The Inhibition of Tissue Acid Proteinases by Pepstatin

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(Received 28 January 1972)

One of the more powerful methods by which to discover the functions of the proteolytic enzymes of living tissues depends on the use of specific inhibitors of these enzymes. We are successfully using inhibitory antisera in a study of the role of cathepsin D in cartilage breakdown (Dingle *et al.*, 1971), but effective inhibitors of low molecular weight might have important additional applications. Cathepsin D is a member of the 'acid proteinase' group of enzymes in which the hydrolysis of peptide bonds is thought to be catalysed by two or more carboxyl groups. Other enzymes in this group are another tissue proteinase, namely cathepsin E, the gastric enzymes pepsin (several forms), gastricsin and rennin, and numerous enzymes secreted by moulds and at least one bacterium. The acid proteinases most rapidly attack peptide bonds linking amino acids with hydrophobic side chains. Among the few known chemical inhibitors of these enzymes the best is probably diazoacetylornithine methyl ester in the presence of Cu^{2+} (Šodek & Hofmann, 1968; Lundblad & Stein, 1969; Keilová & Lapresle, 1970), but this often reacts slowly or incompletely, is unstable and probably is not highly specific. Certainly, it is not suitable for specific inhibition in biological systems, in the way that specific antisera to cathepsin D can be. The discovery of pepstatin in the culture medium of a strain of *Streptomyces* may therefore represent an important new development. Pepstatin has the structure isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid (Morishima *et al.*, 1970), and its discoverers have reported that it inhibits pepsin and gastricsin (Umezawa *et al.*, 1970; Aoyagi *et al.*, 1971) and also acid proteinase activity attributed to cathepsin D in pig liver (Ikezawa *et al.*, 1971) and rabbit macrophages (Dannenberg *et al.*, 1972). The purpose of the experiments now reported was to establish whether this might be a suitable agent with which to investigate the biological functions of the tissue acid proteinases.

Materials

Pepstatin sodium salt, a product of the Banyu Pharmaceutical Co. Ltd., Tokyo, Japan, was kindly given by Dr. A. Dannenberg. Cathepsin D from

liver of rabbit and man was prepared essentially as described by Barrett (1970); for each species an unresolved mixture of isoenzymes was used. A specific antiserum to rabbit cathepsin D was prepared as described by Dingle *et al.* (1971). Cathepsin B1 was isolated from human liver (A. J. Barrett, unpublished work). Trypsin (bovine, 2×crystallized; Sigma Chemical Co., St. Louis, Mo., U.S.A.), α -chymotrypsin (bovine, 3×crystallized; Sigma), rennin (from calf stomach; Koch-Light Laboratories, Colnbrook, Bucks., U.K.), papain (2×crystallized; Sigma) and pepsin (2×crystallized; Sigma) were purchased.

Results and discussion

Specificity of inhibition. The specificity of inhibition of proteolytic enzymes by pepstatin (5 $\mu\text{g}/\text{ml}$) was examined with trypsin (12.5 $\mu\text{g}/\text{ml}$, with α -N-benzoyl-DL-arginine 2-naphthylamide as substrate at pH 7.5), chymotrypsin (50 $\mu\text{g}/\text{ml}$, with α -N-glutaryl-DL-phenylalanine 2-naphthylamide as substrate at pH 7.5), papain (0.75 $\mu\text{g}/\text{ml}$, with α -N-benzoyl-DL-arginine 2-naphthylamide as substrate at pH 6.0 in the presence of 2 mM-cysteine and 1 mM-EDTA) and cathepsin B1 (10 $\mu\text{g}/\text{ml}$, under conditions identical with those used for papain). Pepsin (6 $\mu\text{g}/\text{ml}$), cathepsin D (both rabbit and human) (8 $\mu\text{g}/\text{ml}$) and rennin (1 mg/ml) were assayed with haemoglobin as substrate (Barrett, 1970) at pH 3.5, with 1 μg of pepstatin/ml.

Pepstatin had no effect on the activities of trypsin, chymotrypsin, papain or cathepsin B1, whereas pepsin, cathepsin D (from both species) and rennin were totally inhibited. The inhibition of cathepsin D was not diminished when the inhibitor had first been mixed with a large amount of heat-denatured (100°C, 5 min) cathepsin D. Complete inhibition was also produced by pepstatin at 1 $\mu\text{g}/\text{ml}$ (360 ng/unit), whether or not haemoglobin (20 mg/ml) had first been mixed with the enzyme, at pH 3.5.

