Activation of the Multicatalytic Endopeptidase by Oxidants. Effects on Enzyme Structure†

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ABSTRACT: It is well established that the functional properties of proteins can be compromised by oxidative damage and, in vivo, proteins modified by oxidants are rapidly degraded. It was hypothesized that oxidants may also affect the ability of proteases to hydrolyze peptides and proteins. We therefore examined the effect of oxidants on the endopeptidase activities of the 650 kDa 20S proteasome or multicatalytic endopeptidase (MCP), which is thought to play a central role in nonlysosomal protein breakdown. Treatment of the MCP with the oxidant system, $FeSO_4$−EDTA−ascorbate, stimulated the peptidase activities of the MCP while H_2O_2 treatment showed little or no stimulation. However, treatment of the MCP with $FeSO_4$−EDTA−ascorbate or H_2O_2 stimulated proteinase activity by 480% and 730%, respectively. An endogenous activator of the MCP, PA28, stimulated the acidic, basic, and hydrophobic peptidase activities of the MCP, but had no effect on proteolytic activity. Treatment of PA28 with oxidants in the presence of MCP or alone did not greatly affect PA28's ability to activate the peptidase activities of the MCP. Using nondenaturing polyacrylamide gel electrophoresis, structural alterations in the enzyme which may be responsible for the activation of peptidase and protease activities following exposure to oxidants were investigated. Treatment of the MCP with reagents that activate proteolysis, including H_2O_2, as well as the serine protease inhibitor 3,4-dichloroisocoumarin and the cysteine protease inhibitor *p*-(chloromercuri)benzenesulfonic acid, all caused dissociation of the 650 kDa MCP. However, exposure to $FeSO_4$−EDTA−ascorbate resulted in little or no dissociation of the complex. The MCP complex dissociated by *p*-(chloromercuri)benzenesulfonic acid could be reassociated upon treatment with the reducing agent dithiothreitol, but dithiothreitol failed to completely reassociate 3,4-dichloroisocoumarin- or H_2O_2-treated MCP. Therefore, chemical modification of the MCP can cause activation with varying degrees of complex dissociation. These results suggest that metabolites, such as reactive oxygen species, in addition to endogenous proteins, such as PA28, are capable of modulating MCP activity.

Proteins postsynthetically modified by oxidants are rapidly broken down in cells (Fagan et al., 1986) and are degraded at a faster rate than the native forms of the proteins by various proteolytic enzymes *in vitro* (Rivett, 1985a; Salo et al., 1988; Fagan & Waxman, 1991, 1992). One such enzyme is the multicatalytic endopeptidase (EC 3.4.99.46) (MCP) which has been shown to degrade oxidatively damaged (Ox-) glutamine synthetase (Rivett, 1985b), Ox-hemoglobin (Pacifici, 1989; Fagan & Waxman, 1991), Ox-superoxide dismutase (Salo et al., 1988), and Ox-insulin B chain (Dick et al., 1991). The MCP has also been reported to degrade oxidatively damaged proteins in liver cells in culture (Grune et al., 1995).

The crystal structure of the MCP from archaebacterium has recently been solved, and it indicates that the complex consists of 14 copies of 2 different subunits (Lowe et al., 1995). This enzyme may represent a new class of proteolytic enzymes in which a threonine rather than a serine residue participates in catalysis. The mammalian MCP which is structurally similar, hydrolyzes both proteins and peptides, and, by using a variety of peptide substrates, it has been shown to have five distinct peptidase active sites (Orlowski et al., 1993). The three best characterized peptidase activities are the acid, basic, and neutral activities which are defined by their ability to hydrolyze benzyloxycarbonyl-Leu-Leu-Glu-methoxynaphthylamine (Z-LLE-MNA), benzyloxycarbonyl-Ala-Arg-Arg-aminomethylcoumarin (Z-ARR-AMC), and succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin (Suc-LLVY-AMC), respectively. The peptidase activity of the MCP is stimulated by two cellular proteins known as PA700 (molecular mass ≈700 kDa) (Chu-Ping et al., 1994) and PA28 (molecular mass ≈180 kDa) (Chu-Ping et al., 1992) which have been isolated from red blood cells. PA700 requires ATP to bind to the MCP in order to stimulate peptidase activity while PA28 does not. PA28 is composed of two nonidentical (molecular mass ∼27.3 and 28.6 kDa), but homologous subunits (Mott et al., 1994). Using immunoelectron microscopy, PA28 has been shown to form a regulatory cap on top of the stacked rings of the MCP (Gray et al., 1994). While endogenous peptidase activators of the MCP have been identified, no cellular proteins have been found which stimulate the degradation of proteins. The MCP can, however, be activated to degrade large proteins such as casein by treatment with sodium dodecyl sulfate, fatty acids (Dahlmann et al., 1985), and polylysine (Tanaka et al., 1986),

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by dialysis against water (McGuire et al., 1989), by heat treatment (Mykles, 1989), and by treatment with polyarginine, protamine (Mellgren, 1990), ammonium sulfate (Fagan & Waxman, 1991), and magnesium ions (Pereira et al., 1992b). The serine protease inhibitor 3,4-dichloroisocoumarin (DCIC) (Strack et al., 1992; Cardozo et al., 1992; Pereira et al., 1992a) and the cysteine protease inhibitor *p*-(chloromercuri)benzoate (pCMB) (Figueiredo-Pereira et al., 1994) have also been found to stimulate casein hydrolysis by the MCP. Figueiredo-Pereira et al. established that the MCP complex can be reversibly dissociated by pCMB but that only the native structure possesses catalytic activity (1994). We found that oxidants also activated the MCP and investigated the effect of oxidants and DCIC on the quaternary structure of the MCP as well as the effect of PA28 on the oxidized enzyme.

