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REVIEW ARTICLE

High molecular mass intracellular proteases

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INTRODUCTION

Many of the well-characterized proteolytic enzymes, and particularly those for which X-ray structures are now available, are small monomeric enzymes often having molecular masses in the range of 20–30 kDa. Many of them are extracellular enzymes which are easy to assay and to purify. With a growing awareness of the importance of intracellular protein turnover and mechanisms of intracellular protein breakdown, interest in the proteases responsible has also increased. Although some intracellular proteases, especially those found within the lysosomes in animal cells, are, like extracellular proteases, small and highly active monomeric enzymes, a number of cellular proteases have been described which have molecular masses ranging up to greater than 1000 kDa. Why are they so big? Proteases are of vital importance in the control of a number of cellular processes and the complexity of large intracellular proteases must have important implications for their function, for the regulation of their activity and also for the co-ordination of changes in the level or function of other proteins within the cell. Proteolytic enzymes function both in protein turnover and in a variety of specific peptide and protein processing events. Increasing the length of a proteolytically active polypeptide or making it part of a larger multisubunit complex opens up a number of possibilities for the regulation of its activity. These include binding of small ligands, interaction with inhibitor or activator proteins, and membrane association, and in some cases the large proteases even have more than one type of enzymic activity. In reviewing what is currently known about large intracellular proteases there are several problems, most notably the great variety in their properties and the lack of detailed structural information for many of them. Much of the review is devoted to an intriguing multicatalytic proteinase complex which is widely distributed in eukaryotic cells, but other proteases are also discussed for comparison.

LARGE PROTEASES IN EUKARYOTIC CELLS

The high molecular mass proteases of animal cells are mostly extra-lysosomal, and their recent discovery compared with that of lysosomal proteases can be explained by their relatively low activity which in some cases may lead to difficulties in assaying them in crude cell extracts. Early investigations of protein degradation in mammalian cells concentrated on the lysosomes because lysosomal proteases (often called cathepsins) were found to be highly active against a wide variety of peptide and protein substrates. However, studies using inhibitors of lysosomal function (Seglen, 1983) have demonstrated that intracellular proteolysis is not restricted to the lysosomes. Since a large proportion of intracellular protein breakdown, especially the degradation of proteins with short half-lives, is now known to occur by nonlysosomal mechanisms (Mayer & Doherty, 1986; Bond & Beynon, 1987; Rechsteiner, 1987; Bohley, 1987; Rivett, 1989*b*; Katunuma & Kominami, 1989; Knecht & Grisolia, 1989), there is now a greater interest in nonlysosomal degradation systems and in nonlysosomal proteinases, many of which have large complex structures.

In contrast to the well-known lysosomal proteases, soluble extralysosomal proteases often have multimeric structures. Nonlysosomal proteolytic systems which have received much attention in recent years include the calcium-dependent proteinases called calpains (Murachi, 1983; Pontremoli & Melloni, 1986; Suzuki, 1987; Mellgren, 1987; Suzuki *et al.*, 1987), the high molecular mass multisubunit proteinase now called the multicatalytic proteinase or proteasome (Rivett, 1989*a*), and the proteinase(s) involved in the ubiquitin-dependent pathway of protein degradation (Hershko & Ciechanover, 1986; Rechsteiner, 1987, 1988; Hershko, 1988). Some properties of these and other large mammalian proteases are summarized in Table 1 and considered in more detail below. The precise functions of many of them are still unknown.

The situation for intracellular proteolysis in yeast is similar to that for mammalian cells. The vacuoles (equivalent to lysosomes) were believed to play a major role in protein breakdown and a few years ago the only well-known yeast proteases were all of vacuolar origin (Wolf, 1986). When yeast mutants defective in vacuolar proteases became available the activities of many other proteases could be detected and more than thirty nonvacuolar proteases have now been identified (Wolf, 1986). Some of these are high molecular mass enzymes (Table 2). Proteases in other eukaryotic micro-organisms have been reviewed by North (1982).

The calpains

The calpains (Murachi, 1983), calcium-dependent proteases or calcium-dependent neutral endopeptidases (Suzuki, 1987), are cytosolic cysteine proteinases which require calcium for proteolytic activity. There are two isoenzymes, calpain I and calpain II, which differ in their calcium sensitivity. Calpain I requires micromolar concentrations of calcium for full activity whereas calpain II requires millimolar Ca^{2+} concentrations. Calpains are inhibited by thiol-reactive reagents, Ca^{2+}-chelating agents, leupeptin and E64 (an epoxysuccinyl derivative of microbial origin). The two isoenzymes differ somewhat in their specificity and have distinct preferences for

The term protease is used to include all types of proteolytic enzymes (otherwise called peptidases), whereas the term proteinase is used for endopeptidases only (Barrett, 1986; Rivett, 1989*b*).

