The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L

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The major excreted protein (MEP) purified from Kirsten-virus-transformed 3T3 fibroblasts and mature human cathepsin L were compared in respect to a number of catalytic criteria and found to be similar. The M_r of MEP is 39000, whereas that of mature human cathepsin L is 30000. Sequence data suggested that MEP could be a pro-form of mouse cathepsin L. Both enzymes acted on the synthetic substrate benzyloxycarbonyl-Phe-Arg-7-(4-methyl)coumarylamide with similar catalytic constants and acted optimally at pH 5.5. Both were rapidly inactivated by the active-site-directed inhibitors benzyloxycarbonyl-Phe-Phe-diazomethane and L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane, and furthermore, ^3H -labelled L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-acetamido)butane, which binds covalently to the heavy chain of mature cathepsin L, also bound to MEP. MEP autolyses rapidly at pH 3.0 to give lower- M_r (35000 and 30000) forms, but all forms react with the radiolabelled inhibitor. No autolysis occurred above pH 5.0. MEP hydrolysed azocasein at pH 5.0, demonstrating that it is capable of hydrolysing protein substrates without autolytic activation. Unlike mature forms of cathepsin L, MEP is stable, but not active, at neutral pH. The present work shows that cathepsin L can be secreted as a higher- M_r precursor that is stable in extracellular fluids but only active where local pH values fall below 6.0. These results suggest that the extra *N*-terminal peptide on MEP is not an activation peptide, but is a regulatory peptide affecting the pH-stability and activity of mouse cathepsin L.

INTRODUCTION

The cysteine proteinases cathepsins B and L are located in lysosomes, as demonstrated by subcellular-fractionation studies and cytochemical and immunochemical staining (Kirschke *et al.*, 1977; Graf *et al.*, 1979; Etherington *et al.*, 1984). This may not be the only location of these enzymes, however. One immunochemical study has also shown an extracellular location of cathepsin B around synovial cells (Poole & Mort, 1981). Furthermore, cathepsin B-like activity has been detected in synovial fluid, bronchoalveolar lavage fluid and sputum (Mort *et al.*, 1983; Chang *et al.*, 1986; Burnett *et al.*, 1983). Precursor forms of cathepsin B have been identified immunochemically in ascites fluid, but have not yet been purified to enable a direct demonstration that these forms are proteolytically active (Mort & Recklies, 1986). The function of these secreted enzymes is not known, but extracellular levels of cathepsin B-like activity have been correlated with metastasis (Sloane & Honn, 1984).

A secreted precursor form of a lysosomal cysteine proteinase has now been identified and purified. Kirsten-virus-transformed mouse 3T3 fibroblasts secrete a protein of M_r 39000 that was named 'MEP' (Gottesman, 1978). This purified protein exhibited proteolytic activity against bovine serum albumin at pH 3.0. The incubation conditions used resulted in a decrease in the M_r of MEP

(Gal & Gottesman, 1986a). At pH 3.0, the enzyme also cleaved the B-chain of insulin, cleaving at the same sites as rat liver cathepsin L at pH 6.0 (Gal & Gottesman, 1986b), suggesting that the two enzymes might be related. The protein sequence derived from the cDNA of MEP, and its analogue expressed by mouse peritoneal macrophages, was found to be homologous with the protein sequence of human cathepsin L (Portnoy *et al.*, 1986; Denhardt *et al.*, 1986; Mason *et al.*, 1986; B. Troen, S. Gal & M. M. Gottesman, unpublished work). Over 80% identity of the homologous sequences strongly suggested that these enzymes are species variants of the same protein. MEP has a significantly higher M_r than mature lysosomal forms of cathepsin L and has been shown to be processed to lower- M_r forms similar in size to mature cathepsin L (Gal *et al.*, 1985) and hence most probably represents a precursor form of this enzyme. The present paper describes the characterization of the proteolytic activity of MEP and a comparison of its enzymic activity with that of the mature forms of cathepsin L.

