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The Multicatalytic Proteinase Complex, a Major Extralysosomal Proteolytic System†

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The "multicatalytic proteinase complex" (MPC), first described in bovine pituitaries (Orlowski & Wilk, 1981, 1988; Wilk & Orlowski, 1980, 1983), is an unusually high molecular weight proteinase ($M_r \sim 700000$) composed of a series of low molecular weight nonidentical subunits. The complex exhibits three distinct endopeptidase activities, cleaving bonds on the carboxyl side of hydrophobic, acidic, and basic amino acid residues. Evidence indicates that each of these activities is associated with a distinct component of the complex. It is now well established that the MPC is present in all eukaryotic cells and that it constitutes up to 0.5–1% of the protein in tissue homogenates. There seems to be little doubt that the MPC can be an important factor in intracellular protein turnover, although direct evidence for this function in vivo has not yet been obtained. This review will focus on the biochemistry of the MPC and its relationship to the ubiquitin-dependent proteolytic system. For detailed reviews on the role of ubiquitin in intracellular proteolysis, the reader is referred to Rechsteiner (1987), Hershko (1988), and Hough et al. (1988).

The presence of high molecular weight proteinases in various tissue extracts was noticed by several investigators. Enzymes of this category were found in many tissues including bovine lens (Blow et al., 1975; van Heyningen, 1978), bovine pituitaries (Wilk et al., 1979), fish muscle (Hase et al., 1980; Folco et al., 1988), mouse liver (Rivett, 1985), rat skeletal muscle (Dahlmann et al., 1985a), and human erythrocytes (Edmunds & Pennington, 1982). It soon became evident that all these enzymes are identical with the MPC as defined above. Nevertheless, at least 20 different names have been proposed for these enzymes including high molecular weight proteinase, neutral protease, proteinase *yscE*, ingensin, macropain, and proteasome. A consensus, however, has evolved to retain the term "multicatalytic proteinase" in view of the multicatalytic character of the complex (Dahlmann et al., 1988; Orlowski & Wilk, 1988).

Biochemical Properties

The pituitary MPC can be isolated in good yield as a homogeneous protein after several conventional purification steps. The enzyme migrates in polyacrylamide gel electrophoresis (PAGE) under nondissociating conditions as a single protein band, but in the presence of sodium dodecyl sulfate (SDS), eight main components with molecular masses ranging from 21 to 32 kDa are readily resolved (Orlowski & Michaud, 1989). Some of the main components of the complex show a tendency to separate in high-resolution one-dimensional PAGE into doublets, and two-dimensional PAGE and electrofocusing may resolve as many as 15–20 subunits. Peptide maps of tryptic peptides of subunits isolated by high-pressure liquid chromatography indicated that most differ with respect to primary structure (Tanaka et al., 1988a, 1989). It has, however, not been excluded that some of the subunits might have arisen by posttranslational modifications including limited proteolysis. Indeed, analysis of amino acid sequences of tryptic peptides derived from subunits of human erythrocyte MPC indicated that some of them could represent products of proteolytic degradation, a finding not unexpected in view of the proteolytic activity of the complex (Lee et al., 1990). The presence of thiols is not needed for dissociation, indicating that the subunits are not held together by disulfide bonds and that hydrophobic and charge interactions may contribute to their association. The isoelectric point of the intact complex is about pH 5.0. Electrofocusing experiments on the rat liver enzyme, however, showed that the isoelectric point of the subunits ranges from about pH 4 to pH 9 (Tanaka et al., 1988a), suggesting the importance of charge interactions in subunit assembly.

Highly purified preparations of the MPC with properties nearly identical with those of the bovine pituitary enzyme have

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also been obtained from bovine lens (Ray & Harris, 1985). Furthermore, enzymes with virtually identical immunological and electrophoretic properties were found in all bovine tissues. Minor differences between the pituitary and lens enzyme with respect to the optimal temperature for substrate cleavage and the effect of divalent cations and SDS on activity were interpreted as suggesting the presence of subtle structural differences between enzymes from different organs (Ray & Harris, 1986). Similar enzymes are also present in a variety of human tissues. While the electrophoretic banding pattern for the human enzyme was somewhat different from that of the bovine enzyme, the two enzymes nevertheless showed strong immunological cross-reactivity. Ouchterlony double immunodiffusion experiments, however, of the bovine and human enzyme against an antiserum directed toward the bovine lens enzyme indicated the presence of structural differences between the two enzymes as revealed by spurring of the precipitin lines (Ray & Harris, 1987).

The MPC has now been shown to be a constant component of all eukaryotic cells examined. It is notable that Dahlmann et al. (1989a) isolated an enzyme closely resembling the MPC from the thermoacidophilic archaebacterium *Thermoplasma acidophilum*, a prokaryotic organism with some properties resembling eukaryotic cells. Although this enzyme showed a simpler subunit structure, a different pattern of cyanogen bromide peptides, and the apparent absence of the trypsin-like component, one of the components seemed nevertheless to bind an antibody directed toward the rat enzyme. This finding suggests that enzymes with properties similar to the MPC emerged rather early in evolution. A survey of the literature leads to the conclusion that the subunit and amino acid composition and immunological properties of the MPC isolated from different organs of the same species are generally identical. Though differences can be detected in the subunit composition of complexes isolated from the same tissues of different species, similarities in amino acid composition and immunological reactivity have been noted in complexes from species as diverse as yeast, *Drosophila*, and mammalian species, indicating preservation of the basic properties of the complex in evolution.