Table 1. High molecular mass proteases of mammalian cells

Protease	Class	Subcellular localization	M_r	Subunit composition	Reference
Calpain I/II	Cys	Cytosol	110000	Heterodimer 80 and 30 kDa	Suzuki (1987)
Multicatalytic proteinase	Cys or Ser	Cytosol (+nucleus)	700000	> 10 different types of subunits, 22–34 kDa	Rivett (1989*a*)
Megapain/UCDEN*	?	Cytosol	1500000	Many different subunits, 34–110 kDa	Hough *et al.* (1989), Fagan *et al.* (1987)
Tripeptidyl peptidase II	Ser	Cytosol	> 1000000	Many identical subunits, 135 kDa	Tomkinson *et al.* (1987)
Leucine aminopeptidase	Metallo	Cytosol	360000	Hexamer 6 × 54 kDa or 3 × two subunits, 53 and 65 kDa	Taylor *et al.* (1984) Kohno *et al.* (1986)
ATP-dependent protease	Ser	Mitochondria	550000	?	Desautels & Goldberg (1982),
			650000		Watabe & Kimura (1985)
Meprin	Metallo	Plasma membrane	360000	Tetramer	Bond & Beynon (1986)
Endopeptidase 24.11	Metallo	Plasma membrane	90000	Dimer	Kenny (1986)

* Abbreviation: ubiquitin conjugate degrading endopeptidase.

Table 2. Multisubunit proteases in yeast

More complete lists of proteases in yeast and other eukaryotic micro-organisms are available elsewhere (North, 1982; Wolf, 1986).

Protease	Class	M_r	Subunit structure	References
Proteinase *yscE* (multicatalytic proteinase or proteasome)	Cys / Ser	600000 / 700000	Many different types of subunits 22–33 kDa	Achstetter *et al.* (1984), Kleinschmidt *et al.* (1988) Tanaka *et al.* (1988*a*)
Proteinase *yscY*	?/ATP	> 600000		Wolf (1986)
Aminopeptidase	Metallo	640000		Wolf (1986)

cleavage sites in peptide substrates (Sasaki *et al.*, 1984). Both are widely distributed in mammalian cells. They usually catalyse only limited cleavage of protein substrates and are thus thought to be involved in specific limited proteolytic processes such as the activation of protein kinase C (Suzuki *et al.*, 1987; Murray *et al.*, 1987) and the regulation of cytoskeletal protein function (Mellgren, 1987).

Calpain I and calpain II are each composed of two subunits, an 80 kDa (catalytic) subunit and a 30 kDa subunit. Although the two isoenzymes from the same source have different (but similar in sequence) catalytic subunits, their small subunits are identical. The primary structure of the proteinases isolated from chicken (Ohno *et al.*, 1984), rabbit (Emori *et al.*, 1986*a,b*) and human (Aoki *et al.*, 1986; Emori *et al.*, 1988) calpains have been deduced from the nucleotide sequence of cDNAs. The proposed domain structure is similar in each case (Suzuki, 1987). The larger, catalytic subunit contains four putative domains (I–IV from the *N*-terminus) of which the second is a proteolytic domain showing a high degree of sequence similarity with the plant cysteine protease, papain, and the fourth, the *C*-terminal domain, is a calcium-binding domain which contains four 'E-F hand' structures. The smaller subunit contains a similar calcium-binding domain and, at its *N*-terminal, a hydrophobic membrane-binding domain. The *N*-terminal domain of both subunits is involved in the activation of calpain II by association with cell membranes (Suzuki, 1987; see below).

Calpains interact with a specific endogenous inhibitor protein called calpastatin (Murachi, 1983; Suzuki *et al.*, 1987) and cytoskeletal activator protein(s) (Takeyama *et al.*, 1986; Pontremoli *et al.*, 1988).

The multicatalytic proteinase

The multicatalytic proteinase (molecular mass 700 kDa) has been described under numerous different names (see Rivett, 1989*a*), but there is now general agreement to call it the multicatalytic proteinase, the multicatalytic proteinase complex, or the proteosome. In recent years the proteinase has been purified from a wide variety of sources including many different mammalian tissues (Rivett, 1989*a*), fish (Folco *et al.*, 1988), *Drosophila* (Falkenburg *et al.*, 1988), *Xenopus laevis*

(Kleinschmidt *et al.*, 1988) and yeast (see proteinase *yscE*, below). The basic properties of this widely distributed and abundant proteinase appear to be similar regardless of the source (Tanaka *et al.*, 1988*a*; Rivett, 1989*a*). Recent studies have centred on the proteolytic activities, the structure, and the immunological properties of the complex, as well as on its proposed involvement in ATP-dependent mechanisms of intracellular protein degradation. Controversial issues such as the classification of the proteinase as a cysteine or a serine proteinase, its localization in the cytoplasm or nucleus and cytoplasm, and its RNA content have not been completely resolved.

Studies with polyclonal antibodies (Ray & Harris, 1987; Tanaka *et al.*, 1988*a*) show interspecies cross-reactivity but also suggest some differences in immunological properties of multicatalytic proteinases isolated from different sources. These antibodies precipitate the complex but do not block its proteolytic activities (Rivett, 1989*c*) and few of the subunits are recognized by Western blotting (Falkenburg *et al.*, 1988; Tanaka *et al.*, 1988*a*; Sweeney & Rivett, 1989).