MATERIALS AND METHODS

Materials

Human cathepsin L was purified as described by Mason *et al.* (1985), and MEP was purified as described by Gal & Gottesman (1986a). [^3H]Ac-Ep-459 (sp.

Abbreviations used: Z-, benzyloxycarbonyl; NHMec, 7-(4-methyl)coumarylamide; -CHN₃, diazomethane; MEP, major excreted protein; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; Ac-Ep-459, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-acetamido)butane.

radioactivity 83 Ci/mol) was prepared as described by Parkes *et al.* (1985). Dithiothreitol, bovine serum albumin and E-64 [as L-trans-epoxysuccinyl-leucylamido-(4-guanidino)butane] were from Sigma Chemical Co., Poole, Dorset, U.K. Azocasein was prepared as described by Barrett & Kirschke (1981). Z-Phe-Phe-CHN₂ and Z-Phe-Ala-CHN₂ were gifts from Dr. E. N. Shaw, Friedrich Meischer Institute, Basle, Switzerland. Aminomethylcoumarin substrates were from Cambridge Research Biochemicals, Cambridge, U.K.

Methods

Activity against synthetic substrates. MEP was routinely assayed for activity against Z-Phe-Arg-NHMec at pH 5.5, as described previously for human cathepsin L (Mason *et al.*, 1985). Identical conditions were employed when Z-Arg-Arg-NHMec and Arg-NHMec were used as substrates. Assays were started by adding enzyme, and initial rates were recorded continuously. Catalytic constants were determined by the method of Wilkinson (1961). For pH profiles, buffers used were 100 mM-sodium formate (pH 3.0–4.0), 100 mM-sodium acetate (pH 4.0–6.0) and 100 mM-sodium phosphate (pH 6.0–8.0). For assays of modified (i.e. autolysed) MEP, the enzyme was pretreated at pH 3.0, 37 °C for 2 min immediately before assay.

Stability of MEP. MEP (1 µg) was incubated in 100 µl of a range of buffers, pH 3.0–8.0, at 37 °C for 1 h. Enzyme was then diluted in 100 mM-sodium acetate buffer, pH 5.5, and assayed as described previously (Mason *et al.*, 1985).

Inactivation of MEP by active-site-directed inhibitors. MEP was assayed continuously at pH 5.5 by using standard conditions for the continuous rate assays of human cathepsin L (Mason *et al.*, 1985). After 5 min the inhibitor was added and the rate of inactivation calculated as described previously (Mason *et al.*, 1985). Rates were determined at inhibitor concentrations ranging from 50 to 200 nM in the presence of 1–5 µM-Z-Phe-Arg-NHMec. Rates of inactivation were dependent upon substrate concentration, and replots of reciprocal rate against substrate concentration gave rates independent of substrate and also gave a separate estimate of K_m .

Reaction with [³H]Ac-Ep-459. MEP (3 µg) was incubated with 200 mM-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA and 1 mM-dithiothreitol for 30 min at 37 °C in a final volume of 15 µl. A 5 µl portion of [³H]Ac-Ep-459 (550 µM) was then added and the mixture incubated for a further 30 min. An equal volume of SDS sample buffer (Bury, 1981) was then added and the mixture boiled before it was subjected to 10% (w/v) polyacrylamide-gel electrophoresis as described previously (Bury, 1981). Other samples of MEP (3 µg) were either preincubated with 200 mM-sodium formate buffer, pH 3.0, containing 1 mM-EDTA and 1 mM-dithiothreitol for 2 min at 37 °C before addition of [³H]Ac-Ep-459, or added to this buffer containing the radiolabelled inhibitor.

Gels were sliced for extraction of radioactivity and counted as described by Parkes *et al.* (1985). Efficiency of extraction of labelled proteins was determined by using active-site-titrated papain as a standard. About 50% of

the radioactivity could be extracted with an s.d. of no more than 10%.