MATERIALS AND METHODS

Preparation of Chicken Red Blood Cell Extracts. Fresh erythrocytes (0.5 L) were collected in heparinized (20 USP units/mL) saline (0.5 L) from 4 month old male White Leghorn chickens. Chicken erythrocyte cytoplasm was isolated as described previously (Strack et al., 1991). Blood was centrifuged at 800*g* for 10 min, and the plasma proteins and white blood cells were removed by aspiration. Packed erythrocytes were washed 4 times by resuspending the pellet in cold (4 °C) saline (1:1) and again centrifuged. Cells were lysed in 2 volumes of cold water containing 1 mM dithiothreitol (DTT). Following centrifugation (13500*g*, 1 h), the soluble fraction was decanted, glycerol was added to 20% (v/v), and the pH was adjusted to 7.6 with 1 M Tris base. The cytoplasm was then dialyzed against 20% glycerol, 20 mM Tris-HCl (pH 7.6), 0.1 mM DTT, and 0.1 mM ethylenediaminetetraacetic acid (EDTA).

Separation of PA28 from the MCP. Dialyzed erythrocyte cytoplasm (0.265 L, 14 500 mg of protein) was top-loaded (1.0 mL/min) onto a Q Sepharose Fast Flow (Fast Q, Pharmacia) column (2.5 × 19 cm) equilibrated with 20 mM Tris-HCl (pH 7.6), 20% glycerol, 0.1 mM EDTA, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 20 mg/L sodium azide, and 0.5 mM DTT (Fast Q buffer). The column was washed with 2.2 L of Fast Q buffer, and the bound material was eluted with a 0.5 L NaCl gradient (0.0−0.5 M). Fractions were assayed with the fluorogenic substrate Suc-LLVY-AMC to detect the MCP. Fractions were then reassayed in the presence of 0.5 μg of partially purified MCP to identify the elution position of the PA28 activator. The activator and the MCP were pooled separately. The major cytosolic form of the MCP was purified from chicken red blood cells as described by Strack et al. (1992) and used in the experiments described below.

Treatment of Proteins with Oxidants. The MCP and PA28 were treated with oxidants by a procedure similar to that described by Amici et al. (1989). Proteins were dialyzed (4 °C) against two changes of 50 mM phosphate buffer (pH 7.2), 90 mM potassium chloride, and 10 mM $MgCl_2$ (buffer A). Prior to oxidant treatment, the Fast Q pooled material (1 mg/mL) was pretreated with 0.25 mM sodium azide to inhibit catalase. Fast Q pooled material (1 mg/mL) and purified MCP (0.3 mg/mL) were incubated with either 20 mM H_2O_2, except where indicated, or a mixture of 2 mM iron sulfate ($FeSO_4$), 2 mM EDTA, and 25 mM ascorbic acid (pH 7.2) ($FeSO_4$−EDTA−ascorbate) for 1 h at 37 °C in a shaking water bath. After incubation, the samples (0.25−2.5 mL) were dialyzed using a membrane having a 12−14 kDa cutoff against two changes (125 mL) of 50 mM phosphate buffer (pH 7.4), 1 mM EDTA and then against 50 mM phosphate buffer (pH 7.4).

Assays. α-Casein was tritiated using [^{3}H]formaldehyde and sodium cyanoborohydride (Jenthoft et al., 1983). The degradation of [^{3}H]casein was monitored by measuring the release of acid-soluble counts. Assays contained 50 mM phosphate buffer (pH 7.4), 1 mM DTT, and 5 μg of casein (2 μg of [^{3}H]-α-casein and 3 μg of unlabeled α-casein) in a final volume of 200 μL. The specific details of each assay are provided in the figure and table legends. After 1 h at 37 °C, the assays were terminated by the addition of 25 μL of 10% bovine serum albumin (BSA) and 575 μL of 12% trichloroacetic acid (TCA). Samples were centrifuged at 800*g* for 12 min, and acid-soluble material (400 μL) was removed, 4.0 mL of scintillant (Ecoscint A; National Diagnostics, Atlanta, GA) was added, and the disintegrations per minute (dpm) were measured in a liquid scintillation counter. Fluorogenic peptide hydrolysis was carried out as described for the measurement of casein hydrolysis with the exception that the assay mixture contained 67 μM peptide substrate and the incubation time was 30 min. Assays were terminated by the addition of 0.1 mL of 1% sodium dodecyl sulfate (SDS) and 1.7 mL of 0.2 M sodium borate (pH 9.1). Hydrolytic activity was determined by measuring the increase in fluorescence due to release of the fluorophore from the peptide at 380 nm excitation, 460 nm emission for MNA substrates and 335 nm excitation, 410 nm emission for AMC substrates. Protein was quantitated by the dye binding method of Bradford (1976) with BSA as standard using a kit from Bio-Rad. All assays were carried out in triplicate or quadruplicate. The data were analyzed using ANOVA and a post-hoc Duncan's Multiple Range Test. Significance was set at the 5% level.

Gel Electrophoresis. One-dimension sodium dodecyl sulfate−polyacrylamide gel electrophoresis (1D-SDS−PAGE) was carried out by the method of Laemmli (1970) with a 6% stacking gel and a 12.5% resolving gel using a mini gel format (Hoefer). Gels (8 × 7 cm) were run at constant current (20 mA/gel) until the dye front came within 2 mm of the end of the gel. High and low molecular mass prestained protein standards were purchased from Bethesda Research Laboratories. Gels were silver-stained using a Stain-II Daiichi kit (Integrated Separation Systems). 2D-SDS−PAGE was performed as described by O'Farrell (1975). Nondenaturing gels were run at 50 V constant voltage in a Hoefer mini gel apparatus as described in the legend to Figure 7.