The multicatalytic proteinase degrades a variety of peptide substrates (Wilk & Orlowski, 1983; McGuire & De Martino, 1986; Rivett, 1989*c*) and can catalyse the extensive breakdown of some protein substrates (Rivett, 1985, 1986; Ray & Harris, 1985). The proteinase can cleave peptide bonds on the carboxyl side of basic, hydrophobic or acidic amino acid residues. These proteolytic activities have been referred to as trypsin-like, chymotrypsin-like and peptidyl-glutamyl peptide bond hydrolysing activities, respectively (Wilk & Orlowski, 1983). Based on the differential effect of different inhibitors and activators, it was proposed that these activities of the proteinase are catalysed at distinct sites (Wilk & Orlowski, 1983). Results of later studies (Dahlmann *et al.*, 1985*a*; McGuire & De Martino, 1986; Zolfaghari *et al.*, 1987) confirmed these observations but no attention was paid to the relative K_m and K_i values of substrates and inhibitors. Nonetheless it appears that there are at least two distinct types of catalytic sites. The trypsin-like (-Arg-X or -Lys-X) and chymotrypsin-like (-Leu-X, -Phe-X, or -Tyr-X) activities are easily distinguished. Trypsin-like activity can be almost completely inhibited by concentrations of leupeptin, antipain, or *N*-ethylmaleimide which have no effect on the chymotrypsin-like activity (Wilk & Orlowski, 1983; Rivett, 1989*c*). Moreover, there appears to be little if any competition between the two different types of substrate (Rivett, 1989*c*).

It is not clear from the inhibitor sensitivity of the multicatalytic proteinase (Rivett, 1989*a*) to which class of proteinase it belongs. It has been suggested to be either a cysteine (Dahlmann *et al.*, 1985*a*; Rivett, 1985; McGuire & De Martino, 1986; Hough *et al.*, 1987) or a serine (Tanaka *et al.*, 1986; Mykles, 1989) proteinase. The proteinase is inhibited by thiol-reactive reagents and by peptide aldehyde inhibitors such as leupeptin and chymostatin. However, proteolytic activity is little affected by several other inhibitors of serine and cysteine proteases (Rivett, 1989*a*). The enzyme is activated by low concentrations of sodium dodecyl sulphate or fatty acids (Dahlmann *et al.*, 1985*b*), by polylysine (Tanaka *et al.*, 1988*a*) and by heat treatment (Mykles, 1989). It has been suggested to be present within cells in a 'latent' form.

Generally it has been found that ATP does not stimulate the proteolytic activities of purified multicatalytic proteinases (Rivett, 1989*a*). However, there are now several reports that immunoprecipitation of the multicatalytic proteinase from crude cell extracts reduces ATP-dependent proteolysis in those extracts (McGuire *et al.*, 1988; Tsukahara *et al.*, 1988) and the protease has therefore been suggested to participate in ATP-dependent mechanisms of protein degradation. Moreover the multicatalytic proteinase has recently been isolated in an ATP-dependent form which loses its ATP-dependence and becomes activated upon storage (Driscoll & Goldberg, 1989). Although this proteinase does not degrade ubiquitin–lysozyme conjugates (Hough *et al.*, 1987), it has been implicated in ATP- and ubiquitin-dependent pathways of protein degradation (cf. megapain; see below).

From SDS/polyacrylamide-gel electrophoresis experiments the proteinase appears to be composed of at least ten different types of polypeptide with molecular masses in the range 22–34 kDa (Rivett, 1989*a*). More polypeptides can be distinguished on two-dimensional polyacrylamide gels (Tanaka *et al.*, 1988*b*) and there is some evidence for changes in the *Drosophila* polypeptides during development (Haass & Kloetzel, 1989) including phosphorylation. The major polypeptides of the rat liver proteinase complex are unrelated as judged by peptide mapping (Tanaka *et al.*, 1988*b*) and by their immunological properties (Sweeney & Rivett, 1989).

Electron microscopic studies have shown that the multicatalytic proteinase has a hollow cylindrical structure (Kopp *et al.*, 1986; Baumeister *et al.*, 1988) but there is disagreement about the arrangement of the subunits (Kopp *et al.*, 1986; Tanaka *et al.*, 1988*b*). The proteinase has received much attention recently because its 20 S cylindrical structure is similar to that of a number of other 16–22 S cylindrical particles which have been found in the nucleus and cytoplasm of eukaryotic cells. These have been described as cylinder particles (Kleinschmidt *et al.*, 1983), ring-type particles (Kloetzel *et al.*, 1987), or prosomes (Schmidt *et al.*, 1984; Martins de Sa *et al.*, 1986), or referred to by the name cylindrin (Harris, 1988). Functions suggested for these particles include association with low molecular mass heat shock proteins (Arrigo *et al.*, 1985), 5′-pre-tRNA processing nuclease activity (Castano *et al.*, 1986) and, for prosomes, control of translation of mRNA (Schmidt *et al.*, 1984; Martins de Sa *et al.*, 1986; Arrigo *et al.*, 1987). Some of these particles, including prosomes, have now been shown to have proteolytic activity (Falkenburg *et al.*, 1988; Arrigo *et al.*, 1988; Kleinschmidt *et al.*, 1988) and they also have other properties (multisubunit composition and antigenic cross-reactivity) in common with the multicatalytic proteinase. It is possible that the multicatalytic proteinase has other functions in addition to its proposed role in intracellular protein turnover and that it may be some kind of multifunctional complex. It is also possible that there are several distinct particles. Indeed, cytoplasmic heat shock particles have recently been reported to be distinct from prosomes (Nover *et al.*, 1989). Also, levels of the multicatalytic proteinase complex are unaffected by heat shock, starvation or other stresses (Hendil, 1988).

