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Human kidney cathepsins B and L

Characterization and potential role in degradation of glomerular basement membrane

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Cathepsins B and L were purified from human kidney. SDS/polyacrylamide-gel electrophoresis demonstrated that cathepsins B and L, M_r 27000–30000, consist of disulphide-linked dimers, subunit M_r values 22000–25000 and 5000–7000. The pH optimum for the hydrolysis of methylcoumarylamide (-NHMec) substrates (see below) is approx. 6.0 for each enzyme. K_m and $k_{cat.}$ are 252 μM and 364 s^{-1} and 2.2 μM and 25.8 s^{-1} for the hydrolysis of Z-Phe-Arg-NHMec (where Z- represents benzyloxycarbonyl-) by cathepsins B and L respectively, and 184 μM and 158 s^{-1} for the hydrolysis of Z-Arg-Arg-NHMec by cathepsin B. A 10 min preincubation of cathepsin B (40 °C) or cathepsin L (30 °C) with E-64 (2.5 μM) results in complete inhibition. Under identical conditions Z-Phe-Phe-CHN_2 (0.56 μM) completely inhibits cathepsin L but has little effect on cathepsin B. Incubation of glomerular basement membrane (GBM) with purified human kidney cathepsin L resulted in dose-dependent (10–40 nM) GBM degradation. In contrast, little degradation of GBM (< 4.0 %) was observed with cathepsin B. The pH optimum for GBM degradation by cathepsin L was 3.5. Cathepsin L was significantly more active in degrading GBM than was pancreatic elastase, trypsin or bacterial collagenase. These data suggest that cathepsin L may participate in the lysosomal degradation of GBM associated with normal GBM turnover *in vivo*.

INTRODUCTION

The glomerular basement membrane (GBM), composed primarily of type IV collagen, laminin, fibronectin and proteoglycan, is the primary ultrafiltration barrier of the kidney restricting the passage of plasma proteins into the urine. Isotopic labelling studies (Cohen & Surma, 1980, 1982) have indicated a more rapid turnover of both the collagenous and non-collagenous components of the GBM than previously thought (Price & Spiro, 1977). However, little information is available concerning the enzymes involved in the biosynthesis or degradation of GBM. We have previously reported (O'Connor *et al.*, 1986) that normal glomeruli contain relatively high activities of cathepsins B and L, cysteine proteinases with the ability to degrade a variety of protein substrates *in vitro* (Kirschke *et al.*, 1980, 1982; Barrett & Kirschke, 1981; Mason *et al.*, 1986). Davies *et al.* (1978) have reported that human liver cathepsin B causes the release of hydroxyproline from isolated GBM. However, virtually no information is available concerning renal cathepsins B and L, their ability to degrade intact GBM, or their potential role in GBM turnover. In the present study, we have purified and characterized human kidney cathepsins B and L and examined the ability of the purified enzymes to degrade intact GBM.

EXPERIMENTAL

Materials

Bacterial collagenase (type IV), pancreatic elastase (type IV), trypsin (type XI), L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) and dithiothreitol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were from Cambridge Research Biochemicals, Cambridge, U.K. Z-Phe-Phe-CHN_2 was a gift from Dr. Elliot Shaw, Friedrich-Miescher-Institut, Basel, Switzerland.

Purification of cathepsins B and L

Human kidneys were obtained at post-mortem and stored at −20 °C. Cathepsin B was purified by affinity chromatography using the semicarbazone of Gly-Phe-glycinal linked to Sepharose 4B as previously described (Rich *et al.*, 1986). Cathepsin L was purified essentially as previously described (Mason *et al.*, 1985).

Fluorimetric assay for cathepsin B and L

The activities of cathepsins B and L were measured as previously described (Barrett & Kirschke, 1981) with the fluorogenic substrates Z-Phe-Arg-NHMec (cathepsins B and L) and Z-Arg-Arg-NHMec (cathepsin B).

Determination of K_m and $k_{cat.}$ for cathepsins B and L

By use of the assays described above, K_m and $k_{cat.}$ for the hydrolysis of each peptide methylcoumarylamide substrate were determined by the method of Eisenthal & Cornish-Bowden (1974). The concentrations of purified cathepsins B and L were determined by active-site titration as previously described (Barrett & Kirschke, 1981) with E-64 as the active-site titrant.

Abbreviations used: GBM, glomerular basement membrane; Z-, benzyloxycarbonyl-; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; -NHMec, 7-(4-methyl)coumarylamide.

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GBM isolation

GBM was isolated from bovine kidneys by the method of Blau & Michael (1971) as described in detail in a previous paper (Shah *et al.*, 1987). GBM prepared by this method is essentially free from interstitial and denatured collagen as determined by SDS/polyacrylamide-gel electrophoresis, lack of digestion by thrombin and electron microscopy (Shah *et al.*, 1987).