RESULTS

Separation of PA28 and MCP. Suc-LLVY-AMC is a sensitive substrate for monitoring the activation by PA28 of MCP (Chu-Ping et al., 1992), and it was used to detect both PA28 and MCP when these proteins were fractionated by chromatography on Q Sepharose Fast Flow (Fast Q). In the absence of added MCP (see Materials and Methods), a single peak of Suc-LLVY-AMC hydrolyzing activity was identified

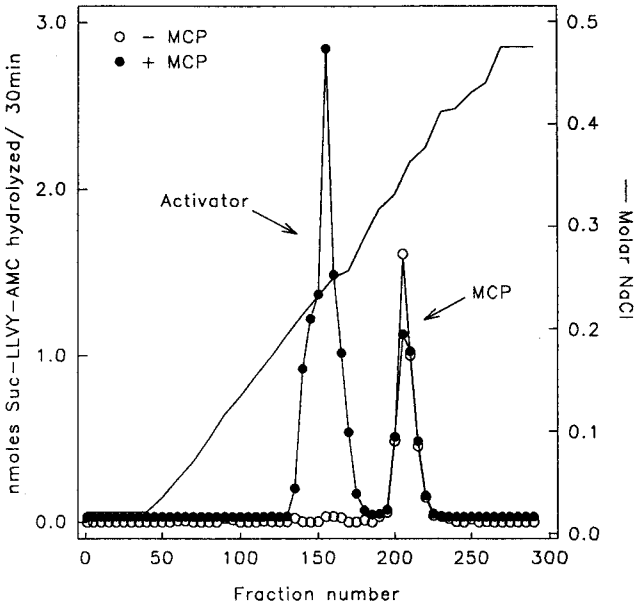

FIGURE 1: Separation of a peptidase activator and MCP present in chicken red blood cell cytoplasm. The hydrolysis of Suc-LLVY-AMC over the Fast Q gradient was measured in assays containing 10 μL of selected fractions, 1 mM DTT, 50 mM Tris-HCl (pH 8.0), 67 μM Suc-LLVY-AMC, and, where indicated, 0.5 μg of purified MCP [480 pmol/h^{-1} (μg of Suc-LLVY-AMC hydrolyzing activity)$^{-1}$] in a final volume of 200 μL. Assays were incubated for 15 min and processed as described under Materials and Methods.

in the fractions from the Fast Q column eluting at 0.35 M NaCl (Figure 1). When the assays were carried out in the presence of added purified MCP (240 pmol hydrolyzed/h), a second peak of activity which eluted earlier in the gradient at 0.25 M NaCl was also detected (Figure 1). The two peaks of activity were pooled separately, and the molecular masses were determined by gel filtration on a calibrated S-300 (Pharmacia) column (results not shown). The peak of activity which eluted first from Fast Q and required the addition of MCP for activity had a molecular mass of ~170 kDa. This activity corresponds to the 180 kDa activator previously described in red blood cells as PA28 (Chu-Ping et al., 1992) and was used in several of the experiments described in this study. The second peak of activity had a molecular mass of ~700 kDa on gel filtration, and following additional purification (Strack et al., 1992) showed ~8 bands ranging from 23 to 32 kDa on 1D-SDS−PAGE. This protein was identified as the MCP and was used in the experiments below following further purification.

Effect of Oxidants and PA28 on the Peptidase and Proteinase Activities of the MCP. The MCP hydrolyzed three fluorogenic peptides known to be substrates for the MCP: Suc-LLVY-AMC (75 pmol h^{-1} μg^{-1}), Z-ARR-AMC (60 pmol h^{-1} μg^{-1}), and Z-LLE-MNA (17 pmol h^{-1} μg^{-1}). Treatment with $FeSO_4$−EDTA−ascorbate increased the hydrolysis of all three peptides by approximately 280%, 180%, and 110%, respectively (Figure 2). However, treatment of the MCP with the oxidant hydrogen peroxide (20 mM) decreased the hydrolysis of Suc-LLVY-AMC by 57%, had no effect on Z-ARR-AMC cleavage, and slightly increased the hydrolysis of Z-LLE-MNA by 38% (Figure 2). Lower concentrations of H_2O_2 (0.2 and 2 mM) had little effect on the rate of hydrolysis of the fluorogenic peptides, whereas higher concentrations (0.2 and 2 M) inhibited hydrolysis (Figure 3; data not shown).

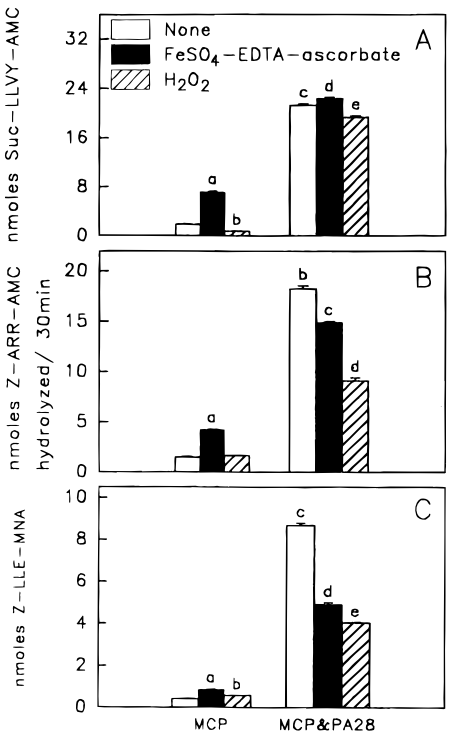

FIGURE 2: Effect of PA28 and the oxidants $FeSO_4$−EDTA−ascorbate and H_2O_2 on the peptidase activities of the MCP. Protein pools from the Fast Q column adjusted to 1.0 mg of protein/mL were treated with oxidants as described under Materials and Methods. After dialysis to remove oxidants, protein concentrations were determined. Assays of PA28 and MCP alone contained 50 μg of each protein specified. Assays containing MCP and PA28 which had been mixed together and then oxidized contained 0.1 mg of protein. Assays were carried out, and hydrolysis of Suc-LLVY-AMC (panel A), Z-ARR-AMC (panel B), and Z-LLE-MNA (panel C) was measured as described under Materials and Methods. Values represent the means ± SEM. Groups showing different letters were significantly different ($p \leq 0.05$) from each other and from untreated MCP.