RNA (repressed mRNA as well as integral RNA species of 80–100 nucleotides in length) has often been found associated with prosome preparations (Schmidt *et al.*, 1984; Martins de Sa *et al.*, 1986; Arrigo *et al.*,

1987) and this has led to suggestions that the multicatalytic proteinase complex (or prosome) represents a new type of ribonucleoprotein particle. Although the multicatalytic proteinase immunoprecipitated from rat liver does have RNA associated with it (Falkenburg *et al.*, 1988), many investigators have questioned the presence of RNA associated with more rigorously purified multicatalytic proteinase preparations (Arrigo *et al.*, 1988; Kleinschmidt *et al.*, 1988). Results of recent studies suggest that some of the discrepancies may be due to differences in methodology (Skilton *et al.*, 1989).

Proteinase *yscE* from yeast

Proteinase *yscE* (Achstetter *et al.*, 1984) is one of the several high molecular mass proteinases which have been identified in yeast (Table 2). This 600 kDa enzyme is similar in several respects to the multicatalytic proteinase (proteasome) found in mammalian cells. Recent studies have demonstrated the similarity of yeast proteinase *yscE* to the 20 S cylinder particles of *Xenopus laevis* (Kleinschmidt *et al.*, 1988) and comparative studies of the multicatalytic proteinases isolated from human, rat, and chicken liver, and from *Xenopus laevis* and yeast, have also shown them to be very similar complexes with similar catalytic properties (Tanaka *et al.*, 1988*a*).

Megapain, a ubiquitin-conjugate degrading endopeptidase

Two high molecular mass complex multisubunit cytosolic proteinases have been purified from reticulocyte lysates (Hough *et al.*, 1987; Waxman *et al.*, 1987). The major protease is the multicatalytic proteinase; the other has a molecular mass of greater than 1000 kDa and is composed of at least nine subunits ranging in molecular mass from 34 to 110 kDa. In some respects this 26 S proteinase, which has been called megapain (Hough *et al.*, 1988), is similar to the multicatalytic proteinase and the two proteinases may even be related (Hough *et al.*, 1987; Matthews *et al.*, 1989). However, unlike the multicatalytic proteinase, megapain degrades lysozyme–ubiquitin conjugates and it does so by an ATP-dependent process. The latter proteinase is therefore of particular interest because of its proposed role in the ubiquitin-mediated pathway of protein degradation (Rechsteiner, 1987; Hough *et al.*, 1988; Hershki, 1988). The ubiquitin system was first elucidated in reticulocyte lysates but is also known to operate in other cell types. The ubiquitin-conjugate degrading endopeptidase has been identified in rat skeletal muscle and liver (Fagan *et al.*, 1987).

Tripeptidyl peptidase II

Tripeptidyl peptidase II is a nonlysosomal aminopeptidase which has been found to release tripeptides from a polypeptide substrate (Bålöw *et al.*, 1983). The enzyme isolated from liver and erythrocytes is composed of many identical subunits of M_r 135000 (Bålöw *et al.*, 1986) and the polymeric form of the protease ($M_r >$ 1000000) appears to be a prerequisite for full activity (Macpherson *et al.*, 1987). Tripeptidyl peptidase II is a serine protease which binds approx. 1 mol of diisopropylfluorophosphate/mol of subunit (Bålöw *et al.*, 1986). Amino acid sequence analysis of a peptide containing the catalytic serine residue has shown tripeptidyl peptidase II to be related to the subtilisin subgroup of serine proteases (Tomkinson *et al.*, 1987).

Leucine aminopeptidase

Leucine aminopeptidase is a zinc metallopeptidase. Both the bovine lens and hog kidney enzymes are composed of six identical bilobal 54 kDa subunits (Taylor *et al.*, 1984). Human liver leucine aminopeptidase on the other hand (Kohno *et al.*, 1986) appears to be composed of two types of subunit (53 and 65 kDa) arranged in a hexameric structure. Leucine aminopeptidase activity is strongly inhibited by bestatin or amastatin and stimulated by metal ions, especially by Mn^{2+} and Mg^{2+}. Detailed kinetic studies (Lin *et al.*, 1988) have shown that activation by metal ions is unlikely to be of physiological significance and, despite its name, the enzyme can cleave almost any amino acid residue from the *N*-terminus of its peptide substrates (Kohno *et al.*, 1986).