The average molecular mass of the complex is about 700 kDa, and the sedimentation coefficient is 19 S, although values from 550 to 900 kDa and from 16 to 20 S for preparations from various species have been reported. The molecular mass of separated subunits varies in the range of 21–34 kDa. In some preparations such as those from yeast (Achstetter, 1984), human lung (Zolfaghari et al., 1987a), and rat liver, higher molecular weight components in the range of 60 000–70 000 have been reported. The presence of these components in some but not all preparations suggests that they are protein contaminants. The MPC is rather stable during several hours of incubation at 37 °C. Dissociation, however, of the complex under a variety of conditions invariably leads to loss of all three proteolytic activities. Therefore, the integrity of the complex seems to be important for preservation of its activities. In the presence of low concentrations of dissociating reagents such as 0.03–0.04% SDS (unpublished observations) or 4 M urea, and in the absence of substrates, there is a loss of activity associated with autolytic degradation of all subunits (Tanaka & Ichihara, 1989a).

Kopp et al. (1986) examined the structure of the MPC from rat skeletal muscle by electron microscopy and also by dynamic laser light scattering. Both methods showed the presence of monodisperse particles. The particles had a cylindrical shape with a water-filled tunnel. The length of the particles was

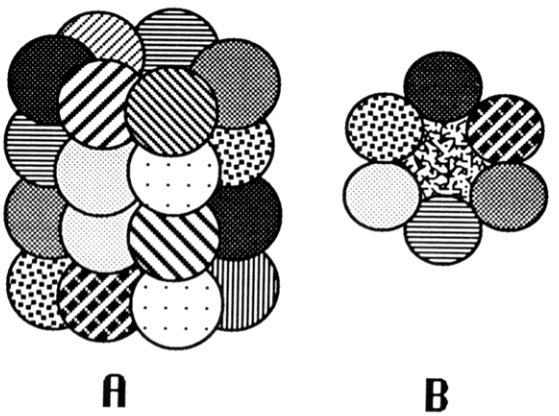

FIGURE 1: Schematic representation of the structural model of the multicatalytic proteinase complex based on the electron microscopic data of Kopp et al. (1986). The particle is composed of four stacked rings, each containing six components surrounding a central canal. The complex contains more than 10 nonidentical subunits. (A) Front view; (B) view from above.

about 16 nm in negatively stained specimens with the tunnel having a diameter of about 4 nm. The diameter varied from 11 to 16 nm depending on the method of preparation of the specimen (Baumeister et al., 1988). A model was proposed (Figure 1) whereby each particle is composed of four stacked rings, each ring having six arranged subunits surrounding a central canal. In view of the presence of more than six nonidentical subunits, the composition of the ring structures is apparently not uniform. This model has been generally confirmed by other investigators, although the presence of an 8-fold radial symmetry rather than six mass centers in a ring was also proposed (Arrigo et al., 1988). A somewhat different interpretation of the structure of the MPC is given by Tanaka and co-workers (1988a). The overall shape of the particles examined by small-angle X-ray scattering is described as appearing ellipsoidal with an ellipsoid cavity in the center.

Evidence that the pituitary enzyme is a multicatalytic proteinase came from studies in which it was shown that the enzyme cleaves bonds on the carboxyl side of hydrophobic, basic, and acidic amino acid residues (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983). Thus, each of the three model substrates, Cbz-Gly-Gly-Leu-pNA (pNA = *p*-nitroanilide), Cbz-D-Ala-Leu-Arg-2NA (2NA = 2-naphthylamide), and Cbz-Leu-Leu-Glu-2NA, was cleaved between the aromatic amine and the adjacent amino acid. Similar cleavage sites were also observed in natural peptides such as neurotensin, LH-RH, substance P, and the B chain of insulin. On the basis of the structure of the amino acid in the P_1 position, the activities were designated as chymotrypsin-like (cleavage on the carboxyl side of a hydrophobic residue), trypsin-like (cleavage on the carboxyl side of a basic residue), and peptidylglutamyl-peptide hydrolyzing (cleavage on the carboxyl side of a glutamate residue). Further examination of the specificity of the latter component led to the conclusion that this component also cleaves bonds on the carboxyl side of aromatic amino acid residues, thus exhibiting chymotrypsin-like activity, even though it did not attack the Leu-pNA bond in Cbz-Gly-Gly-Leu-pNA (Orlowski & Michaud, 1989).

The specificity of the complex could have been interpreted as being an expression of a single enzyme with broad specificity or as a result of the presence within the complex of three distinct proteinases, each attacking a different peptide bond. In the latter case it was reasonable to assume that each of the three activities must be associated with a separate subunit of the complex. The molecular mass of the subunits alone (21–34 kDa) seemed to exclude the possibility that more than one

Table I: Effect of Inhibitors and Activators on the Activities of the Components of the Pituitary Multicatalytic Proteinase Complex[a]

additions	concn	relative activity: chymotrypsin-like	relative activity: peptidyl-glutamyl-peptide hydrolyzing	relative activity: trypsin-like
none		100	100	100
N-ethylmaleimide	1.0 mM	60	66	4
leupeptin	0.16 mM	93	100	9
Cbz-Gly-Gly-leucinal	2.0 mM	12	88	170
SDS	1.38 mM	63	1490	3
palmitic acid	4.32 mM	133	369	1
linoleic acid	2.85 mM	29	524	33
heparin	4 units/mL	139	70	69
spermidine	0.2 mM	94	293	99
3,4-dichloroisocoumarin[b]	0.004 mM	15	54	260

