THE ROLES OF CATHEPSINS B1 AND D IN THE DIGESTION OF CYTOPLASMIC PROTEINS IN VITRO BY LYSOSOMAL EXTRACTS

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Summary

By means of the inhibitors leupeptin and pepstatin it is shown that both thiol proteinases (such as cathepsin B1) and the acid proteinase cathepsin D play substantial roles in the degradation in vitro of cytoplasmic proteins by mixtures of lysosomal enzymes.

Recent direct evidence has supported the suggestion that lysosomes are important agents of degradation of intracellular (1) as well as endocytosed (2) proteins. In the cases of digestion of endocytosed haemoglobin and immunoglobulin by macrophages, cathepsin D is implicated (2). On the other hand, in vitro studies indicate that both cathepsins D and B1 can degrade several purified proteins (albumin, haemoglobin) (3,4), and the use of proteinase inhibitors in such work implies that thiol proteinases, particularly cathepsin B1 may be of prime importance (5). By extension, it has been argued that cathepsin B1 is the major proteinase active on intracellular proteins (cf exogenous purified proteins). Some studies of protein turnover in cultured fibroblasts and macrophages are consistent with this, amongst other hypotheses (6). That cytosol proteins are susceptible to both purified cathepsins D and B1 has been shown recently (7), in confirmation of earlier work on digestion of cytosol proteins by partially purified mixtures of lysosomal enzymes (8). The present work demonstrates that during in vitro degradation of cytosol proteins by extracts of purified lysosomes laden with Triton WR-1339

('tritosomes') both cathepsin D and B1 are of considerable importance

Methods

Rat liver tritosomes were isolated as previously (9), and lysed by treatment with 0.1% Triton X-100. The resulting suspension was used for *in vitro* digestions. A cytosol protein mixture (dialysed against 50 mM-potassium phosphate buffer, pH7.0) was obtained from the liver of a rat injected 24h before death with 50μCi[^{3}H]-Valine as described previously (7). Digestions were performed at 37°C, 100mM-sodium acetate buffer, pH5.0, 0.1% Triton X-100, 2mM-dithiothreitol, in a final volume of 3ml. Each contained 9mg tritosomal protein, and 60mg dialysed cytosol protein (20,000 d.p.m.). Where indicated the following inhibitors were used at the concentrations specified: pepstatin (10^{-5}M, added in ethanol:methanol, 1:1); leupeptin (3.3×10^{-5}M); iodoacetic acid (10mM). In some experiments the effect of the addition of chloride ions (10mM-NaCl) was tested. Digestions were performed in triplicate, and samples of 0.5ml were taken at 1, 2, 4 and 24 h for measurement (by scintillation counting) of radio-activity soluble in 5% trichloracetic acid-1mM Valine, produced by proteolysis. Results presented are means of the three determinations; values differed from the mean by up to 6%. Release of radio-activity was not linear with time for more than 60 min.; values from early time points are excluded from Fig. 1 for clarity.

Results and Discussion

The levels of leupeptin (an inhibitor of cathepsin B1 and other thiol proteinases (5)) and pepstatin (a specific inhibitor of carboxyl proteinases (10)) used here are sufficient for complete inhibition of rat liver cathepsin B1 and D respectively, under assay conditions (5). However, in the *in vitro* digestions of protein substrates by the mixture of lysosomal enzymes, dithiothreitol is required, to activate cathepsin B1 (11), whereas in the normal assay of cathepsin D, acting on haemoglobin, dithiothreitol is omitted, and may be inhibitory to the enzyme from some sources (12, 13). It is possible that such inhibitions involve a conformational change or subunit dissociation, and this might result in an insensitivity to pepstatin. This was investigated for cathepsin D (the only carboxyl proteinase known to occur in rat liver (1)) present in the tritosomal extract, acting on haemoglobin: there was

no inhibition by 2mM-dithiothreitol of digestion and cathepsin D remained sensitive to pepstatin. Thus the use of this combination of reagents was validated. Iodoacetic acid is a general inhibitor of proteinases with essential thiol groups, whereas at the concentration used leupeptin may not act on all thiol proteinases (5). Chloride ions are required for the activity of the exopeptidase cathepsin C (14), and thus the importance of this enzyme was assessed by parallel incubations with and without chloride ions.