Mitochondrial ATP-dependent protease

Following the discovery of the ATP-dependent Lon protease of *Escherichia coli* (see below), Desautels & Goldberg (1982) demonstrated the presence of ATP-dependent proteolytic activity in rat liver mitochondria and purified the protease (M_r 550000) responsible for this activity. Later, Watabe & Kimura (1985) partially purified what appears to be the same ATP-dependent protease (reported M_r 650000) from bovine adrenocortical mitochondria and confirmed its presence in mitochondria from a variety of other tissues. However, little structural information is available regarding this enzyme.

Other high molecular mass mammalian proteases

A few other large cellular proteases have been identified. There are several peptidases located in the plasma membrane of mammalian cells. These include two metalloproteases, endopeptidase 24.11 (Kenny, 1986) and meprin (Bond & Beynon, 1986), and an aspartic protease called cathepsin E (Yamamoto *et al.*, 1987; Jupp *et al.*, 1988). All are multisubunit enzymes.

Endopeptidase 24.11 (Kenny, 1986) is a widely-distributed dimeric enzyme of subunit M_r 45000 which degrades a variety of peptide substrates. The peptidase from kidney microvilli has been studied most extensively but the enzyme is also found in some regions of the central nervous system where it may be involved in the inactivation of neuropeptide transmitters (Kenny, 1986).

Meprin, on the other hand, has only been found in significant amounts in the kidney and liver of mice and rats (Bond & Beynon, 1986). An inherited deficiency of meprin has been discovered in some inbred mouse strains and the gene that controls meprin (*Mep-1*) has been found to be located near the major histocompatibility complex (Bond *et al.*, 1984). The four subunits in the meprin molecule are linked by disulphide bridges (Bond & Beynon, 1986). Each subunit contains 1 mol of Zn^{2+}/mol and 3 mol of Ca^{2+}/mol. The protease is inhibited by metal chelators but, unlike many other metalloproteases, including endopeptidase 24.11 (Kenny, 1986), is not inhibited by phosphoramidon, a sugar-*N*-phosphoramidate compound of microbial origin.

Cathepsin E, a dimeric aspartic proteinase of M_r 80000, appears to be identical to the aspartic protease found associated with the cytoplasmic surface of erythrocyte

membranes (Yamamoto *et al.*, 1987; Jupp *et al.*, 1988). The enzyme has been purified from rat spleen and identified in polymorphonuclear leukocytes, in macrophages and in rabbit bone marrow.

In addition to the large intracellular proteases described above, a number of extracellular mammalian proteases have complex structures. Several of the blood coagulation proteases have serine protease domains in longer polypeptides (Furie & Furie, 1988) and the subunit of nerve growth factor is an example of an extracellular proteolytic activity associated with a multisubunit complex (Thomas & Bradshaw, 1981).

BACTERIAL PROTEASES

Microbial proteases can be either intracellular or extracellular enzymes (Holzer *et al.*, 1975). The extracellular proteases are small stable enzymes which are active in the growth medium and are generally easy to assay and purify. The intracellular proteases have been investigated only recently. By 1975, only a few had been identified and, with the exception of aminopeptidases, all were of $M_r < 100000$ (Holzer *et al.*, 1975). However, it is now clear that *E. coli* contains at least twenty soluble proteases (Goldberg *et al.*, 1981; Katayama *et al.*, 1988). Two of the larger proteases, Lon protease and the multicomponent protease Clp (Table 3) are particularly intriguing because their degradation of protein substrates is dependent upon the hydrolysis of ATP.

Lon protease (protease La) from *Escherichia coli*

Lon protease is the product of the *Lon* gene in *E. coli* and has been alternatively referred to as protease La (Goldberg *et al.*, 1981). It is a tetrameric protein of subunit M_r 87000 as calculated from the DNA sequence (Chin *et al.*, 1988) although earlier reports suggested a molecular mass of 380–450 kDa (Waxman & Goldberg, 1982). The protease contains an ATPase site as well as a serine protease site within each subunit and proteolytic activity is ATP-dependent. Lon protease apparently cleaves peptide bonds and hydrolyses ATP in a linked process (Waxman & Goldberg, 1982) with two ATP molecules hydrolysed per peptide bond cleaved for a protein substrate (Menon *et al.*, 1987). Interestingly, both the ATPase and protease activities are stimulated by protein substrates (Waxman & Goldberg, 1986). Lon protease is a heat shock protein which is under the control of the *htpR* gene (Phillips *et al.*, 1984). It is also a DNA-binding protein (Zehnbauer *et al.*, 1981) and proteolytic activity is stimulated by DNA (Chung & Goldberg, 1982). It plays an important role in the degradation of several short-lived normal regulatory proteins (Mizusawa & Gottesman, 1983; Maurizi, 1987) and in the degradation of abnormal polypeptides (Goff & Goldberg, 1985, 1987). *Lon*$^-$ mutants show a decreased capacity for degrading these proteins, whereas cells with an increased number of copies of the *Lon* gene are able to degrade them more rapidly.