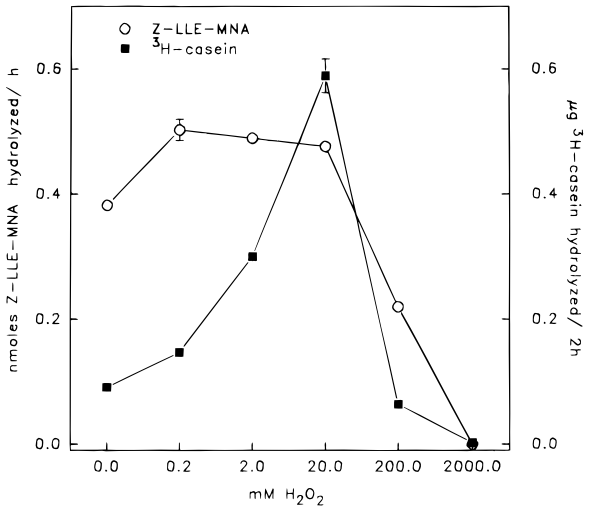

FIGURE 3: H_2O_2 concentration dependence on the peptidase and proteinase activities of MCP. Purified MCP was treated as described in Figure 5 with various concentrations of H_2O_2. Assays contained 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 5 μg of [^{3}H]casein (78 100 dpm/μg). Z-LLE-MNA hydrolysis was carried out for 1 h and [^{3}H]casein for 2 h.

The effect of oxidants on the casein degrading activity of the MCP was also examined. The MCP hydrolyzed [^{3}H]-casein to acid-soluble fragments [20 μg h^{-1} (μg of MCP)$^{-1}$], and this activity was stimulated by treatment with $FeSO_4$−

EDTA−ascorbate (480%) and 20 mM H_2O_2 (730%) (data not shown). [^{3}H]Casein degradation was also stimulated by lower concentrations (0.2 and 2 mM) of H_2O_2, while higher concentrations either inhibited (0.2 M H_2O_2) or resulted in a complete loss of activity (2 M H_2O_2) (Figure 3).

Since PA28 has been shown to stimulate the peptidase activities of the MCP (Chu-Ping et al., 1992), we examined the effect of oxidants on this process. The PA28 activator, which was partially purified and separated from the MCP on Fast Q Sepharose, had no detectable peptidase activity (Figure 1), nor could any be detected after oxidant treatment (data not shown). In the absence of oxidants, addition of 50 μg of partially purified PA28 to 50 μg of MCP was found to maximally stimulate the hydrolysis of all three substrates: Suc-LLVY-AMC by 1030%, Z-ARR-AMC by 1110%, and Z-LLE-MNA by 1980%. When the MCP and PA28 were incubated together and then treated with $FeSO_4$−EDTA−ascorbate, the hydrolysis of Z-ARR-AMC and Z-LLE-MNA was diminished by 18% and 43%, respectively, while Suc-LLVY-AMC hydrolysis remained unchanged. The effect of H_2O_2 on the stimulation of MCP peptidase activity by PA28 was similar to that seen with $FeSO_4$−EDTA−ascorbate: the hydrophobic peptidase activity was slightly inhibited while the acidic and basic peptidase activities were decreased by half (Figure 2).

The effect of oxidation of PA28 on peptide hydrolysis by the MCP was also examined by incubation with MCP which had either been oxidized or left untreated. Oxidant treatment of either PA28, the MCP, or both decreased the level of stimulation of Suc-LLVY-AMC hydrolysis by the activator by only 6%. On the other hand, oxidant-treated PA28 stimulated MCP hydrolysis of Z-ARR-AMC and Z-LLE-MNA about two-thirds as well as untreated PA28, while the stimulation of oxidized MCP by untreated PA28 was reduced by about 10%. The greatest reduction (~50%) in the stimulation by PA28 of Z-ARR-AMC and Z-LLE-MNA hydrolysis was observed when both components had been oxidized, either in separate incubations or together.

Unlike its general stimulatory effect on peptidase activity, PA28 had no effect on casein hydrolysis, in accord with prior studies (Chu-Ping et al., 1992). Moreover, oxidant treatment of the MCP in the presence of PA28 had no effect on the stimulation of casein hydrolysis by either $FeSO_4$−EDTA−ascorbate or H_2O_2, nor did addition of PA28 to MCP which had been previously oxidized (data not shown).

Effect of Oxidants on Subunit Sizes of the MCP. The MCP isolated from chicken red blood cytoplasm displayed the characteristic pattern of ~8 bands ranging in size from 23 to 32 kDa on 1D-SDS−PAGE (Figure 4, lane 2). Treatment of the MCP with the oxidant generating system $FeSO_4$−EDTA−ascorbate (Figure 4, lane 3) had little effect on the subunit banding pattern between 23 and 32 kDa. However, a small amount of protein aggregation was detected between 40 and 68 kDa following treatment with this oxidant or when the MCP was treated with H_2O_2 (Figure 4). When the subunits of the MCP were separated by 2D-SDS−PAGE, the control and oxidant-treated enzyme showed the same pattern of ~28 spots determined previously (Strack et al., 1992) (results not shown).