Since cathepsin E was not available to use in pure form, its sensitivity to inhibition was determined by use of a homogenate of rabbit bone marrow, a tissue known to contain cathepsin E (Lapresle, 1970). Portions (3.0 ml) of the homogenate (1 g of tissue in 20 ml of 1% NaCl, pH 6.8) were treated with sheep anti-(rabbit cathepsin D) serum (0–300 μl), stored

Fig. 1. Effect of increasing cathepsin D concentration on the digestion of haemoglobin in the presence of pepstatin

The digestion of haemoglobin was measured as the increase in E_{280} of trichloroacetic acid-soluble products in the absence of inhibitor (○) or in the presence of 50ng (●), 100ng (▲) or 200ng (■) of pepstatin. Each assay was made in a total volume of 1.0ml at pH3.5 (as described by Barrett, 1970), the enzyme and inhibitor being mixed at this pH. The results were obtained from two separate experiments.

overnight at 4°C and centrifuged, to remove cathepsin D. The proteolytic activity of the supernatant fluids (0.5 ml) was determined at pH3.5, with haemoglobin as substrate, in the presence or absence of pepstatin (200ng/ml). It was found that 48% of the acid proteinase activity was not precipitable by the antiserum and this was attributed to the immunologically distinct cathepsin E. The entire acid proteinase activity of the extract was inhibited by pepstatin, however, and it was therefore concluded that cathepsin E is susceptible to inhibition by pepstatin.

The finding that pepstatin had no effect on the 'serine' or 'cysteine' proteinases, but was a potent inhibitor of the four acid proteinases tested, confirms and extends the results obtained by Aoyagi *et al.* (1971).

Kinetics of inhibition of cathepsin D. A method described by Ackerman & Potter (1949) (see also Khoo & Russell, 1970) was used to determine whether the inhibition of human cathepsin D by pepstatin is reversible, 'pseudo-irreversible' or

irreversible (Fig. 1). The results are characteristic of pseudo-irreversible or 'tight-binding' inhibition, in that the curves obtained in the presence of inhibitor tend to parallel the control curve obtained in its absence at high enzyme concentrations, but approach the origin, rather than intersecting the abscissa, at low concentrations of the enzyme. It is shown by extrapolation that, in the presence of excess of enzyme, 25 ng of pepstatin completely inhibits 1 unit of human cathepsin D. On the basis that the specific activity of pure human cathepsin D is 600 units/mg and its molecular weight 45000 (Barrett, 1970), and that the molecular weight of sodium pepstatin is 708 (from Umezawa *et al.*, 1970), and assuming that the pepstatin preparation was pure, 25 μg of pepstatin/unit of enzyme represents a molar ratio of 0.95:1.0. Equimolar binding of pepstatin to pepsin has been reported by Aoyagi *et al.* (1971). Our results do not permit an accurate calculation of the dissociation constant, but it appears to be less than 10^{-8} M. This finding is comparable with the value of less than 3×10^{-9} M for pepstatin with pepsin (Aoyagi *et al.*, 1971).

As would be expected from the extremely small dissociation constant, the inhibition of cathepsin D by pepstatin is very difficult to reverse experimentally; thus incomplete recovery of enzymic activity was achieved during dialysis for several days against a large volume of buffer.

Samples of human cathepsin D without inhibitor, or with 15, 30, 60 or 120 ng of pepstatin/unit of enzyme, were subjected to analytical isoelectric focusing in polyacrylamide gel (essentially by the method of Barrett, 1970), and the gels were split longitudinally so that half could be stained for protein and the corresponding profile of enzymic activity could be determined by assays made with sections cut transversely from the other half. The samples containing pepstatin showed the same degree of inhibition as had been determined before isoelectric focusing, and there was no detectable difference in degree of inhibition between the several separated isoenzymes. No shift in the positions of the bands (attributable to the binding of pepstatin, a monobasic acid) was detected, even at the highest concentrations of inhibitor.

The antigenic properties of rabbit cathepsin D were shown quantitatively to be unaltered by the presence of a high concentration of pepstatin, in that the inhibitor did not affect the characteristics of binding of 125 I-labelled enzyme to an antibody attached covalently to Sepharose 4B (Dr. Zena Werb, personal communication).

It seems most probable that pepstatin has a very high affinity for the active site of acid proteinases. The predominantly hydrophobic nature of its side chains is compatible with the affinity of the acid proteinases for substrates in which the bond to be hydrolysed is flanked by non-polar residues. From the data of

Aoyagi *et al.* (1971) it seems that the hydroxyl group of the non-terminal residue of 4-amino-3-hydroxy-6-methylheptanoic acid is of great functional importance in the binding of pepstatin, perhaps through the formation of a hydrogen bond.

On the basis of the above results, pepstatin seems to be a valuable group specific inhibitor for the acid proteinases. It produces complete inhibition of cathepsins D and E at low concentration, binding in equimolar ratio to cathepsin D. In view of the high potency and low toxicity (Umezawa *et al.*, 1970) of pepstatin, it may well be of use in establishing the function of tissue acid proteinases in tissue systems.

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