The multicomponent ATP-dependent protease of *Escherichia coli* (Clp protease, protease Ti)

The second ATP-dependent protease to be isolated from *E. coli* (Katayama-Fujimura *et al.*, 1987) was a multicomponent one which is now referred to as Clp protease (Katayama *et al.*, 1988) or protease Ti (Hwang *et al.*, 1987). It is composed of two different types of subunit, neither of which has proteolytic activity by itself. One is an ATP-binding regulatory subunit (A) of M_r 81000; the other type of subunit (P) of M_r 23000 contains the proteolytic site (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1988). There is disagreement about the effect of serine protease inhibitors on protease activity (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987) and therefore about the classification of the protease. The precise ratio of the two different types of subunits in the active M_r 370000 complex is also uncertain.

Katayama *et al.* (1988) have recently cloned the *clp A* gene which encodes the A subunit. Unlike Lon protease, Clp protease is not a heat shock protein (Katayama *et al.*, 1988; Hwang *et al.*, 1988). It appears to be a constitutive protease being present at similar levels in cells grown under a variety of different conditions. Moreover, it does not share the regulatory protein substrates of Lon protease (Katayama *et al.*, 1988) and probably has distinct functions within the cell.

PROTEASE STRUCTURES

Some of the smallest known proteases (M_r 15000–18000) are cysteine proteinases and dimeric aspartic proteinases of viral origin (Graves *et al.*, 1988; Hansen *et al.*, 1988). Ubiquitin (M_r 8500) has recently been reported to have proteolytic activity (Fried *et al.*, 1987) but this observation does not appear to have been confirmed by other investigators (Rechsteiner, 1987; Hershko, 1988). Most of the simple monomeric proteases of animals, plants or micro-organisms have a molecular mass in the range 20–30 kDa (North, 1982; Bond & Butler, 1987) but, as described above, proteases can have much larger and more complex structures. The larger proteases may consist of longer single polypeptides or be composed of two, four, or even more identical subunits, or possess two or even numerous different types of

Table 3. Examples of multisubunit proteases in bacteria

Protease	Class	Source	M_r	Subunit structure	References
Lon protease (protease La)	Ser/ATP	*E. coli*	340000	Tetramer, identical subunits	Menon & Goldberg (1987*a*,*b*), Maurizi (1987), Chin *et al.* (1988)
Multicomponent protease (Clp, Ti)	?/ATP	*E. coli*	360000	Two types of subunit, 81 and 23 kDa	Katayama *et al.* (1988), Hwang *et al.* (1988)
Aminopeptidase I	?	*E. coli*	323000	Hexamer, identical subunits	Holzer *et al.* (1975)

subunit in complexes varying in size from just over 100 kDa to greater than 1000 kDa. Individual polypeptide chains can vary from 9 kDa to 135 kDa. There is considerable variety in the structure of proteolytic enzymes isolated from any given cell type and presumably the different structures reflect different functions within the cell.

Within each of the well-defined catalytic classes, namely those of serine, cysteine, aspartic or metallo proteases, the well-characterized small proteases have often been found to have a high degree of structural similarity (Neurath, 1984; Barrett, 1986) and they fall into only one or two different superfamilies of enzymes with homologous primary structures with the sequences around catalytic residues being particularly highly conserved. Based on their inhibitor sensitivity, most of the larger proteases which have been identified can be assigned to one of the four major catalytic classes noted above. In view of this one might expect that high molecular mass proteases could contain either proteolytic domains or proteolytic subunits which were structurally related to well-characterized smaller proteases. In cases where the amino acid sequence has been deduced, this has indeed been found to be true for both the more complex intra- and extra-cellular proteases. For example, the proteolytic domain of the calpains shows similarity to that of papain (Suzuki, 1987) and also to those of the M_r 25000–30000 lysosomal cysteine proteases, cathepsins B, H and L. Similarly, the amino acid sequence of a peptide containing the catalytic serine residue of tripeptidyl peptidase II ($M_r > 1000000$) has shown it to belong to the subtilisin subgroup of serine proteases (Tomkinson *et al.*, 1987). Lon protease, on the other hand, bears no sequence similarity to any other known serine protease structures (Chin *et al.*, 1988).

Notwithstanding the lack of detailed structural information available for many of the larger proteases, it is clear that nonproteolytic domains of the high molecular mass proteases also show homology with domains having a similar function in other proteins. For example, the calcium-binding domains of calpains are similar in sequence to those found in calmodulin and some muscle proteins (Suzuki, 1987), and sequences which are closely related to the ATP-binding domains of other ATPases have been identified in Lon protease (Chin *et al.*, 1988). Therefore, it seems likely, at least in some cases, that the multidomain structures of polypeptide chains of the larger intracellular proteases have evolved as a result of gene fusions, a phenomenon well-recognized for the serine proteases of the blood coagulation cascade (Furie & Furie, 1988).