Effect of pCMBS, DCIC, and Oxidants on the Peptide and Protein Hydrolyzing Activity of the MCP. It has been suggested that activation of the MCP requires a rearrangement of the subunits in the complex (Figueredo-Pereira et al., 1994). This model was based on the observation that the mercurial pCMB dissociated the MCP, resulting in loss of activity, and following the addition of DTT, the complex reassembled and proteolytic activity was stimulated. This was tested by comparing the effect of DTT on enzyme treated with pCMBS, the oxidants H_2O_2 and $FeSO_4$−EDTA−ascorbate, and DCIC, a serine protease inhibitor shown previously to stimulate casein hydrolysis by the MCP (Strack et al., 1992; Pereira et al., 1992a). Treatment of MCP with DTT had little effect on the Z-LLE-MNA and [^{3}H]casein hydrolyzing activities (Table 1). Treatment of MCP with 50 μM pCMBS completely inhibited the Z-LLE-MNA hydrolyzing activity, and the addition of 1 mM DTT restored only 36% of this activity (Table 1). When examined over a broad range of concentrations, it was found that 10 μM pCMBS inhibited ~90% of the Z-LLE-MNA hydrolyzing activity (Figure 5). At the highest pCMBS concentration tested (400 μM), DTT (1 mM) was unable to restore this activity (Figure 5). pCMBS also inhibited [^{3}H]casein hydrolysis in the absence of DTT by 85% (Table 1 and Figure 6). These results are in contrast to those of Figueiredo-Pereira et al. (1994), who showed that pCMB stimulated casein hydrolysis in the absence of DTT. This difference may be attributable to the incorporation of negative charges into the protein which would occur when cysteine residues react with pCMBS but not pCMB. However, addition of DTT to pCMBS-treated MCP resulted in a stimulation (171%) of [^{3}H]casein degrading activity (Table 1 and Figure 6). This stimulatory effect of DTT occurred at pCMBS concentrations above 10 μM and maximally at 100 μM (Figure 6). This is in contrast to DCIC treatment, which stimulated the [^{3}H]casein hydrolyzing activity by 238% independent of subsequent treatment with DTT (Table 1).

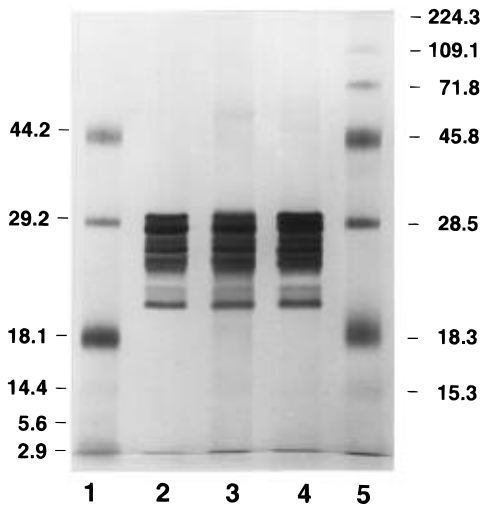

FIGURE 4: One-dimensional polyacrylamide gel electrophoresis of the MCP treated with oxidants. Lane 1, low molecular mass protein standards (apparent molecular mass): ovalbumin, 44.4 kDa; carbonic anhydrase, 29.2 kDa; α-lactoglobulin, 18 kDa; lysozyme, 14 kDa; bovine trypsin inhibitor, 5.6 kDa; insulin A chain, 2.9 kDa. Lane 2, 1 μg of chicken red blood cell MCP. Lane 3, 1 μg of MCP treated with $FeSO_4$−EDTA−ascorbate as described under Materials and Methods. Lane 4, 1 μg of MCP treated with H_2O_2 as described under Materials and Methods. Lane 5, high molecular mass protein standards (apparent molecular mass): myosin (H-chain), 224.3 kDa; phosphorylase *b*, 109.1 kDa; bovine serum albumin, 71.8 kDa; ovalbumin, 45.8 kDa; carbonic anhydrase, 28.8 kDa; β-lactoglobulin, 18.3 kDa; lysozyme, 15.3 kDa. The proteins were stained with silver.

Table 1: Effect of pCMBS, DCIC, and Oxidants on the Peptide and Protein Hydrolyzing Activity of the MCP[a]

addition	Z-LLE-MNA nmol hydrolyzed	Z-LLE-MNA % activity	[^{3}H]casein μg hydrolyzed	[^{3}H]casein % activity
none				
−DTT	0.93	100	0.34	100
+DTT	0.95	102	0.33	97
pCMBS				
−DTT	0.01	1	0.05	15
+DTT	0.34	37	0.92	271
DCIC				
−DTT	0.57	61	1.16	338
+DTT	0.87	94	1.15	338
$FeSO_4$−EDTA−ascorbate				
−DTT	2.03	218	0.59	174
+DTT	2.11	227	0.76	224
H_2O_2				
−DTT	0.58	62	0.52	153
+DTT	0.57	61	0.59	174

[a] Assays were carried out as described under Materials and Methods containing 1.5 μg of purified MCP, 50 mM Tris-HCl (pH 8.0), and preincubated at 25 °C for 10 min as indicated with either 50 μM DCIC, 50 μM pCMBS (solubilized in DMSO), or oxidants as described. All assays contained 0.63% DMSO. DTT (1 mM) was then added where indicated, and the mixtures were further incubated at 25 °C for 30 min. Z-LLE-MNA or [^{3}H]casein (80000 dpm/μg) was added, and assays were carried out as described under Materials and Methods.

