator becomes an active protease.

A number of approaches were employed to examine these possibilities. We first repeated an experiment presented in the preceding paper (28). Purified human MCP was incubated with increasing amounts of regulator, and the mixtures were electrophoresed on native gels. The activated, and hence slower migrating, MCP was excised from the gel, and its polypeptide composition was examined by SDS-PAGE. The subunit patterns in Fig. 1, panel D, confirm that activation is accompanied by the appearance of novel 30–32-kDa proteins.
22372

11 S Regulator of MCP

TABLE I
Purification of an 11 S regulator of MCP

Complete details of the purification steps are provided under “Experimental Procedures.”

| Fraction               | Protein |
|------------------------|---------|
|                        | Total   |
|                        | activity|
|                        | Recovery|
|                        | %       |
| DEAE pool 1            | 42.0    |
|                        | 11.50   |
|                        | 16,800  |
|                        | 483.0   |
|                        | 705,600 |
|                        | 1,388   |
|                        | 100%    |
| HW55 pool              | 160.0   |
|                        | 1.10    |
|                        | 3,300   |
|                        | 176.0   |
|                        | 528,000 |
|                        | 5,030   |
|                        | 3,000   |
|                        | 75      |
| DEAE pool 2            | 46.0    |
|                        | 0.66    |
|                        | 3,320   |
|                        | 30.4    |
|                        | 152,720 |
|                        | 5,030   |
|                        | 22%     |
| Glycerol gradient      | 9.9     |
| pool                   | 0.50    |
|                        | 11,300  |
|                        | 4.9     |
|                        | 111,384 |
|                        | 22,600  |
|                        | 16      |

* Since our assay for regulator cannot be used in crude extracts, we have arbitrarily designated the DEAE pool as 100% recovery.

Only 25 ml of the HW55 pool was subjected to full purification. Accordingly, the entries for DEAE pool 2 have been multiplied by 6.4 to provide a consistent set of values.

Because DEAE pool 2 represents half of the activity (fractions 100–104 in Fig. 4), the apparent recovery is artificially low.

Because only a portion of DEAE pool 2 was subjected to glycerol gradient centrifugation, tabular entries have been adjusted to reflect further purification of the entire DEAE pool 2.

![Fig. 6. Two-dimensional PAGE of MCP regulator.](image)

at the electrophoretic position of MCP. The experiment does not, however, reveal whether the new 30-kDa polypeptides are modified MCP subunits or derived from the regulator. This was addressed in the following experiment.

A relatively impure preparation of regulator (see “Experimental Procedures”) was radiolabeled with 125I-Bolton-Hunter reagent and mixed with purified human MCP. The combined MCP regulator fraction and radiolabeled regulator alone were then analyzed on 10–30% glycerol gradients. If activation of peptide hydrolysis results from association of regulator with MCP, one might expect an iodinated component from the regulator preparation to co-sediment with activated MCP. The upper panel in Fig. 9 presents the distribution of 125I across both gradients. Mixing MCP and regulator produced a shoulder on the radioactive regulator profile, and the position of the shoulder exactly matched the distribution of MCP activity. SDS-PAGE analyses of individual fractions from the glycerol gradient (lower panel of Fig. 9) revealed that among the 15 or so radiolabeled proteins in the regulator preparation, only a pair of 30-kDa proteins significantly altered their sedimentation in the presence of the multicatalytic protease.

**Activation and Covalent Modification of Subunits**—We conclude from the experiment just described that increased hydrolysis of sLLVY-MCA by MCP results from the binding of 30-kDa regulatory subunits to the large protease. However, it could not be determined from one-dimensional SDS-PAGE whether any covalent modifications occurred after binding. For this reason, two-dimensional PAGE was used to examine the patterns of each component before and after activation. The gel presented in panel A of Fig. 10 shows a two-dimensional pattern typical for the regulator. Although the pattern is slightly complicated because of contaminating proteins, the regulator subunits are clearly resolved (arrows). Panel B shows a two-dimensional pattern for MCP. Panels C and D represent mixtures of regulator and MCP in which components were incubated together for 10 min under conditions promoting activation and then quenched with IEF buffer (panel C), or each component was dissolved in IEF sample buffer, which contains 9 m urea, prior to mixing (panel D). Since the patterns in panels C and D are virtually identical, activation does not appear to be accompanied by modification of MCP or regulator subunits.