[a] Activity was determined in 0.05 M Tris-HCl buffer, pH 8.0. Activity in the absence of additions was arbitrarily set at 100. [b] Enzyme was preincubated with the inhibitor for 75 min.

active site could be located on any single component of the complex. Indeed, studies on the effect of inhibitors and activators provided evidence that each of the three activities is associated with a separate component of the complex. Table I shows some of the data supporting this conclusion. Thus, for example, leupeptin selectively inhibited the trypsin-like activity, and this activity was also most sensitive to inhibition by thiol blocking agents. The chymotrypsin-like activity was selectively inhibited by Cbz-Gly-Gly-leucinal, a transition-state analogue of the substrate Cbz-Gly-Gly-Leu-pNA, and it was selectively activated by heparin. Low concentrations of SDS (0.02–0.04%) selectively activated the peptidylglutamyl-peptide hydrolyzing activity (Orlowski & Wilk, 1981; Wilk & Orlowski, 1980, 1983) while inhibiting the other two activities. Fatty acids such as linoleic acid, arachidonic acid, palmitic acid, and stearic acid had a similar effect, and in some preparations both the peptidylglutamyl-peptide hydrolyzing and the trypsin-like activities were stimulated (Dahlmann et al., 1985b). The large activation by SDS and fatty acids induced many authors to propose that most of the intracellular proteolytic activity of the complex is latent and that the full intracellular activity of the complex is only expressed under not yet clearly defined conditions. Mixed substrate experiments provided additional evidence that substrates with a Phe and Arg residue in the P_1 position are hyrolyzed at different sites (Rivett, 1989b). 3,4-Dichloroisocoumarin, a general serine proteinase inhibitor, inhibited all three activities, but the pseudo-first-order constants of inactivation were distinctly different for each of the three activities. Of interest was the finding that Cbz-Gly-Gly-leucinal, while inhibiting the chymotrypsin-like activity, activated the trypsin-like activity. A similar effect was also seen with 3,4-dichloroisocoumarin. At low concentrations (2 μM) this inhibitor inactivated the chymotrypsin-like activity while markedly activating the trypsin-like activity (Orlowski & Michaud, 1989). These findings suggest cooperative interactions between the components of the complex, since they implied that occupation of the active site of the chymotrypsin-like component promotes activity at the trypsin-like site.

The MPC degrades a number of proteins including α- and β-crystallin (van Heyningen, 1978), [methyl-^{14}C]globin, ^{125}I-hemoglobin, oxidatively changed hemoglobin, oxidized glutamine synthetase from *Escherichia coli*, casein and dephosphorylated casein, bovine serum albumin, and lysozyme with a pH optimum of 7.5–9.5 (Rivett, 1985, 1989a). Examination of the effect of SDS on proteolysis indicates that the main component responsible for degradation of proteins is the peptidylglutamyl-peptide hydrolyzing activity (Orlowski & Michaud, 1989).

Interest in the MPC was further enhanced when it was noticed that the electron microscopic appearance of the MPC is remarkably similar to that of prosomes and when the identity of the two particles was independently confirmed by two groups (Falkenburg et al., 1988; Arrigo et al., 1988). The name "prosome" was proposed (Schmid et al., 1984) as a designation for previously observed 19S ribonucleoprotein particles present in supernatants of nucleated animal and plant cells but not present in *E. coli* (Shelton et al., 1970). Prosomes were isolated by density gradient centrifugation from sources as diverse as wheat germ, yeast, *Drosophila melanogaster* tissue culture cells, chicken embryo fibroblasts, and a variety of mammalian tissues and cells (Arrigo et al., 1985, 1987; Domae et al., 1982; Schuldt & Kloetzel, 1985). The identity of the MPC with prosomes is indicated by the cylindrical shape and dimensions of both particles, by identity of the sedimentation coefficients and molecular weights, by the same composition characterized by the presence of multiple nonidentical subunits with molecular masses in the 19–35-kDa range, by the presence of identical proteolytic activities, and finally by immunological cross-reactivity between particles isolated from diverse species.

Prosomes were considered to represent ribonucleoprotein particles containing small RNA molecules consisting of 60–200 nucleotides (Schuldt & Kloetzel, 1985; Martins et al., 1986). In some preparations RNA was reported to constitute up to 15% of the mass of the particle. However, estimates of the number of RNA molecules per particle varied from a single RNA species (Falkenburg et al., 1988) to as many as 10 small RNAs per particle (Akhayat et al., 1987). Others found little or no RNA (Castano et al., 1986: Kleinschmidt et al., 1988; Tanaka et al., 1986b, 1988b). The presence or absence of RNA in prosomes may depend on the method of isolation, for highly purified preparations have been reported to be free of RNA (Arrigo et al., 1988). It is noteworthy that a 700-kDa multienzyme complex containing at least eight different aminoacyl-tRNA synthetases with molecular masses ranging from 56 to 160 kDa is present in mammalian cells and that the sedimentation coefficient of this particle is almost identical with that of the prosome (Godar et al., 1988). Indeed, hollow cylindrical particles identical with those seen in prosome preparations are usually seen as contaminants of preparations of this complex. It can therefore be expected that preparations of the MPC obtained by density gradient centrifugation may be contaminated by particles and dissociated protein components derived from the aminoacyl-tRNA synthetase complex.