Autolysis of MEP. MEP was incubated in 100 mM-sodium formate buffer, pH 3.0, containing 1 mM-EDTA at 37 °C for 2 min. The reaction mixture was then diluted with 100 mM-sodium acetate buffer containing 1 mM-EDTA, pH 5.5, and stored at 4 °C until required.

Activity against bovine serum albumin. MEP (0.3 µg, 1 µl) was added to either 100 mM-sodium formate, pH 3.0, containing 1 mM-EDTA and 1 mM-dithiothreitol (5 µl) or 100 mM-sodium acetate, pH 5.0, containing 1 mM-EDTA and 1 mM-dithiothreitol (5 µl), and incubated at 37 °C. After 1 min, bovine serum albumin (3 µg, 5 µl) was added and incubated at 37 °C for 1 h. A sample of enzyme was preincubated at pH 3.0 with the formate buffer and E-64 (10 µM final concn.) and assayed similarly. The reaction was stopped by boiling in SDS sample buffer and samples run in 10%-(w/v)-polyacrylamide gels as described by Bury (1981). Human cathepsin L (10 ng of active enzyme) was assayed similarly.

Activity against azocasein. MEP (0.3 µg) was incubated with azocasein (0.1%) dissolved in buffer containing 1 mM-EDTA and 1 mM-dithiothreitol in a total volume of 1.0 ml. Buffers used were 100 mM-sodium acetate (pH 4.5–6.0) or 100 mM-sodium phosphate (pH 6.0–7.5). Incubation was for 2 h at 30 °C, and the reaction was stopped by adding 200 µl of 20% (w/v) trichloroacetic acid. Soluble fragments of azocasein were measured by their A_{366} as described previously (Barrett & Kirschke, 1981). For comparison, human cathepsin L (30 ng of active enzyme) was assayed similarly.

RESULTS

Activity against synthetic substrates

MEP was active against the synthetic cathepsin L substrate Z-Phe-Arg-NHMec. The initial activity of MEP against this substrate was found to be maximal at pH 5.5 (Fig. 1). There was a second peak of activity at pH 3.0. This pH-activity profile differs from that of mature human liver cathepsin L, which has a broad initial pH-activity profile, with activity at least 50% of maximal over the range pH 4.0–7.5 (Mason *et al.*, 1985). Treatment of MEP at pH 3.0 has been shown to cause autolysis to yield a smaller protein, similar in size to the mature lysosomal form of the enzyme (Gal & Gottesman, 1986a). To determine if autolysed MEP has the same pH-activity curve as cathepsin L, MEP was incubated at pH 3.0 and 37 °C for 2 min and its initial activity against Z-Phe-Arg-NHMec measured (Fig. 1). The pH-activity profile of this acid-treated protein was similar to that of mature human cathepsin L, and did not exhibit the biphasic profile seen for non-autolysed MEP. The high activity seen for 'intact MEP' at pH 3.0 most probably reflects activity of the protein autolysed during the assay.

When activity was measured continuously at pH 4.0, activity slowly increased until the rate was almost equivalent to that of the enzyme assayed at pH 3.0 (Fig. 2). The most likely interpretation of this result is that MEP is rapidly converted into a lower- M_r catalytically

Fig. 1. pH-activity profile of MEP against Z-Phe-Arg-NHMec

MEP was diluted in pH 5.5 activating buffer and stored on ice for 10 min. The initial activity of enzyme was measured at a range of pH values as described in the Materials and methods section (○). MEP was autolysed to a lower- M_r form, and the initial activity of the modified enzyme was measured at a range of pH values as described in the Materials and methods section (△).

Fig. 3. pH-stability of MEP

MEP was preincubated for 1 h at 37 °C in a range of buffers (pH 3.0–8.0) as described in the Materials and methods section. Activity remaining was assayed at pH 5.5 with Z-Phe-Arg-NHMec as substrate and is expressed as a percentage of activity measured without preincubation (○). The stability of human cathepsin L is shown for comparison (△).