GBM degradation

GBM degradation was measured as the release of non-sedimentable hydroxyproline during incubation of GBM with purified enzyme as follows. GBM (approx. 12 μg of hydroxyproline) was incubated with the enzyme indicated in 400 μl (final volume) of buffer (see below). Incubations were carried out for 24 h at 32±2 °C in a Dubnoff shaking water bath and terminated by centrifuging each tube at 10000 *g* for 10 min. Hydroxyproline was then determined in each supernatant and pellet after hydrolysis with 6 M-HCl. Results are expressed as the mean±S.E.M. percentage hydroxyproline release [100 × (hydroxyproline in supernatant)/(hydroxyproline in supernatant+hydroxyproline in pellet)] for triplicate determinations. Unless specified otherwise, the following buffers, each containing 1 mM-EDTA and 1 mM-dithiothreitol (freshly added), were used: 100 mM-sodium acetate, pH 5.0, for cathepsin L; 100 mM-sodium phosphate, pH 6.0, for cathepsin B. In experiments comparing GBM degradation by various proteinases (Table 2) the following buffers were used: 100 mM-Tris/HCl, pH 8.0, containing 0.36 mM-$CaCl_2$ for bacterial collagenase; 100 mM-Tris/HCl, pH 8.8, for pancreatic elastase; 100 mM-Tris/HCl, pH 8.0, for trypsin. Concentrations of these enzymes were estimated on the basis of M_r and 100% active enzyme.

Other methods

Hydroxyproline was determined by the method of Woessner (1961) as described in detail in a previous paper (Shah *et al.*, 1987).

SDS/polyacrylamide-gel electrophoresis was performed as previously described (Mason *et al.*, 1985).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Biochemical and physical properties of human kidney cathepsins B and L

In our initial studies we examined some physical and biochemical properties of cathepsins B and L purified from human kidney. The specific activity of the purified proteinases was 368 units/μmol for cathepsin B (Z-Arg-Arg-NHMec) and 1700 units/μmol for cathepsin L (Z-Phe-Arg-NHMec). SDS/polyacrylamide-gel electrophoresis under non-reducing conditions revealed a single band, M_r approx. 25000–30000, for each enzyme. SDS/polyacrylamide-gel electrophoresis under reducing conditions demonstrated that human kidney cathepsins B and L are disulphide-linked dimers, each consisting of a heavy chain (M_r 22000–25000) and a light chain (M_r 5000–7000). Similar pH optima, approx. 6.0±0.5, were observed for the hydrolysis of Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec by cathepsin B, and of Z-Phe-Arg-NHMec by cathepsin L. The Michaelis constant, K_m, and the catalytic rate constant, $k_{cat.}$, for the individual methylcoumarylamide substrates are presented in Table 1. Human kidney cathepsins B and L were inhibited 100% by a 10 min preincubation (40 °C for cathepsin B; 30 °C for cathepsin L) with 2.5 μM-E-64. Under identical conditions, cathepsin L was also completely inhibited (100%) by 0.56 μM-Z-Phe-Phe-CHN_2. In contrast, the same concentration of Z-Phe-Phe-CHN_2 produced little inhibition (8.0%) of cathepsin B. These results are in general agreement with previously published data for mammalian cathepsins B and L (Barrett & Kirschke, 1981; Mason *et al.*, 1985) and indicate that there are no major differences in physical or biochemical properties between the enzymes in human kidney and those in other mammalian tissues.

Table 1. Kinetic constants for hydrolysis of peptide methylcoumarylamide substrates by human kidney cathepsins B and L

K_m and $k_{cat.}$ were determined by the method of Eisenthal & Cornish-Bowden (1974). The concentrations of cathepsins B and L were determined by active-site titration with E-64. See the Experimental section for further details.

Enzyme	Substrate	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)
Cathepsin B	Z-Phe-Arg-NHMec	252	364	1.4×10^6
	Z-Arg-Arg-NHMec	184	158	0.9×10^6
Cathepsin L	Z-Phe-Arg-NHMec	2.2	25.8	11.7×10^6

Role of kidney cathepsins B and L in GBM degradation

Incubation of GBM (12 μg of hydroxyproline) with purified cathepsin L (16 pmol) resulted in significant GBM degradation: 59.7±4.8% hydroxyproline released (mean±S.E.M., $n = 6$). In contrast, only small amounts of hydroxyproline (3.3±1.4%, $n = 5$) were released during incubation of GBM with cathepsin B, even when relatively large amounts of cathepsin B (187 pmol) were used. GBM degradation by cathepsin L increased with increasing cathepsin L concentrations in the range 10–40 nM (Fig. 1). The pH optimum for GBM degradation by cathepsin L was 3.5. However, significant GBM degradation, about 50% of maximum, occurred in the pH range 4.0–5.5 (Fig. 2), the pH range of the lysosomal vacuole *in vivo* (Reijngoud & Tager, 1977). No GBM degradation by cathepsin L was observed at pH 6.0 or higher, possibly owing to the instability of cathepsin L at neutral pH (Mason *et al.*, 1985). In order to gain some insight into the relative potency of cathepsin L in GBM degradation, we simultaneously examined GBM degradation by cathepsin L and several well-characterized proteinases. As shown in Table 2, cathepsin L was significantly more active in degrading GBM than was pancreatic elastase, trypsin or bacterial collagenase.