Several functions and enzymatic activities have been claimed to be associated with prosomes. They include aminoacyl transferase I activity (Shelton et al., 1970), pre-tRNA 5′ processing endonuclease activity, repression of messenger RNA translation, inhibition of protein synthesis (Schmid et al., 1984; Castaño et al., 1986; Martins et al., 1986), posttranslational protein modification, association with specific RNA species or with certain heat shock proteins, and aminoacyl-tRNA synthetase activity. Hybridization of prosomal RNA to mRNA was also reported. It has not been established that these functions are an integral part of the complex; it is likely that they represent contaminating activities in preparations of doubtful purity obtained by density gradient centrifugation. RNase activity was claimed to be part of the complexes by some authors (Tsukahara et al., 1989) and denied by others

(Arrigo et al., 1987). Experiments suggested that prosomes are not induced by heat shock or by cell crowding in culture (Hendil, 1988). Tracer studies have indicated that prosomes are rather stable metabolically and that their half-life is about 5 and 12–15 days in HeLa cells and rat liver, respectively (Hendil, 1988; Tanaka & Ichihara, 1989b).

Like the MPC, prosomes are mainly localized in postribosomal supernatants of cell homogenates; studies using immunofluorescent antibody techniques also showed prosomes in nuclei of eggs and embryos of the sea urchin and in nuclei of mammalian cells (Akhayat et al., 1987; Arrigo et al., 1988). In fat cells of *Drosophila* prosomes were reported to be localized exclusively in nuclei (Haass et al., 1989). Studies on the subcellular distribution of the MPC showed that the enzyme is mainly localized in the soluble fraction of cell homogenates (Dresdner et al., 1982). Small activities occasionally found in mitochondrial or microsomal fractions can be attributed to contamination with cytoplasmic proteins. The presence of the enzyme in nuclear fractions (Akhayat et al., 1987; Tanaka et al., 1988b) and its association with the polyribosomal fraction (Kloetzel et al., 1987) were also reported. Immunocytochemical localization of the MPC in liver showed its presence in hepatocytes, Kupfer cells, bile duct epithelial cells, and vascular endothelial cells. In skeletal muscle the enzyme was localized in intermyofibrillar spaces and vascular endothelial cells (Tanaka et al., 1986a). In the rat central nervous system the MPC was visualized in the cytoplasm of motor neurons, glial cells, Purkinje cells, and granular cells of the hippocampus and in axons (Kamakura et al., 1988). The complex can already be detected in early embryos of *Drosophila*, and the subunit pattern seems to undergo developmental changes becoming more complex with development (Haass & Kloetzel, 1989). The MPC is estimated by immunochemical analysis to constitute 0.5–1% of the soluble fraction of cellular homogenates (Hendil, 1988). Although purifications of up to 2000-fold are frequently needed for the isolation of a homogeneous MPC, this cannot be used as a basis for calculation of the amount of enzyme in cells, since in crude tissue homogenates the MPC is apparently significantly inhibited by the presence of high protein concentrations. Ray and Harris (1987) showed that among bovine tissues the lung has the highest MPC activity, followed in decreasing order of activity by the pituitary, kidney, liver, muscle, heart, brain, lens, and red blood cells.

Gel electrophoresis, N-terminal amino acid sequence analysis, and partial sequence analysis of tryptic peptides derived from separated subunits of the MPC from human erythrocytes indicated the presence of 13 subunits with different primary structures. Eight of the subunits had blocked N-termini. At least 10 major components had different amino acid sequences, but homologies among the subunits indicated that they are products of a related family of genes (Lee et al., 1990). The complete amino acid sequence of two major subunits of the MPC from rat liver was deduced from the nucleotide sequence of recombinant cDNA clones (Fujiwara et al., 1989; Tanaka et al., 1990). The larger component (C2) consisted of 263 amino acids with a molecular weight of 29 516. No homology was found between this amino acid sequence and any of the known sequences of proteases, but a stretch of 48 amino acids showed some homology to the amino acid sequence of the large subunit of chicken but not human calpain. No evidence was obtained that the isolated component represents a proteolytically active subunit. Northern blot hybridization analysis showed the presence of the mRNA encoding the component in all rat tissues and also in other mammals and species of amphibians and birds. The second, smaller component (C3) consisted of 234 amino acids with a molecular weight of 25 925. The distribution of the mRNA encoding the component was similar to that of C2. It is of interest that a 70 amino acid stretch of the sequence showed 30% identity with sequences of tyrosine kinases and included a possible tyrosine phosphorylation site similar to that found in chicken Src protein ($pp60^{c\text{-}src}$), epidermal growth factor receptor, human insulin receptor, and mouse platelet-derived growth factor receptor. The amino acid sequence of component C3 showed 31% identity with the amino acid sequence of component C2 and also similarity to the amino acid sequence of a 35-kDa component from *Drosophila* prosomes (Haass et al., 1989). The primary translation product of this component contained 279 amino acids with a molecular mass of 31.4 kDa and a consensus sequence for a tyrosine phosphorylation site.