PROTEASE FUNCTION

It is difficult to establish the precise function of a protease from its specificity since, with a few exceptions, proteases are generally not specific for particular peptide or protein substrates. Nonetheless, the catalytic properties as well as the structure and localization of proteolytic enzymes can give some indication of possible function. Proteinases which catalyse the extensive degradation of protein substrates may play an important role in protein turnover, whereas proteinases catalysing only limited cleavage of proteins possibly play a role in specific protein modification events. In the latter case, colocalization with potential substrates would be expected.

Both tissue distribution and subcellular localization studies may provide clues to the function of proteolytic enzymes. The most incisive studies to date have been carried out by using antibodies directed against the protease of interest. Many intracellular mammalian proteases are found in virtually all types of cells and presumably play an important role in basic cellular functions. These include the lysosomal proteases, calpains (Murachi, 1983), tripeptidyl peptidase II (Bålöw & Eriksson, 1987) and the multicatalytic proteinase (Tanaka *et al.*, 1986; Ray & Harris, 1987; Rivett, 1989*a*). Other proteases may have characteristic tissue distributions, indicating that they are likely to have specialized functions unrelated to gross protein turnover or other basic cellular processes.

The subcellular localization of a proteolytic enzyme can often be a direct consequence of its function. For example, a prohormone processing protease has been isolated from bovine pituitary secretory granules (Parish *et al.*, 1986), signal peptidases are present in the mitochondria (Hawlitschek *et al.*, 1988) and calpains have been co-localized with potential cytoskeletal protein substrates (Beckerle *et al.*, 1987).

The function of an individual proteolytic enzyme may also be deduced by comparing proteolytic processes in mutant cells with those of parental types. In *E. coli* the degradation of abnormal proteins and of some other protein substrates by Lon protease *in vivo* has been demonstrated by studies with *lon*$^-$ cells and cells having extra copies of the *lon* gene (Goff & Goldberg, 1985, 1987). The functions of several yeast proteinases have also been identified by using mutants (Wolf, 1986). One known example of a natural deficiency of a mammalian protease is in the *mep-1* gene which encodes the membrane peptidase, meprin (Bond & Beynon, 1986). Further studies in mammalian cells may be dependent on the development of techniques for inactivating the specific gene of interest (Frohman & Martin, 1988).

REGULATION OF PROTEASE ACTIVITY

Importance of regulation of extralysosomal proteolysis

The action of the small but highly active lysosomal proteases in mammalian cells is controlled by compartmentalization, and intralysosomal protein degradation is limited by the uptake of proteins into the lysosomes. The lysosomal proteases have low pH optima to suit the acidic environment of the lysosome and perhaps to limit their activity outside it. For example, cathepsin D, an aspartic proteinase, is inactive at neutral pH, and there is a protein inhibitor of the lysosomal cysteine proteases (cathepsins B, H, and L) in the cytoplasm possibly to protect cells against any leakage of these enzymes from the lysosomes. The inhibitor (M_r 11000) is one of the cystatin family of cysteine protease inhibitors (Barrett, 1987). For nonlysosomal proteolysis, the requirement for regulation of the activity of individual proteases is more critical (except for specific processing proteases within secretory vesicles), because proteolytic activity must be intimately linked to the metabolic state of the cell.

Mechanisms of regulation

The most important function of the complex structures of some extralysosomal cellular proteinases must be to provide the potential for regulation of their activity to

Table 4. Catalytic properties and regulation of some large intracellular proteases

See the text for references.

Protease	Property
Mammalian proteases	
Calpains	Ca^{2+}-dependent activity
	Catalyse limited proteolysis
	Endogenous inhibitor, calpastatin
	Activator protein
	Activation by membrane association and autolysis
Multicatalytic proteinase	At least two distinct types of catalytic site
	'Latent' form?
	Activation by fatty acids
	Possible ATP-dependence
Megapain	Degrades ubiquitin–protein conjugates
	ATP-dependent
Bacterial proteases	
Lon protease (La)	ATP-dependent degradation
	Activation by protein substrates
	Heat shock protein
	DNA-binding protein
Clp protease (Ti)	ATP-dependent degradation
	Isolated proteolytic subunit inactive

protect the cell against unwelcome proteolysis. Mechanisms suggested from studies *in vitro* are listed in Table 4 and, in some cases, there is also evidence for their operation *in vivo*.

The role of **endogenous inhibitors and activators** is best illustrated by the calpains which are regulated, not only by Ca^{2+} concentration, but also by the interaction with a specific endogenous inhibitor protein called calpastatin (Murachi, 1983). This inhibitor is widely distributed in the cytosol together with the calpains. The structure of calpastatin has been determined recently and it has been found to contain four internal repeating units, each of which can inhibit calpain activity independently (Emori *et al.*, 1987). It is the central conserved region of each of these inhibitor units which is important for the interaction with calpain (Maki *et al.*, 1988). The precise role of a recently described calpain activator protein has not been established. This small protein is a component of the cytoskeleton but there is disagreement about its molecular mass and its effect on the Ca^{2+} sensitivity of the proteinases (Takeyama *et al.*, 1986; Pontremoli *et al.*, 1988). Calpain II is not active *in vivo* in the form in which it requires millimolar concentrations of Ca^{2+}. Activation occurs by association with the cell membrane (Pontremoli & Melloni, 1986; Suzuki, 1987; Mellgren, 1987) which leads to autolysis and release from the membrane in an active form. The latter involves cleavage at the *N*-termini of both the 80 kDa catalytic subunit and the 30 kDa subunit (Suzuki, 1987), effecting an increase in Ca^{2+} sensitivity for the larger subunit and loss of membrane binding for the smaller one.