It is currently thought that GBM degradation *in vivo* occurs in at least two steps: an initial extracellular step catalysed by a neutral proteinase(s) that generates phagocytosable GBM fragments, followed by phagocytosis of these fragments and subsequent degradation within the lysosomes. Our results establish that human kidney cathepsin L is a potent GBM-degrading pro-

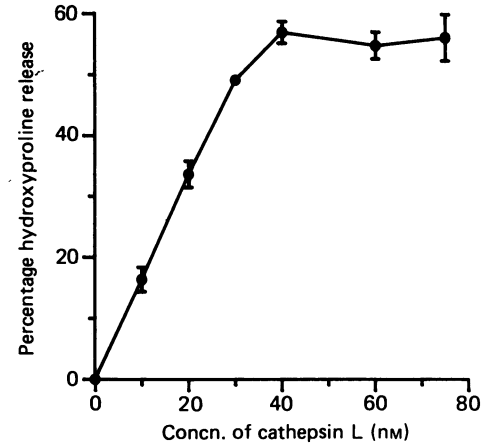

Fig. 1. GBM degradation as a function of cathepsin L concentration

GBM (approx. 12 μg of hydroxyproline) was incubated with the concentration of cathepsin L indicated for 24 h at 32±2 °C in 100 mM-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA and 1 mM-dithiothreitol (added fresh). Results are expressed as the mean±S.E.M. percentage hydroxyproline release for triplicate determinations. Further details are given in the Experimental section.

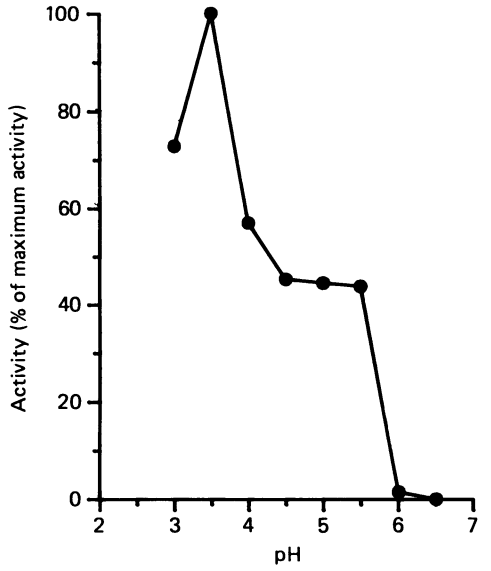

Fig. 2. Effect of pH on GBM degradation of purified human kidney cathepsin L

GBM (approx. 12 μg of hydroxyproline) was incubated with purified cathepsin L for 24 h at 32±2 °C in the following buffers, each containing 1 mM-EDTA and 1 mM-dithiothreitol (added fresh): pH 3.0–4.0, 100 mM-sodium citrate; pH 4.0–5.5, 100 mM-sodium acetate; pH 5.5–6.5, 100 mM-sodium phosphate. Results were calculated as percentage hydroxyproline release for two separate determinations carried out in triplicate and expressed as percentages of maximal activity. Further details are given in the Experimental section.

teinase. The ability of purified cathepsin L to degrade GBM combined with the high activity of cathepsin L in glomeruli suggests that this enzyme may participate in

Table 2. Comparison of GBM degradation by selected proteinases

Each enzyme was incubated for 24 h at 32±2 °C with GBM (12 μg of hydroxyproline) in 400 μl of buffer. See the Experimental section for further details. Results are expressed as the means for duplicate determinations corrected for hydroxyproline release from GBM alone incubated in the appropriate buffer.

Enzyme	Amount of enzyme (pmol)	Percentage hydroxyproline release
Cathepsin L	4	8.0
	16	40.3
Cathepsin B	187	1.0
Bacterial collagenase	95	22.8
Pancreatic elastase	8	5.0
Trypsin	105	6.9

the lysosomal phase of GBM turnover *in vivo*. The lack of GBM degradation at pH values above 6.0 suggests that cathepsin L does not participate in the extracellular phase of GBM degradation, which, under normal conditions, would take place at near neutral pH. However, the ability of viable cells to release active forms of cathepsin L (Mason *et al.*, 1987), coupled with their ability to acidify at least small amounts of their extracellular microenvironment (Schenk, 1974; Dingle, 1975; Etherington, 1980), raises the possibility that cathepsin L could also participate in extracellular GBM degradation associated with either normal GBM turnover or pathological states such as glomerulonephritis.

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