The time courses of digestion in the presence of the various inhibitors are shown in Fig. 1. The substantial inhibition

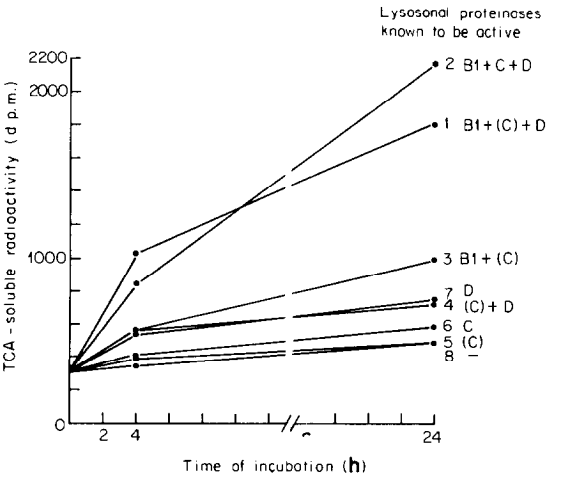

Figure 1. Inhibition by pepstatin, leupeptin and iodoacetic acid of digestion *in vitro* of cytoplasmic proteins by lysosomal enzymes.

Incubations were under the conditions described in 'Methods', with inhibitors and activators (at the concentrations specified there) as follows; (1) Control, no additions; (2) + chloride; (3) + pepstatin; (4) + leupeptin; (5) + pepstatin + leupeptin; (6) + pepstatin + leupeptin + chloride; (7) + iodoacetate; (8) + pepstatin + iodoacetate. The right-hand column lists the characterised lysosomal proteinases (cathepsins B1, C and D) which are active under the incubation conditions. Brackets round cathepsin C, indicate that the enzyme is probably incompletely active, because of inadequate chloride concentration.

by pepstatin alone (incubation 3 vs 1) and the considerable activity remaining when thiol proteinases are inhibited (lines 7 & 4) indicate that cathepsin D plays an important part in digestion *in vitro* of this substrate. This is in agreement with its importance in cartilage autolysis (15), and in contrast to its insignificant role in proteolysis *in vitro* of albumin (5).

Cathepsin B1 seems to play a somewhat greater role than cathepsin D, in view of the considerable inhibition by leupeptin alone (4 vs. 1). However, no greater inhibition is obtained with iodoacetate (non-specific: incubation 7) than with leupeptin, and so some of the reduction in activity by leupeptin may be due to inhibition of enzymes other than cathepsins B1 or D (5) which are at present incompletely characterised.

At the high concentrations used leupeptin also inactivates cathepsin C (5). But comparison of incubations 2 and 1 (control with or without chloride) and of 6 and 5 (tritosomes plus leupeptin and pepstatin, with or without chloride) shows that this enzyme plays only a minor role in generation of TCA-soluble fragments. Time course 2 suggests that this exopeptidase may be most important after initial attack by cathepsins D and B1.

Virtually no digestion occurred when the combinations leupeptin and pepstatin or iodoacetate and pepstatin were present (incubations 5 and 8 respectively). Autoproteolysis of cytosol proteins under similar conditions (without the tritosomal extract) gave only 400 d.p.m. TCA-soluble radioactivity after 24h. Thus digestion of cytosol proteins under these conditions is mainly due to cathepsin D and thiol proteinases, of which cathepsin B1 is the main example.

Synergism between cathepsin D and the leupeptin-sensitive enzymes is indicated by the present results also: the liberation of radio-activity at 24h in incubation 1 is much greater than the sum of the releases in 3 and 4 . This is consistent with much earlier data (see 16), though it was not detected in experiments with purified heterologous cathepsins D and B1 acting *in vitro* on the same substrate (7).

Direct extrapolation from these results to *in vivo* digestion is not valid. However, evidence for a major role of cathepsin D in turnover of intracellular proteins in perfused rat liver has been presented (1): pepstatin directed to lysosomes by presentation in liposomes suitable for endocytosis, caused inhibition of their degradation. In similar experiments in this laboratory on turnover in perfused liver, Dorling (unpublished) has found that leupeptin supplied free in the perfusate causes an equivalent inhibition. As leupeptin does not seem to permeate liposomes, its main route of entry into the perfused liver may be by endocytosis and thus its inhibitory effect may be on intralysosomal cathepsin B1. Cathepsins B1 and D are near equals in proteolysis *in vitro*, and probably, therefore, *in vivo*.

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