Several proteinases in addition to calpain II have been suggested to be present in the cell as **latent proteinases** which can be activated under appropriate conditions. However, there is little solid evidence for this. Cathepsin E may be inactive when associated with the erythrocyte membrane but active when present in a soluble form (Yamamoto *et al.*, 1987). Although the multicatalytic proteinase has been referred to as a latent multifunctional protease complex (Tanaka *et al.*, 1986), there is no evidence to date that the observed activation of the proteinase by heat treatment (Mykles, 1989), ATP, polylysine or fatty acids is of physiological significance.

In addition to the direct effects of activators or inhibitors on protease activity by interaction with protease molecules, **recognition of substrate proteins** may play an important role in the regulation of intracellular proteolysis. Substrate protein structure has an important influence on the susceptibility to proteolytic attack both for specific cleavage events (Parish *et al.*, 1986; Hawlitschek *et al.*, 1988) and for extensive proteolysis (Rechsteiner *et al.*, 1987; Beynon & Bond, 1986; Dice, 1987). Moreover, the proteolytic susceptibility of proteins can be dramatically affected by specific covalent modification reactions (Stadtman, 1986; Rivett, 1986). In one case **allosteric activation of a proteinase by substrate proteins** has been proposed. Peptide and ATP hydrolysis by Lon protease is activated by protein substrates but not by non-substrate proteins (Menon & Goldberg, 1987*b*).

ATP-dependent proteolysis

Although it is now clear that proteins can be degraded by ATP-independent mechanisms (Rechsteiner, 1987; Rivett, 1989*b*), the purification of ATP-dependent proteinases (Goldberg *et al.*, 1981; Desautels & Goldberg, 1982; Hough *et al.*, 1987; Katayama-Fujimura *et al.*, 1987) has provided strong evidence for the occurrence of ATP-dependent pathways of intracellular proteolysis. A clear distinction should be made between proteases which are ATP-dependent in a mechanistic sense and those which are merely stimulated or stabilized by ATP. The general characteristics of protein degradation by ATP-dependent proteases are the absolute requirement for MgATP, inhibition by vanadate and lack of activity in the presence of nonhydrolysable ATP analogues. Lon protease (La), Clp protease (Ti), megapain, and the mitochondrial ATP-dependent protease all fall into this category (Menon & Goldberg, 1987*a,b*; Katayama-Fujimura *et al.*, 1987; Hough *et al.*, 1987; Desautels & Goldberg, 1982) and activity of the multicatalytic proteinase may also be ATP-dependent *in vivo* (McGuire *et al.*, 1988; Driscoll & Goldberg, 1989). The requirement of ATP-dependent proteases for ATP hydrolysis for the degradation of synthetic peptide substrates is not as strict as for protein substrates and in some cases such activity is only slightly stimulated by ATP (Maurizi, 1987; Hough *et al.*, 1987). ATP hydrolysis is often not required for the degradation of small peptide substrates (Woo *et al.*, 1989).

Not enough is known about the detailed mechanisms of these ATP-dependent proteases to understand why ATP hydrolysis should be required for the cleavage of peptide bonds in protein substrates. Since peptide bond hydrolysis is exergonic and most proteases do not require ATP, it seems likely that the ATP dependence is for the regulation of proteolytic activity or even for protein unfolding or translocation of the substrate relative to the proteinase. Some mechanistic studies have been carried out with the best characterized of the ATP-dependent

proteases, Lon protease (Menon & Goldberg, 1987*a*,*b*; Maurizi, 1987). The protease appears to require the hydrolysis of two molecules of ATP for each peptide bond cleaved in a protein substrate. A processive mechanism of protein degradation has been suggested (Menon *et al.*, 1987) but other results (Maurizi, 1987) do not support this proposal.

ATP-dependent mechanisms of protein degradation seem to be particularly important under conditions of stress when there is a large amount of abnormal protein in cells (Goff & Goldberg, 1985; Rechsteiner, 1987; Rivett, 1989*b*).

CONCLUDING REMARKS

Over the past decade there has been considerable progress in the identification and characterization of cellular proteases. Novel types of high molecular mass proteases have been described and, where available, information about structure and catalytic properties has helped to understand the functional significance of the variation in protease structure and the regulation of proteinase activity. Characterization of intracellular proteases is an essential step towards understanding mechanisms of intracellular proteolysis. Obviously there is still much to be learned, and determination of the molecular structure and mechanisms of regulation of the more complex intracellular proteases promises to be an exciting area for future research.

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