Fig. 2. Activity of MEP against Z-Phe-Arg-NHMec at pH 4.0

MEP was assayed continuously in the presence of 5 μ M-Z-Phe-Arg-NHMec in 100 mM-sodium formate buffer, pH 4.0, containing 1 mM-dithiothreitol and 1 mM-EDTA.

active form at pH 3.0, but is activated by autolysis much more slowly at pH 4.0. Although the autolysed form of MEP is catalytically similar to mature forms of cathepsin L, the higher- M_r form has a much more restricted pH-activity range.

With respect to proteolytic activity, MEP was found to

be stable over the pH range 4.5–8.0, but unstable below pH 4.5 (Fig. 3). This contrasts with the activity of the mature forms of cathepsin L, which are unstable above pH 6.5 and below pH 4.0 (Mason, 1986).

Having established that the activity of MEP against Z-Phe-Arg-NHMec was greatest and stable at pH 5.5, we were then able to determine the K_m of MEP for this substrate. A value of $1.28 \pm 0.28 \mu$ M was determined by the method of Wilkinson (1961), a value similar to that found for all mature forms of cathepsin L (Mason, 1986). The k_{cat} of the untreated purified enzyme was determined to be 1.6 s^{-1} , on the basis of total protein. This is approx. 10 times less than that found for mature cathepsin L on the basis of active-site-titrated enzyme. This suggests that either MEP is catalytically less efficient than the mature form of human cathepsin L or that, under these assay conditions (incubation for 30 min at pH 5.5), the purified MEP contains 90% inactive protein. The latter was found to be the case when the enzyme was allowed to react with [3 H]Ac-Ep-459 (see below).

The cathepsin B and H substrates Z-Arg-NHMec and Arg-NHMec are poor substrates for cathepsin L (Mason *et al.*, 1985). This was also true for MEP (results not shown).

It has therefore been shown that MEP has a similar catalytic activity to cathepsin L against synthetic substrates. The major difference is that mature cathepsin L is active, but unstable, at pH 8.0, whereas MEP is inactive but stable.

Inactivation by active-site-directed inhibitors

A significant characteristic of mature human cathepsin L is its rate of inactivation by the active-site-directed

Fig. 4. Autolysis of MEP and its reaction with [^3H]Ac-Ep-459

Lane 1, MEP treated at pH 3.0 for 2 min at 37 °C; lane 2, MEP treated at pH 5.0 for 30 min at 37 °C; lane 3, MEP + [^3H]Ac-Ep-459 (550 μM) treated at pH 3.0 for 2 min at 37 °C; lane 4, M_r standards. After pretreatment, enzyme fractions were allowed to react with an excess of [^3H]Ac-Ep-459 before running samples in 10 %-(w/v)-polyacrylamide gels in SDS under reducing conditions. Radioactivity (d.p.m.) recovered from each band was as follows: lane 1: band 1, 159; band 2, 267; band 3, 1246; lane 2: band 1, 1519; band 2, 99; band 3, 12; lane 3: band 1, 1025; band 2, 318; band 3, 585.

inhibitors Z-Phe-Phe- CHN_2 and E-64. These compounds react covalently with the active-site cysteine residue of cysteine proteinases (Leary & Shaw, 1977; Barrett *et al.*, 1982). They are thought to react with active enzyme and are used to titrate cysteine proteinases (Barrett & Kirschke, 1981). The rates of inactivation of MEP by these compounds were determined at pH 5.5 and found to be $180\,000 \pm 18\,000$ and $110\,000 \pm 20\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. Z-Phe-Ala- CHN_2 also rapidly inactivated MEP, with a rate constant of $150\,000 \pm 22\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$. K_m values for Z-Phe-Arg-NHMec determined from re-plots of the inverse rate of inactivation against substrate concentration, fell into the range 1–2 μM , confirming the results obtained by using the Wilkinson (1961) plots (see above). The rates of inactivation were similar to those of species variants of mature cathepsin L (Mason, 1986).