**Reversibility of Activation**—This question of covalent modification was addressed in a second experiment. We reasoned...
that if activation involved events such as phosphate removal, peptide cleavage, and deubiquitination, it would be an irreversable process, at least using highly purified components. Hence, mixing experiments were performed at vastly differing ratios of regulator to MCP to effect complete activation of MCP or complete binding of regulator to the protease. In the experiment shown in Fig. 11, MCP and regulator were mixed at a molar ratio of 6 (MCP/regulator) based on molecular masses of 700 kDa for MCP and 200 kDa for regulator. The samples were then incubated at 37 °C for 10 min and sedimented on 15–40% glycerol gradients for 40 h. Gradients were collected and analyzed for protease or regulator activities; individual gradient fractions were also examined by SDS-PAGE to determine the location of regulator or protease subunits.

It is clear from a comparison of regulator activities in panels B and C of Fig. 11 that adding a large excess of MCP did not permanently consume the regulator; that is, about 50% of the regulator activity was recovered from the gradient containing both components. However, consistent with the results in Fig. 9, the multicatalytic protease markedly influenced the sedimentation of regulator. Alone, the regulator sedimented to fractions 18 and 19; when mixed with MCP the regulator was found as a broad peak centered on fraction 14. The SDS-PAGE gels in panels E and F reinforce the connection between 30-kDa subunits and regulator activity since the 30-kDa species are the only proteins with altered sedimentation in the presence of MCP.

We interpret these results as follows. MCP and regulator are in dynamic equilibrium during the centrifugation run. As sedimentation proceeds, the larger protease eventually separates from regulator, and the net effect is displacement of the regulator to a location in the glycerol gradient between MCP and its position when centrifuged alone. The fact that all regulator subunits exhibited altered sedimentation shows that sufficient MCP was present to bind every regulatory complex. Recovery of regulator activity (closed circles, panel B), in turn, demonstrates that regulator molecules are not permanently inactivated by binding MCP. The equivalent experiment in which the ratio was 10 regulators/MCP resulted in the recovery of a fully reactivatable MCP following sedimentation (data not shown). Thus, activation of MCP does not consume the regulator or convert MCP to a nonactivatable state.

On the surface, the results of Fig. 11 are seemingly inconsistent with those in Fig. 9; that is, in Fig. 9 the regulator appears to remain fully associated with MCP, whereas the regulator has clearly dissociated from the protease in the experiment shown in Fig. 11. Although the apparent discrepancy can be explained by both dilution and increased sepa-
FIG. 11. Sedimentation analyses of the association between MCP regulator and MCP. Reaction mixtures (2 ml) contained (panel A) 4.4 mg of MCP, (panel B) 4.4 mg of MCP + 0.13 mg of regulator, and (panel C) 0.13 mg of regulator in TSD containing 10% glycerol. These solutions were incubated at 37 °C for 10 min and then layered atop 28 ml of 15–40% glycerol gradients and centrifuged for 40 h at 25,000 rpm in an SW 28 rotor. Gradients were collected from the bottom, and each 1-ml fraction was assayed for protein, MCP, and regulator. Closed boxes denote MCP activity in the absence of regulator; open boxes signify MCP activity observed after adding 30 µl of crude activation. The dashed line denotes A280. Silver-stained SDS-PAGE gels of relevant fractions from the gradients are shown in panels D–F.

ration of MCP and regulator during the longer centrifugation in Fig. 11, a competition experiment was performed to confirm that regulator and MCP are in dynamic equilibrium. A crude regulator fraction was radioiodinated and incubated with MCP for 10 min at 37 °C. A 500-fold excess of unlabelled regulator fraction was added to half of this reaction mixture. Both samples were then analyzed on glycerol gradients, and their sedimentation profiles were compared with that of regulator alone. It can be seen in Fig. 12 that in the absence of MCP the labeled 30-kDa regulator subunits sedimented to fractions 23–28. By contrast, in the presence of MCP most radioiodinated regulator subunits were found in fractions 17–20. Addition of excess unlabeled regulator prior to sedimentation resulted in the labeled 30-kDa subunits again being found in fractions 23–28. The ability of unlabeled regulator subunits to displace their iodinated counterparts from MCP demonstrates that regulator and MCP are in dynamic equilibrium. It should also be noted in panel B that the labeled regulator subunits are shifted by one to two fractions from the peak of MCP activity shown in panel D. Thus, by collecting smaller fractions (e.g. 0.5 ml in Fig. 12 versus 0.8 ml in Fig. 9), one can observe dissociation of MCP regulator complexes even at shorter times of sedimentation.

Peptide Specificity of Activation—The analyses presented thus far utilized an excellent fluorogenic substrate for MCP, Suc-Leu-Leu-Val-Tyr-MCA. Several additional fluorogenic peptides were examined to determine whether activation is specific for individual active sites, e.g. the so-called trypsin versus chymotrypsin sites, or whether it involves a global property of MCP. Hydrolysis rates for four fluorogenic peptides were measured in the presence of increasing amounts of regulator. According to the data in Fig. 13, cleavage of sLLVY-MCA and LLE-pNA was increased 50–70-fold, whereas hydrolysis of PFR-MCA was activated 10-fold and breakdown of GGF-MCA increased only 3-fold.