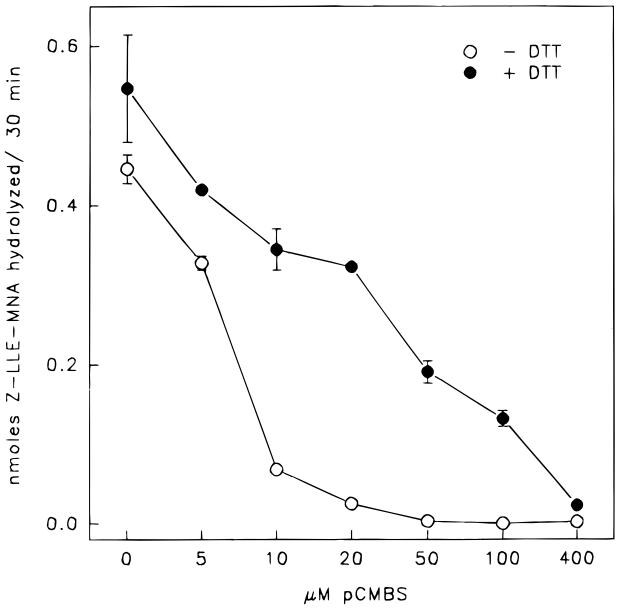

FIGURE 5: Effect of pCMBS on the MCP's peptidase activity. Assays were carried out as described in Table 1.

H_2O_2 treatment of the MCP decreased Z-LLE-MNA hydrolysis (39%), and activity could not be restored by the addition of DTT (Table 1). [^{3}H]Casein hydrolysis was stimulated 53% by H_2O_2 treatment and 74% upon the addition of DTT. These results are similar to what was observed with DCIC and pCMBS. However, the degree of stimulation of peptide and protein hydrolysis by MCP treated with H_2O_2, DCIC, and pCMBS differed from that measured with the oxidant system $FeSO_4$−EDTA−ascorbate. $FeSO_4$−EDTA−ascorbate stimulated both Z-LLE-MNA (118%, 127%) and [^{3}H]casein (74%, 124%) in the absence and presence of DTT, respectively.

Effect of Oxidants, pCMBS, and DCIC on the Structure of the MCP Complex. Electrophoresis of the MCP under

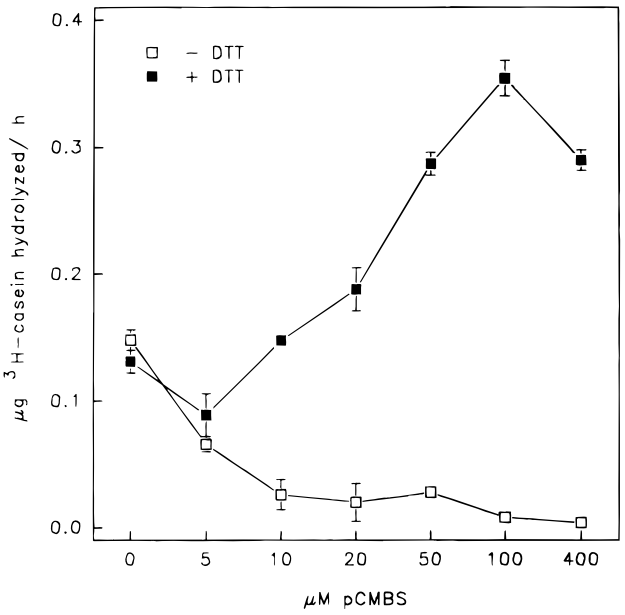

FIGURE 6: Effect of pCMBS on the MCP proteinase activity. Assays were carried out as described in Table 1.

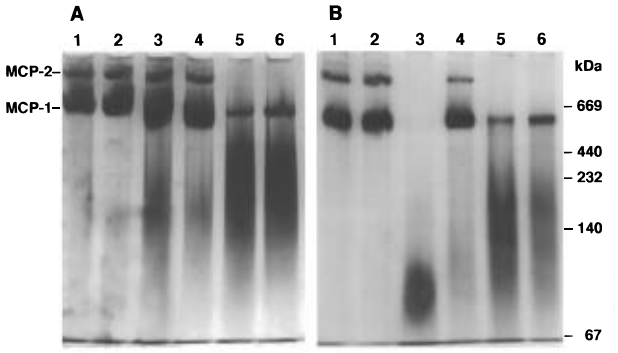

FIGURE 7: Nondenaturing polyacrylamide gel electrophoresis of MCP treated with oxidants, pCMBS, and DCIC. MCP (1.5 μg) was treated either with oxidant (A) as described under Materials and Methods or with protease inhibitors (B) for 10 min at 23 °C and for another 30 min with dithiothreitol (1 mM) where indicated. Protease inhibitors were dissolved in dimethyl sulfoxide (DMSO), and all samples contained 0.19% DMSO. Proteins were resolved on 5% polyacrylamide gels run at 50 V, 4 °C, using a Tris (25 mM)−glycine (192 mM) buffer. Molecular mass standards (Pharmacia) (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; and bovine serum albumin, 67 kDa) were used only as a means of reference and not to determine molecular mass since electrophoretic mobility is a function of protein size and charge. The gels were stained with silver.

nondenaturing conditions showed two distinct protein bands, one having a mass of 650 kDa corresponding to the native size of the MCP (MCP-1) and a second with an apparent mass of ~1300 kDa (Figure 7A, lane 1) (MCP-2). No protein bands were present at masses below 650 kDa. Although it is possible that the 1300 kDa band is a dimer of the purified MCP, this species is not detected on gel filtration (data not shown). Since nondenaturing gels separate proteins based on size as well as charge, it is likely that MCP-2 is a charge variant of MCP-1. This conclusion is supported by SDS−PAGE of the material applied to the nondenaturing gel which shows only the subunits which are characteristic of the MCP (Figure 4). In addition, activity against Z-LLE-MNA was found in the region of the gel containing MCP-1 and MCP-2, and polyclonal antibodies raised against the