The mechanistic classification of the proteolytic activities of the MPC has been a subject of controversy. Inhibition by some thiol blocking agents led to the suggestion that the enzyme is a thiol proteinase (Dahlmann et al., 1985a; Rivett, 1985; Wagner et al., 1986). An expression of this classification was a proposal to name the enzyme "macropain" (McGuire & DeMartino, 1986), apparently to emphasize that the enzyme may be related to papain, a thiol proteinase. This classification seemed to be supported by the finding that only the chymotrypsin-like activity of the complex was inhibited by DFP (diisopropyl fluorophosphate) and only at concentrations of 2 mM and above (Wagner et al., 1985, 1986). Doubts, however, concerning this classification were introduced by the finding that E-64 and EP-475, cysteine proteinase inhibitors, had no effect on activity. Though *p*-mercuribenzoate inhibited all three activities, other thiol blocking agents such as *N*-ethylmaleimide, iodoacetic acid, and iodoacetamide either inhibited, had no effect, or even activated the MPC from different sources. DFP inhibited only the chymotrypsin-like activity in some preparations whereas in others it inhibited all three activities. The MPC from lobster claw and abdominal muscles was inhibited by both DFP and PMSF (phenylmethanesulfonyl fluoride) (Mykles, 1989). Furthermore, several of the major MPC subunits could be labeled with [^{3}H]DFP (Arrigo et al., 1988; Tanaka et al., 1986a). Dahlmann et al. (1989a) reported that the enzyme from archaebacteria was very sensitive to DFP inhibition and less so to PMSF. Pepstatin and thiol blocking agents had no effect. Orlowski and Michaud (1989) reported that all three activities of the MPC are inactivated by low concentrations of 3,4-dichloroisocoumarin, a general serine protease inhibitor (Harper et al., 1985), and that the pseudo-first-order rate constants of inactivation of the three components differ within a wide range. Collectively, these data seem to indicate that all three catalytic components of the complex belong to the class of serine proteinases, although final classification must await elucidation of their structure and identification of residues involved in peptide bond cleavage.

Among other inhibitors chymostatin inhibited the activity toward substrates with a hydrophobic group in the P_1 position, hemin (50 μM) inhibited the caseinolytic activity and both the chymotrypsin- and trypsin-like activities, and aprotinin inhibited the trypsin-like activity (Rivett, 1985). A consistent property of the MPC from various sources is its activation by low concentrations of SDS and fatty acids (Orlowski & Wilk, 1981; Dahlmann et al., 1985b). The magnitude of the activation varies in different preparations, and can be as high as 30-fold, and is apparently associated with conformational

changes (Saitoh et al., 1989). Activation by SDS is reversible and removal of the detergent decreases the activity to basal levels. Incubation, however, of the enzyme with SDS in the absence of substrate leads to a progressive, irreversible loss of activity associated with autolytic degradation of the complex. Initial studies on the bovine pituitary enzyme showed that all three proteolytic components are inhibited by relatively low concentrations of monovalent cations such as Na^+ and K^+. This observation led to the initial designation of the enzyme as "cation-sensitive endopeptidase" (Wilk et al., 1979; Wilk & Orlowski, 1980) and to the avoidance of salt gradients during purification. This property was also reported for the MPC from human lung and kidney and for several other complexes (Zolfaghari et al., 1987a,b; Seol et al., 1989). In many preparations part of the proteolytic "latency" of the MPC can be attributed to the presence of salts used in the elution of the complex from ion-exchange columns. This is indicated by the finding that such preparations can be "activated" by dialysis against distilled water.

Different extents of activation of the bovine lens enzyme were noticed by Mg^{2+} and Ca^{2+}. Ray and Harris (1987) reported that this activation progressively decreased during purification and disappeared altogether during storage. Dahlmann et al. (1989a) reported that the enzyme from archaebacteria was strikingly activated by Ca^{2+}. The peptidylglutamyl-peptide hydrolyzing activity of the pituitary enzyme is activated by Ca^{2+} and either inhibited or activated by Mg^{2+} depending on the concentration. The presence of monovalent cations changes significantly the response to either Mg^{2+} or Ca^{2+}. The activity of the MPC could be under regulation by metal ions, although the nature of this regulation remains to be defined.

The Multicatalytic Proteinase Complex and the Ubiquitin System

Intracellular protein degradation seems to require energy input. This conclusion is supported by the finding that depletion of cellular ATP slows degradation of proteins and prolongs their half-lives (Simpson, 1953; Goldberg & St. John, 1976; Hershko & Ciechanover, 1982; Gronostajski et al., 1985). Direct involvement, however, of ATP in the mechanism of peptide bond hydrolysis has not been demonstrated. At least part of the ATP requirement was elucidated when work by Hershko, Ciechanover, Rose, and others (Hershko et al., 1980; Hershko, 1988) led to the discovery of a multienzyme system that catalyzed the conjugation of ubiquitin to proteins in an ATP-requiring reaction. It was subsequently postulated that ubiquitination of proteins marks these conjugates for proteolytic degradation and that the degradation process itself is also catalyzed by an ATP-dependent proteolytic system (Ciechanover, 1987). However, whereas the role of ATP in the mechanism of the conjugation reaction was established, the role of ATP in the subsequent proteolytic degradation of the conjugates remains enigmatic, especially since peptide bond cleavage requires no energy input and proteinases, including the MPC, are generally active in the absence of ATP.