Protein Breakdown by Activated MCP—Based on equivalent extents of activation by detergent, Orłowski et al. (34) have suggested that the pepitdyglutamyl peptide hydrolyzing site is responsible for protein breakdown by MCP. Because the regulator markedly stimulates LLE-pNA hydrolysis by MCP, we examined the degradation of several proteins including ubiquitin-lysozyme conjugates. The data in Table I show that the regulator does not significantly stimulate proteolysis of bovine serum albumin, casein, lysozyme, or ubiquitin-lysozyme conjugates; nor does it have much effect on the response of MCP to nucleotides. In almost every case, ATP inhibits peptide or protein hydrolysis by 10–50%.

Inhibitor Sensitivity of Latent and Activated Forms of MCP—Although it seems reasonable to assume that hydrolysis of say, sLLVY-MCA, occurs at the same catalytic site in
Fig. 13. Activation of peptide hydrolysis by MCP regulator. Reaction mixtures contained 3 μg of MCP, 0–20 μl of TSK-HW55-purified regulator in a final volume of 50 μl of RB. Enzyme reactions were initiated by the addition of 50 μl of 200 μM fluorogenic peptide in RB and continuously monitored for 1–2 min in a Perkin-Elmer fluorometer. The ordinate is initial velocity in the presence of regulator divided by the initial velocity observed for MCP alone.

Table II
Effect of regulator on the substrate specificity of MCP

| Substrate      | Degradation, MCP alone | Degradation, MCP + regulator |
|----------------|------------------------|------------------------------|
|                | -ATP +ATP              | -ATP +ATP                    |
| 125I-Bovine serum albumin | 0 | 0 | 0 | 0 |
| 125I-Lysozyme  | 1 | 1 | 1 | 1 |
| 125I-Casein    | 19 | 10 | 25 | 16 |
| 125I-Ubiquitin/lysozyme conjugates | 2 | 2 | 4 | 3 |
| Suc-RPFLLYV-MCA | 1 | 0 | 2 | 1 |
| Suc-GPLGP-MCA  | 0 | 0 | 1 | 1 |
| Suc-LLVY-MCA   | 12 | 9 | 311 | 325 |
| Z-GGR-MCA      | 1 | 1 | 3 | 2 |
| Glut.GGF.MCA   | 7 | 8 | 25 | 16 |
| PFR-MCA        | 1 | 1 | 23 | 15 |
| Cbz-LLE-pNA    | 8 | 4 | 84 | 74 |

Table II shows that both latent and activated MCP complexes, this need not be the case. Binding of regulator subunits to MCP could activate a cryptic protease site in the large complex. In fact, subunits of the regulator might be latent proteases that become active after binding to MCP. Since protease inhibitors can be useful diagnostic tools, we examined the sensitivity of latent and activated MCPs to various inhibitory compounds. The results, presented in Table III, are consistent with the idea that the regulator increases catalysis at sites preexisting in MCP; that is, there is good agreement between inhibitor profiles for latent and activated species of MCP. Other than increased sensitivity to most compounds, activated and latent MCPs respond similarly to each inhibitor. Moreover, this equivalence is observed with specific fluorogenic peptides despite the fact that some inhibitors are more effective with a given substrate, e.g. leupeptin and PFR-MCA.

DISCUSSION

On first principles, one might expect that cells would possess efficient mechanisms for regulating the activity of potentially lethal cytosolic proteases; and in fact, a decade of studies on the calpains has revealed a variety of methods by which these Ca2+-activated proteases are controlled. Besides calcium availability, control mechanisms include removal of a propeptide from the 80-kDa heavy chain (35), interaction of the enzyme with membranes or phospholipids (36, 37), interaction of the protease with calpastatin, its well characterized inhibitor (38, 39), and the existence of calpain activators (40–43). The calpains thus appear to be subject to various forms of regulation (for reviews see Refs. 44 and 45).

MCP is likewise an abundant cytosolic protease capable of degrading certain proteins such as oxidant–damaged hemoglobin, caseins, and the B chain of insulin (46–48). Etlinger and his colleagues have described two intracellular protein inhibitors of the large protease (49, 50). The results reported here and those of Yukawa et al. (51), who described an activator of MCP present in human platelet extracts, suggest that activation might also be a major regulatory mechanism. The existence of MCP activators is not surprising considering its structure. If the proteolytic active sites line the cylinder's pore, degradation of intact proteins requires mechanisms for unfolding and inserting polypeptides into the pore or ways to expose the buried active sites. In the preceding paper (28), we isolated a multisubunit complex, the ball, that stimulates hydrolysis of sLLVY-MCA by MCP. The 26 S protease so formed degrades ubiquitin-lysozyme conjugates in the presence of ATP, and we believe that energy is used to move polypeptides into the pore of MCP.