Table 2: Densitometric Scan of Nondenaturing Gels of MCP Treated with Oxidants, pCMBS, and DCIC[a]

	lane densities, area (AU mm)											
lane:	1A	2A	3A	4A	5A	6A	1B	2B	3B	4B	5B	6B
treatment:	none	none	$FeSO_4$−EDTA−ascorb	$FeSO_4$−EDTA−ascorb	H_2O_2	H_2O_2	none	none	pCMBS	pCMBS	DCIC	DCIC
DTT:	−	+	−	+	−	+	−	+	−	+	−	+
MCP-2	0.28	0.44	0.37	0.39	0.00	0.00	0.49	0.61	0.00	0.23	0.00	0.00
MCP-1	1.00	1.69	2.15	1.90	0.64	0.74	1.73	1.85	0.00	2.21	0.32	0.65

[a] The gels shown in Figure 7 were silver-stained, and density readings of the MCP bands in each lane were determined using an LKB densitometer. Densitometer readings were linear up to 1.5 μg of protein.

Table 3: Catalytic Activities of DCIC- and pCMBS-Treated MCP Subjected to Gel Filtration

		[^{3}H]casein degradation		Z-LLE-MNA hydrolysis	
	protein (μg)	ng degraded/h	ng degraded (μg of protein)$^{-1}$ h^{-1}	nmol hydrolyzed/h	nmol hydrolyzed (μg of protein)$^{-1}$ h^{-1}
MCP + DTT	30.2	5570	184	68.80	2.28
MCP + DCIC	16.8	7030	419	43.90	2.61
MCP + DCIC + DTT	21.1	6630	314	125.40	5.94
MCP + pCMBS	2.4	0	0		
MCP + pCMBS (+DTT)[a]	2.4	3280	1367	0.16	0.07

[a] (+DTT) indicates 1 mM DTT added to assays after MCP was separated on gel filtration. 69 μg of MCP was incubated 10 min at 23 °C with either 50 μM pCMBS or DCIC and for an additional 30 min in the absence or presence of 1 mM DTT where indicated. Approximately 52 μg was applied to a Superose 6 (Pharmacia) gel filtration column (1 cm × 30 cm) equilibrated with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. 0.5 mL fractions were collected and assayed with Z-LLE-MNA and [^{3}H]casein. The peak of activity in all cases eluted in fractions 27−29 which were combined. The pooled fractions were then assayed for total protein, [^{3}H]casein, and Z-LLE-MNA hydrolysis.

MCP from rat liver recognized both bands. The addition of DTT, which has been shown to cause reassociation of MCP subunits dissociated by treatment with mercurials (Figueiredo-Pereira et al., 1994), did not change the banding pattern but caused a slight increase in the staining intensities of the MCP-1 and MCP-2 bands (Table 2). Treatment of the MCP with the $FeSO_4$−EDTA−ascorbate oxidant generating system increased the intensity of the 650 kDa band (Table 2) and generated diffuse staining in the region below this band (Figure 7A). The addition of DTT to the $FeSO_4$−EDTA−ascorbate-treated MCP did not alter the staining intensities or change the banding pattern (Figure 7A, Table 2). Treatment of the MCP with the oxidant H_2O_2 produced a very different banding pattern. The MCP-2 band was not present in the H_2O_2-treated MCP, and the intensity of the 650 kDa band was decreased (Figure 7A, Table 2). H_2O_2 also increased the staining intensity in the region below 650 kDa. There was no change in the banding pattern or in band intensities when the H_2O_2-treated MCP was subsequently exposed to DTT (Figure 7A, Table 2). Treatment of the MCP with the thiol reagent pCMBS (Figure 7B, Table 2) or the serine protease inhibitor DCIC (Figure 7B, Table 2) resulted in the disappearance of the MCP-2 band and partial to almost complete loss of the MCP-1 band accompanied by increased staining below 650 kDa. The addition of DTT to MCP treated with DCIC resulted in only a slight increase in the MCP-1 band with no reassociation of the MCP-2 band (Figure 7B, Table 2). DTT, on the other hand, appeared to restore both bands of the pCMBS-treated MCP (Figure 7B, Table 2), which is in good agreement with the effect of DTT on pCMB-treated MCP reported recently (Figueiredo-Pereira et al., 1994).

DCIC treatment resulted in a significant loss of the MCP-1 and MCP-2 protein bands (Figure 7B, Table 2) but only a 40% decrease in peptidase activity and a 3-fold increase in proteolytic activity (Table 1). To address whether the residual full-sized enzyme is more active (i.e., has a higher specific activity) or the dissociated subunits are catalytically active, MCP was treated with DCIC or with DCIC followed by DTT and fractionated by gel filtration on Superose 6 in order to resolve native MCP from any partially dissociated species. Column fractions were assayed for their ability to hydrolyze casein and Z-LLE-MNA. Irrespective of the treatment, the only activity detected was in the elution position of native MCP with no evidence for any activity eluting at lower molecular weight (data not shown). The peak fraction was then combined with adjacent side fractions, and peptide and protein hydrolysis was measured. The specific activity of [^{3}H]casein degradation by the DCIC-treated MCP was more than 2-fold higher than the control (Table 3). DCIC-treated enzyme which had been incubated with DTT prior to gel filtration also had a specific activity higher than the control enzyme (Table 3), in agreement with the results shown in Table 1. DCIC treatment resulted in a loss of Z-LLE-MNA hydrolyzing activity, similar to the results shown in Table 1, although the specific activity of the enzyme against this substrate was slightly higher (Table 3). However, when DCIC-treated MCP was exposed to DTT prior to gel filtration, the enzyme after gel filtration had nearly twice the total activity and greater than 2.6-fold higher specific activity against the peptide substrate. Since the effect of DTT on DCIC-treated MCP was to return Z-LLE-MNA hydrolyzing activity nearly to control levels (Table 1), the enhanced rate of peptide hydrolysis after gel filtration suggests that this step removes an inhibitory component. To obtain a direct comparison with the results of Figueiredo-Pereira et al. (1994), MCP was treated with pCMBS and subjected to gel filtration. Only 8% of the protein and no activity were recovered in the position of the native MCP compared to the control, which is in good agreement with analysis of the same experiment on nondenaturing PAGE (Figure 7). Although this material had no detectable proteolytic activity, the specific activity of the casein-degrading component following treatment with DTT was 7.5-fold higher than that of the control (Table 3). In contrast,

DTT failed to regenerate Z-LLE-MNA hydrolyzing activity (Table 3).