Autolysis of MEP and its reaction with a radiolabelled inhibitor

It has previously been demonstrated that MEP autolyses at pH 3.0 to yield lower- M_r forms (Gal & Gottesman, 1986a). In order to determine whether this could be happening at pH 5.0, MEP was preincubated at this pH for 30 min at 37 °C, and then a large excess of [^3H]Ac-Ep-459 was added and allowed to react with the enzyme as described in the Materials and methods section. The labelled protein had an M_r identical with that of the purified enzyme (lane 2, Fig. 4). The radioactivity extracted from this band indicated that under these reaction conditions the amount of labelled MEP was only 10 % of the total protein. When MEP was pretreated at pH 3.0 for 2 min at 37 °C (lane 1, Fig. 4), most of the MEP was converted into a lower band of

M_r 30 000. Total recovery of radioactivity was similar to that seen with enzyme preincubated at pH 5.0.

In order to observe any intermediate forms of MEP, inhibitor and pH 3.0 buffer were added to the MEP simultaneously. In this instance, bands of M_r 39 000 and 30 000 were seen and, in addition, a band of M_r 35 000. Again all three bands incorporated radioactivity, indicating that they contained active enzyme.

Thus the secreted form of cathepsin L, like the mature form, is labelled by an irreversible active-site-directed inhibitor. The specific activity of intact MEP was determined by using incorporated radioactivity to estimate the concentration of active enzyme and activity against Z-Phe-Arg-NHMec measured as described above. Only 10 % of the MEP reacted with the inhibitor, providing an estimate for k_{cat} of 16 s^{-1} . This is similar to values obtained for mature forms of cathepsin L (Mason, 1986).

These results indicate that 90 % of the purified MEP is inactive. This inactive protein does not appear to be latent enzyme, and hence most probably represents either irreversibly oxidized enzyme or denatured protein. This is a common problem when purifying cysteine proteinases by conventional chromatographic techniques. Homogeneous preparations of human cathepsins B, L and H were found to be 50, 60 and 90 % inactive respectively by titration with E-64 (Barrett & Kirschke, 1981; Mason *et al.*, 1985).

Activity of MEP against bovine serum albumin

MEP was described as an acid-activatable cysteine proteinase on the basis of its ability to cleave bovine serum albumin at pH 3.0 (Gal & Gottesman, 1986a). We

Fig. 5. Activity of MEP and cathepsin L against bovine serum albumin

Enzyme and bovine serum albumin were incubated together at 37 °C for 1 h as described in the Materials and methods section. Lane 1, MEP and albumin, pH 3.0; lane 2, MEP, E-64 and albumin, pH 3.0; lane 3, albumin alone, pH 3.0; lane 4, MEP and albumin, pH 5.0; lane 5, albumin alone, pH 5.0; lane 6, human cathepsin L and albumin, pH 3.0; lane 7, human cathepsin L, E-64 and albumin, pH 3.0; lane 8, albumin alone, pH 3.0; lane 9, human cathepsin L and albumin, pH 5.0; lane 10, albumin alone, pH 5.0.

Fig. 6. Activity of MEP against azocasein

Activity of MEP against azocasein was assayed at a range of pH values as described in the Materials and methods section. ○, MEP; △, human cathepsin L (for comparison).

have now compared the proteolysis of albumin by MEP directly with that by human cathepsin L (Fig. 5). Activity of MEP at pH 3.0, but not at pH 5.0, against albumin was confirmed. The activity at pH 3.0 was blocked by E-64, proving that the proteolytic activity was due to a cysteine proteinase. Albumin was similarly resistant to

hydrolysis by mature human cathepsin L at pH 5.0, but was degraded at pH 3.0. Many proteins are resistant to proteolysis in their native state, but are readily hydrolysed when denatured. This most probably explains why this protein is particularly well cleaved by cathepsin L and MEP at low pH and hence it is not a particularly good substrate for measuring the proteolytic activity of MEP at higher pH values. This experiment cannot distinguish between activity of intact or hydrolysed MEP at pH 3.0.