In contrast, several pieces of evidence suggest that a simpler mechanism is involved in the stimulation of MCP peptidase activity by the regulator. First, the regulator does not enable MCP to degrade intact proteins or ubiquitin-lysozyme conjugates (Table II). Second, the regulator does not appear to generate any novel protease activity; that is, peptides that are not substrates in the absence of regulator, such as Suc-GPLGP-MCA, remain undegraded after addition of regulator. Similarly, the inhibitor studies presented in Table III are consistent with the idea that peptide hydrolysis occurs at sites preexisting in MCP. Third, kinetic studies indicate that the regulator increases Vmax rather than altering Km.

2 Taken together, these observations indicate that binding of the regulator induces a conformational change in MCP that allows better access of small substrates and/or release of products. Finally, the role of ATP provides a further distinction between activation of MCP by the 20 S ball component and by the 11 S regulator. Whereas ATP is necessary both for 20 S protease formation and function (52), it is not needed for activation of the 20 S protease by the regulator. From the inhibitor data in Table III, we concluded that the regulator does not contribute new active sites to MCP. This conclusion should be viewed with caution for the following reason. In Fig. 4 of the preceding paper (28), gel overlays demonstrated that MCP alone (20 S) can cleave peptides, albeit at very slow rates. We are not certain that the activity exhibited by “MCP” in Table III is actually contributed by MCP devoid of any bound regulator. Indeed, the gel overlay in Fig. 1C suggests that even in the

2 Y. Yoo and M. Rechsteiner, manuscript in preparation.
apparent absence of regulator, the weak residual activity migrates as 20 S_m. Until we are able to make kinetic measurements on MCP particles known to be free of regulator, our conclusion that regulator subunits do not contribute protease active sites must remain a tentative one.

Although the 30-kDa size of regulator subunits immediately suggests that they might be members of the MCP family, two observations argue against this idea. First, the positions of regulator subunits on two-dimensional polyacrylamide gels do not correspond to that of any described MCP subunits. Second, and much more telling, we have sequenced six peptides comprising 92 amino acids from the lower member of the regulator doublet; they exhibit no homology to MCP subunits or any known proteases. The sequence data strengthen, but do not prove, our inference that regulator subunits do not confer additional protease sites to MCP.

Several experiments indicate that the mechanism underlying increased peptide hydrolysis is the reversible formation of MCP regulator complexes. The sedimentation analysis in Fig. 9 shows that a pair of 30-kDa subunits in a crude regulator fraction binds to and sediments with MCP. A repeat of this experiment using large amounts of unlabeled components not only confirms that MCP alters the sedimentation of regulator but also shows that both MCP and regulator can be recovered in active form (Fig. 11). The results in Fig. 12 demonstrate that excess unlabeled regulator subunits can displace their radioiodinated counterparts from MCP. Finally, the two-dimensional PAGE analyses in Fig. 10 do not reveal any modifications to MCP or regulator subunits. In aggregate, these experiments strongly suggest that activation of peptide hydrolysis is the result of the reversible binding of 30-kDa regulator subunits to MCP.

It is notable that the activation of peptide hydrolysis varies so much among the fluorogenic substrates (Fig. 13). This would seem to argue against a global conformational change induced by regulator binding unless one proposes that specific active sites are differentially masked in “latent” MCP. For example, one could hypothesize that the chymotrypsin site (sLLVY-MCA) and the peptidyl glutamyl site (LLE-MCA) are somehow “buried” deeper within the cylinder than the PFR-MCA (trypsin) site. This reasoning does not, however, explain why cleavage of GGF-MCA is stimulated so little since it, too, should be a substrate for the chymotrypsin site.

The data can also be explained by assuming that the regulator enhances product release and that peptides with adjacent leucines (e.g. LLE and LLVY) are particularly difficult to release. In any event, it is clear that the regulator has markedly different effects depending upon the substrate.

In summary, we have identified and substantially purified an 11 S complex that activates peptide hydrolysis by MCP. All available evidence indicates that reversible binding of regulator to MCP is the mechanism for increasing peptide hydrolysis. Still, a number of important questions remain. What is the configuration of regulator subunits within the 11 S complex? Are they arranged as a ring? Does binding of regulator displace the higher molecular mass subunits from MCP? Is the ultrastructure of MCP significantly altered upon binding regulator? Why are some peptides affected to greater extents than others? Does the regulator affect substrate selection in vivo? These questions provide clear focus for future experiments.

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