Rechsteiner and co-workers (Hough et al., 1986, 1987) reported the purification from rabbit reticulocytes of two high molecular weight proteinases that cleaved ^{125}I-casein and synthetic substrates with an Arg or a hydrophobic group in the P_1 position. One enzyme had a sedimentation coefficient of 26 S and a molecular mass of over 1000 kDa, while the other had a molecular mass of 700 kDa and a sedimentation coefficient of 20 S. Both enzymes were inhibited by thiol blocking agents, leupeptin, chymostatin, and hemin, but only the larger enzyme degraded ^{125}I-lysozyme–ubiquitin conjugates, and this activity, as well as the activity toward synthetic substrates, was stimulated 2–3-fold by ATP. The enzyme did not degrade free lysozyme. Both enzymes were multisubunit complexes, but only the smaller enzyme showed the subunit composition and independence of ATP typical of the MPC from bovine pituitaries (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983). The 26S enzyme contained a set of subunits with molecular masses between 34 and 110 kDa but also contained a set of subunits typical for the 20S enzyme. Hydrolysis by the larger enzyme of a synthetic substrate containing a Tyr residue in the P_1 position was stimulated by ATP and also by other nucleoside triphosphates and, to a lesser extent, by ADP and the ATP analogue β,γ-CH_2-ATP. ATP protected the larger enzyme against thermal inactivation since preincubation in the absence of ATP led to rapid loss of activity. The smaller enzyme was quite stable during such preincubations. While the two enzymes shared many properties such as subunit composition, activity toward synthetic substrates, and sensitivity to some inhibitors, they differed with respect to other properties. Thus, the larger enzyme was more sensitive to inhibition by PMSF, and it was not activated by SDS. Nevertheless, Rechsteiner et al. considered the possibility that the larger enzyme may represent a modified form of the 20S enzyme since complete separation of the two complexes was not achieved. Indeed, a model was proposed whereby the 26S enzyme would contain subunits of the 20S complex in addition to higher molecular weight components (Hough et al., 1988).

Partial purification of two similar high molecular weight proteinases from rabbit reticulocytes was also reported by Waxman et al. (1987). The large enzyme having a molecular mass of about 1500 kDa was stabilized against thermal inactivation by nucleotides and was inhibited by hemin, various peptide chloromethyl ketones, 3,4-dichloroisocoumarin, and low concentrations of thiol blocking agents. The enzyme was necessary for ATP-stimulated proteolysis and was inactive toward substrates that could not be conjugated to ubiquitin. A smaller 20S enzyme also present in reticulocyte lysates had properties identical with those of the MPC. Unlike, however, in the report by Hough et al. (1986, 1987) ATP stimulated the degradation of ^{125}I-lysozyme, and the subunits with a molecular mass of 22–32 kDa seen in the smaller enzyme were presumably not present in the large proteinase. Inasmuch as the two enzymes were not sufficiently purified for detailed characterization, it was considered that they could nevertheless be structurally related. A similar high molecular mass, ATP-stimulated proteinase was also purified from the cytosol of bovine brain (Azaryan et al., 1989). The presence of "ATP-dependent" proteolytic systems was also reported in several preparations including erythroleukemia cells (Rieder et al., 1985) and BHK 21/C13 fibroblast cells (McGuire et al., 1988a) and in extracts from liver and skeletal muscle (Fagan et al., 1987). Surprisingly, however, the complex from the latter sources was completely inhibited by egg white cystatin, an inhibitor of thiol proteinases. It should be emphasized that in none of those preparations has it been demonstrated that cleavage of peptide bonds is accompanied by cleavage of ATP. It is appropriate to avoid interchanging the terms ATP stimulated and ATP dependent; the latter term should be restricted to reactions absolutely requiring ATP.

The presence of the high molecular mass 26S proteinase in reticulocyte lysates and also in skeletal muscle extracts and in BHK cells as a distinct ATP-dependent entity was brought into question by reports that immunoprecipitation of the MPC by specific antibodies seemed to completely remove the

ATP-stimulated activity against lysozyme and lysozyme–ubiquitin conjugates (McGuire et al., 1988a, 1989b; McGuire & DeMartino, 1989; Matthews et al., 1990). Immunoprecipitation of the MPC from reticulocyte extracts also removed the ATP-stimulated degradation of [^{3}H]methylcasein (Tanaka & Ichihara, 1988). These findings were in apparent contradiction with the assertion that the MPC does not degrade ubiquitin–lysozyme conjugates. Furthermore, purified preparations of the MPC were reported to degrade, in an ATP-stimulated manner, protein substrates in which free amino groups were blocked by reductive methylation, guanidination, or carbamoylation (McGuire et al., 1988b). These findings indicated that the MPC has a decisive role in the degradation of protein–ubiquitin conjugates or that at least some of its subunits must be associated with the 26S complex.

Cleavage of a synthetic substrate by the MPC can also be occasionally stimulated by ATP. For example, extracts of erythroleukemia cells (K562) are stimulated up to 10-fold by ATP in their cleavage of a substrate containing a Tyr residue in the P_1 position (Tsukahara et al., 1988). The stimulation, however, by ATP in the presence of EDTA indicates that the nucleoside triphosphate is not hydrolyzed during this reaction. Indeed, it was shown that the effect of ATP depended on the protection of the proteinase from inactivation at 37 °C. Purification of the enzyme led to disappearance of the ATP effect. Driscoll and Goldberg (1989) reported that the MPC can be isolated from skeletal muscle in an ATP-dependent form provided that isolation is carried out rapidly and that the enzyme is protected by the presence of glycerol. Such preparations hydrolyzed succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide in a reaction that was stimulated 12-fold by ATP. Storage at 4 °C converted the enzyme to an ATP-independent form. Nonhydrolyzable analogues of ATP also stimulated activity, and ATP stimulation was again seen even in the presence of EDTA.