Activity against azocasein

It has been reported that secreted forms of cathepsin B, a proteinase related to cathepsin L, can hydrolyse synthetic substrates, but not proteins (Mort & Recklies, 1986). We therefore examined the ability of MEP to hydrolyse azocasein, a chromogenic denatured protein substrate. Activity was measured over the pH range 4.5 (the minimum value for solubility of the substrate)–7.5. The optimal pH for hydrolysis of azocasein by MEP was pH 5.0, with no significant activity being measurable above pH 6.0 (Fig. 6). By contrast, human and rabbit cathepsin L are at least 50% as active at pH 6.0 as at pH 5.0 (Mason *et al.*, 1984, 1985). The specific activity of MEP for the hydrolysis of azocasein was compared directly with that of titrated human liver cathepsin L at pH 5.0, and the mouse fibroblast enzyme was found to be twice as active. It has already been demonstrated that MEP does not autolyse significantly at pH 5.0, and we therefore conclude that intact MEP degrades this protein. The data provide further evidence that MEP is an active form of cathepsin L, but with a narrower pH range.

DISCUSSION

These results show that MEP is catalytically similar to cathepsin L. Taken with the sequence homology and M_r differences, it is now clear that MEP (Gottesman, 1978) is a precursor form of mouse cathepsin L (Mason

et al., 1986). The *N*-terminal extension peptide does not act as a true activation peptide, however, as the precursor enzyme is active. A precursor form of cathepsin D secreted by human breast-cancer cells has also been found to be active (Capony *et al.*, 1987). Thus two of the major lysosomal proteinases have *N*-terminal extension peptides which do not require cleavage in order for the enzyme to exhibit proteolytic activity. It is likely that these *N*-terminal extension peptides are regions of the proteins which are sensitive to hydrolysis within the lysosome and hence are not seen on the isolated stable forms of the mature enzymes.

The *N*-terminal extension peptide of mouse cathepsin L (MEP) does seem to have some effects on the enzyme, however. Our results suggest that this peptide stabilizes mouse cathepsin L at neutral pH. Such stability may be required during the normal biosynthesis of cathepsin L as it passes through the Golgi apparatus before transport to lysosomes. The exact pH in the Golgi is uncertain, but is generally considered to be less acidic than in lysosomes, where the mature enzymes are found. Extracellular body fluids are at neutral pH or above, and only the precursor form of cathepsin L, which is the form secreted by transformed mouse cells, would be stable in these fluids.

If stabilization were the only function of the *N*-terminal extension, then the secreted enzyme might be expected to be active at neutral pH, as is the case for the unstable mature form of cathepsin L. The precursor was in fact found to be inactive at pH 7.0, and therefore, although stable at neutral pH, is not functional. This restricts action of the enzyme to sites of low pH within and around cells and tissues (Etherington *et al.*, 1981). Within cells, MEP would be active in acid compartments such as lysosomes, endosomes and some secretory granules, but would be inactive if accidentally released into the cytoplasm.

It now appears that cathepsin L may function both intracellularly and extracellularly. The site of degradation of proteins hydrolysed by this enzyme will now have to be reconsidered. Chapman & Stone (1984) found that Z-Phe-Ala-CHN₂, which has now been shown to be a potent inhibitor of both lysosomal and secreted forms of cathepsin L, could inhibit elastin degradation by human alveolar macrophages, whereas chloroquine, which only blocks the activity of lysosomal enzymes, was less effective. These results therefore suggest a role for a secreted form of cathepsin L in elastin turnover.

Alternatively, the secreted enzyme may have no extracellular proteolytic function and may only be activated when taken up by other cells and packaged into lysosomes. The secreted enzyme can be taken up by other cells with mannose 6-phosphate receptors and be processed intracellularly (S. Gal & M. M. Gottesman, unpublished work). Thus the secreted precursor of cathepsin L could affect proteolysis within cells at some distance from the secreting cell.

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