DISCUSSION

The finding that oxidants can stimulate the degradation of proteins by the MCP may be physiologically relevant in that the cell has a mechanism for the efficient removal of postsynthetically modified proteins. Over the last decade, numerous studies have shown that oxidatively damaged proteins are more rapidly degraded than their native counterparts and that these proteins are targeted for rapid degradation by various intracellular proteases. In red blood cells, it was found that the metalloinsulinase played a significant role in the degradation of proteins damaged by oxidants (Fagan & Waxman, 1991, 1992). We reported that the MCP played a relatively minor role in this process since red cell extracts (that had not been exposed to oxidant) degraded oxidatively damaged hemoglobin equally well in the presence or absence of the MCP (Fagan & Waxman, 1992). Exposure of skeletal muscle extracts to H_2O_2 both increased the supply of substrates for the ATP-independent degradative system and appeared to activate the proteases which participate in the ATP-independent degradation of oxidatively damaged proteins (Gecha & Fagan, 1992). Proteolysis of cellular proteins, as well as oxidatively-modified superoxide dismutase and hemoglobin, was greatly increased in the lysate of cultured liver cells treated with mild oxidants (Grune et al., 1995). Therefore, exposure of cells and tissues to active oxygen species not only may postsynthetically modify proteins and make them better substrates for degradation but also may modulate the activity of specific proteases, either directly or indirectly, by their effect on endogenous activators or inhibitors.

While oxidants stimulated the hydrolysis of [^{3}H]casein by the MCP, they had little effect on the ability of PA28, an endogenous activator of the MCP, to stimulate peptidase activity. In addition, while some stimulation of peptidase activity following treatment of the MCP with oxidants was observed, it was much less effective (3−6-fold) than seen with PA28. However, the addition of PA28 to oxidant-treated MCP resulted in no greater stimulation of peptidase activity than with untreated MCP. Therefore, the effects of oxidant treatment and PA28 on peptidase activity are not synergistic. The observation that oxidant treatment failed to abolish the positive interaction of PA28 with the MCP also suggests that the regions of the MCP and PA28 involved in binding appear not to be susceptible to modification by oxidants.

Conformational changes brought about by oxidative modification of specific amino acids on subunits of the MCP may result in activation of the enzyme. Although the residues have not been identified, we suggest that cysteines may play a key role. *N*-Ethylmaleimide and dithiobis(2-nitrobenzoic acid) (Tanaka et al., 1986), as well as pCMB (Figueiredo-Pereria et al., 1994), all thiol-specific reagents, have been shown to activate the proteolytic activity of the MCP. While the oxidants H_2O_2 and $FeSO_4$−EDTA−ascorbate are less specific than the first three reagents, they can also oxidize thiol groups on proteins. However, unlike pCMB, their effects cannot be reversed by treatment with DTT. Finally, although the serine proteinase inhibitor DCIC is believed to inhibit the peptidase activities of the MCP by reacting with catalytic threonine residues (Lowe et al., 1995), this reagent can also modify protein sulfhydryl groups (L. Waxman, unpublished results). Because of the large size of the MCP, identification of those key amino acids, particularly the cysteine residues, which participate in the activation process presents a formidable challenge.

It has been proposed that relaxation of the structure of the MCP complex is essential for activating the MCP's proteolytic activity based on the finding that MCP exposed to the mercurial pCMB dissociated into inactive subcomplexes which could be reassociated in the presence of DTT, with recovery and activation of proteolytic activity (Figueiredo-Pereira et al., 1994). Similarly, in the studies presented here, pCMBS dissociated the complex and inhibited MCP's protease and peptidase activity. The complex reassembled following addition of DTT concomitant with stimulation of [^{3}H]casein degrading activity and partial restoration of peptidase activity. We have also shown that the serine protease inhibitor, DCIC, causes partial dissociation of the MCP and stimulation of casein hydrolysis. However, unlike the effect of DTT on pCMB- or pCMBS-treated MCP, the addition of DTT to DCIC-treated MCP was unable to facilitate reassociation of the complex. Furthermore, no evidence could be obtained in our studies for catalytically active subcomplexes (unpublished observations). Thus, our results support the suggestion that structural integrity is required for expression of the MCP's enzymatic activities (Figueiredo-Pereira et al., 1994).

Treatment of the MCP with the oxidant system $FeSO_4$−EDTA−ascorbate stimulated protein and peptide hydrolysis, but appeared to have little effect on the size of the MCP complex based upon nondenaturing PAGE. However, treatment of MCP with H_2O_2 resulted in subunit dissociation which could not be reversed with DTT, little change in the peptidase activity, and a stimulation of proteolytic activity. Similar to the effect of DCIC, neither dissociation nor reassociation of the complex appear to be absolutely required for activating MCP's proteolytic activity. On the basis of our observations, we propose that at least two pathways can lead to activation of proteolysis. In the first, modification by oxidants can induce conformational changes in MCP subunits similar to the rearrangement of the subunits promoted by a cycle of dissociation−reassociation. Alternatively, oxidants as well as other agents could exert their effect by compromising the binding of inhibitory subunits. Both possibilities are open to experimental verification.

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