The relationship between the 26S and 20S complexes became somewhat more clear when Hershko and co-workers (Eytan et al., 1989) reported that the 20S complex is one of three factors necessary for the assembly of the 26S proteinase in an ATP-requiring reaction. This proteinase acquires thereby the ability to degrade ubiquitin–protein conjugates. Indeed, it was postulated that the 20S proteinase constitutes the "catalytic core" of the 26S complex. Incubation of the 20S complex (CF3) with two other factors (CF1 and CF2 in the nomenclature of Hershko and co-workers) in the presence of ATP and Mg^{2+} shifted the 20S MPC into the 26S form. Ishiura et al. (1989) reported that molecular sieving chromatography of the MPC from rat liver and procine brain in the presence of ATP shifted its molecular mass from 700 kDa to more than 1000 kDa. This ATP effect was not seen in purified enzyme preparations. Dialysis of the high molecular weight form against an ATP-free buffer caused a partial decrease in molecular mass to that of the native MPC.

The results summarized above indicate that ATP is apparently needed for the assembly of the 26S complex, although the role of the nucleoside triphosphate in this process is not known. According to the ubiquitin-marking hypothesis, ATP is also needed in the subsequent proteolytic degradation of ubiquitin–protein conjugates (Hough & Rechsteiner, 1984; Hershko et al., 1984b). As in the assembly process, however, the role of ATP remains undefined. Similarly, the nature and role of the components (beyond the MPC) needed for assembly of the 26S complex also need definition. ATP could exert an influence in several different ways. Thermal stabilization of the 26S complex by ATP seems to constitute an important factor. ATP might also be needed for inducing conformational changes in ubiquitin–protein conjugates that are necessary for interaction of the conjugates with the proteinase. Both binding to and release of the protein from the active site of the proteinase might be influenced by ATP. Another role may involve the removal of an endogenous inhibitor that binds to the active site of the proteinase, as proposed by Murakami and Etlinger (1986). Resolution of these and other possibilities requires the isolation of all components of the 26S complex and examination of their function in well-defined systems. Of all the components of the 26S complex, only the MPC has been isolated as a homogeneous protein.

A note of caution in attributing all observed ATP stimulation of proteolytic events to a cytoplasmic proteolytic system was introduced by Dahlmann and co-workers (Dahlmann et al., 1989b; Kuehn et al., 1989). They isolated a high molecular weight proteinase from skeletal muscle and also from rabbit reticulocytes that catalyzed the hydrolysis of [^{14}C]methylcasein and also synthetic substrates in an ATP-stimulated reaction. Further analysis provided evidence that the enzyme is a complex of α_1-macroglobulin and the cysteine proteinases cathepsins B, L, and H. Similar complexes were also obtained when some procedures used for the isolation of high molecular weight ATP-stimulated proteinases from liver (DeMartino & Goldberg, 1979) and heart muscle were followed. Such enzyme preparations contained protein bands typical of the MPC but also material immunoreactive with antibodies against α_1-macroglobulin. That cathepsins can be stimulated by ATP had been previously demonstrated (McKay et al., 1984; Bond & Butler, 1987). The possibility must therefore be considered that at least part of the ATP-stimulated proteolytic activity of some not sufficiently purified high molecular weight complexes might actually be attributed to α_1-macroglobulin–cathepsin complexes.

Function

The use of inhibitors of lysosomal proteolysis clearly showed that cytoplasmic protein degradation plays a major role in intracellular protein turnover. Tracer techniques and intracellular protein microinjections showed that the half-life of different proteins can vary from a few minutes to many days [for reviews see Rechsteiner (1987) and Rechsteiner et al. (1987)]. Short-lived proteins are primarily degraded by cytoplasmic proteolysis. Proteins that are changed conformationally as a result of either errors in transcription or translation, mutations, posttranslational covalent modifications, denaturation, or incorporation of amino acid analogues are frequently rapidly degraded. The rate of proteolytic degradation also seems to be influenced by the presence of certain specific amino acid sequences within the protein and by the nature of the α-amino terminus. Studies on ubiquitin–β-galactosidase fusion proteins containing various amino acids at the junction between ubiquitin and β-galactosidase showed that in *Saccharomyces cerevisiae* ubiquitin is rapidly cleaved off these proteins in vivo and that the nature of the exposed amino acid at the N-terminus has a pronounced influence on the half-life of the protein (Bachmair et al., 1986). It is of interest that a fusion protein containing a Pro residue at the ubiquitin–β-galactosidase junction was resistant to deubiquitination but was degraded with a half-life of only about 7 min, suggesting that an N-terminal stable ubiquitin–protein conjugate could serve as a signal for protein degradation. That ubiquitination of the amino terminus of proteins might be essential for their degradation was proposed on the basis of in vitro experiments which showed that acetylation of the N-terminus of proteins slows their degradation in an ubiquitin-dependent

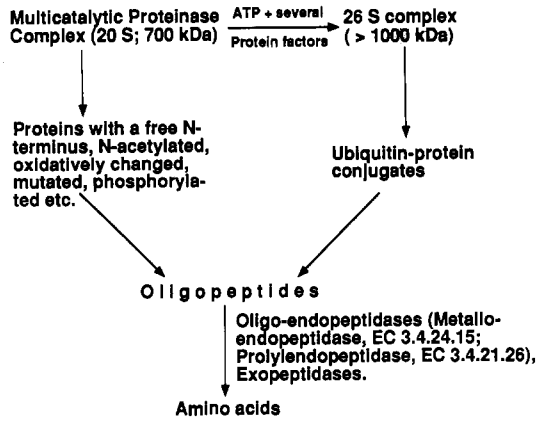

FIGURE 2: Scheme of the proposed function of the multicatalytic proteinase complex in the ubiquitin-dependent and ubiquitin-independent cytoplasmic pathways of protein degradation.

proteolytic system (Hershko et al., 1984a; Hershko, 1988). Conjugation of multiple ubiquitin moieties to ϵ-amino groups of lysine in the process of degradation of proteins is apparently also an important factor in intracellular proteolysis (Rechsteiner, 1987; Hough et al., 1988; Bachmair et al., 1986). In addition, intracellular oxidations of certain amino acids such as Met, His, and Cys by mixed-function oxidase systems may also render those proteins susceptible to proteolytic degradation (Stadtman, 1986). Protein phosphorylation is another factor that could either accelerate or slow proteolytic degradation. Thus, it was shown that the rate of degradation and the nature of proteolytic products formed by the action of the MPC on casein and dephosphorylated casein differ markedly (Orlowski & Michaud, 1989).

The main cytoplasmic proteinases identified so far as being capable of degrading proteins include two calcium-dependent proteinases (the calpains) and the MPC. This complex seems also to constitute the main proteolytic nucleus of the 26S complex shown to be critical for the degradation of ubiquitin–protein conjugates. Current evidence indicates that the MPC is involved in both ubiquitin-dependent and ubiquitin-independent proteolytic pathways. The MPC can degrade proteins with a free amino terminus, as well as proteins in which the N-terminus and also the internal amino groups are blocked. A variable number of peptides and small RNAs are associated with the complex when isolation is carried out by density gradient centrifugation. This association may be an expression of binding of these molecules to the MPC in the process of degradation of proteins or ribonucleoproteins. The presence of three proteolytic activities in a single particle probably represents a functional advantage, since this can assure cleavage of most peptide bonds in a protein. Degradation of proteins by the MPC would be expected to generate smaller peptides that could be resistant to further degradation. Indeed, some peptide bonds, such as those involving proline residues, are resistant to hydrolysis by the MPC. It is therefore of significance that cytoplasmic fractions contain two potent endopeptidases that attack only oligopeptides and are inactive toward large peptides and proteins. The two enzymes, endopeptidase 24.15 (Orlowski et al., 1983, 1989) and prolyl endopeptidase (Walter et al., 1971), the first a zinc metalloendopeptidase and the second a serine endopeptidase, could degrade those peptides that escape degradation by cytoplasmic proteinases. Further degradation of small peptide fragments would be accomplished by the action of several cytoplasmic exopeptidases (Figure 2). Indeed, all or none degradation of proteins seems to be typical of cytoplasmic proteolysis (Rechsteiner, 1987). The finding that the MPC is associated with nuclei and polyribosomes indicates that its function may extend beyond the cytoplasmic compartment and that it may involve posttranslational protein modifications and processing and/or degradation of biologically active peptides and prohormone precursors (Neurath & Walsh, 1976).

PERSPECTIVES

Identification of factors determining intracellular protein turnover is attracting intense efforts from researchers in many laboratories. The aim is to identify the enzymes involved and to elucidate not only their structure, mechanism of action, and specificity but also those structural determinants and post-translational modifications of proteins that influence their rate of degradation. Intracellular proteolysis must be a highly controlled and regulated process. This process can be expected to affect the orderly functioning of cellular metabolism. It also carries the potential of causing considerable damage if not properly controlled. The ubiquitous distribution and the high concentration of the MPC in cells indicate that the complex represents a major extralysosomal proteolytic system which, in concert with cytoplasmic oligoendopeptidases and exopeptidases, has the potential to degrade proteins to simple amino acids. In vitro studies indicate that the MPC functions in the ubiquitin-dependent and ubiquitin-independent pathways of protein degradation and that its function can extend beyond the cytoplasmic compartment, notably into the cell nucleus. Though research on the biochemistry and function of the complex has advanced in recent years, we are only beginning to understand its complexities. Many questions remain to be answered. What is the precise composition and structure of the complex and its components? How many genes encode the different subunits, and how are the subunits assembled into the complex? Which of the components are proteolytically active, and what is the function of the other subunits? Are there other, still undetected enzymatic activities in the complex? Studies on the mechanism of action and specificity of the proteolytic components should lead to the synthesis of specific inhibitors that could be used to reveal the role of the complex in intracellular proteolysis. Physiological studies must address the role of the MPC in cellular function, regulation of its expression and function, and its adaptation to changing metabolic and developmental requirements. Some answers to these questions are only beginning to emerge, and major efforts are needed to answer others. It can be expected that research on the MPC will bring with it a better understanding of major aspects of intracellular proteolysis.

Registry No. Proteinase, 9001-92-7; ubiquitin, 60267-61-0.

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