The effects of lactoyl-pepstatin and the pepsin inhibitor peptide on pig cathepsin D

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Lactoyl-pepstatin (an acylated tetrapeptide) is much more readily soluble in aqueous media than the more common isovaleryl- and acetyl-pepstatis (acylated pentapeptides). However, the K_i value for inhibition of cathepsin D by lactoyl-pepstatin at pH 3.5 is approx. 10^{-7} M, some two to three orders of magnitude weaker than has been obtained previously for isovaleryl- or acetyl-pepstatis. One of the peptides released during activation of pig pepsinogen is known to be an effective inhibitor of pig pepsin, but it does not alter the activity of the similar aspartic proteinase, pig cathepsin D.

Pepstatin is an *N*-acylated pentapeptide: isovaleryl-Val-Val-statyl-Ala-statine where statine is (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid. The uses of this specific inhibitor of aspartic proteinases as a tool in cell biology experiments *in vivo* and as a therapeutic agent for treatment of ulcers, hypertension and muscular dystrophy are hampered by its poor solubility in aqueous media and the consequent need to introduce organic solvents. Attempts to improve the water-solubility have concentrated on covalent modification of the free CO₂H group of the second statine residue with hydrophilic moieties (Knight & Barrett, 1976; Campbell *et al.*, 1980; Eid *et al.*, 1981). The relative unimportance of this second statine residue has been confirmed by the synthesis of a series of analogues of pepstatin (Marciniszyn *et al.*, 1976; Rich *et al.*, 1980). These studies indicated that the Val-statyl-Ala combination together with the 3*S*,4*S* configuration at the first statine residue (Gunn *et al.*, 1981) are the vital factors for inhibitor potency. By contrast, relatively little attention has been paid to the effects of changes at the *N*-terminal end of the molecule. Acetyl-pepstatin is as potent an inhibitor as the isovaleryl derivative (Nakatani *et al.*, 1980; Satoi & Murao, 1971) and the shortened tetrapeptides, acetyl-Val-statyl-Ala-statine and isovaleryl-Val-statyl-Ala-statine are also very-tight-binding inhibitors of pig pepsin (Marciniszyn *et al.*, 1976; Rich *et al.*, 1980). These acylated tetrapeptides are just as poorly soluble in water as their

parent pentapeptides, whereas by the simple expedient of introducing a hydrophilic lactoyl residue as the acylating group, the resultant blocked tetrapeptide (lactoyl-pepstatin) is rendered totally water-soluble. The present paper compares the inhibitory potency of lactoyl-pepstatin with the effects of isovaleryl- and acetyl-pepstatis on cathepsin D, an enzyme which is believed to play a major role in tissue protein breakdown and in various inflammatory and neoplastic diseases.

In addition, it has been suggested that cathepsin D is synthesized as a precursor, cathepsinogen D (Puizdar & Turk, 1981). Conversion of the zymogen into the active enzyme was postulated to occur by an activation process similar to that of the better-characterized protein pepsinogen [for a review, see Kay (1980)]. One of the peptides released in the first step of activation of pig and bovine pepsinogens is known to be an effective inhibitor of pig and cow pepsins (Kay & Dykes, 1977; Dunn *et al.*, 1978). It was found by Puizdar & Turk (1981) that the peptides released on conversion of cathepsinogen D into cathepsin D were very similar to their pepsinogen counterparts and, indeed, one (or more) of the cathepsinogen peptides was considered to inhibit cathepsin D. Thus we decided to examine the effect of the well-characterized pepsin inhibitor peptide on the activity of cathepsin D.

Materials and methods

Isovaleryl-pepstatin and acetyl-pepstatin (*Streptomyces* pepsin inhibitor) were generously given by Professor H. Umezawa and Professor S. Murao respectively. Lactoyl-pepstatin was prepared as

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follows: Val-statyl-Ala-statine was prepared from isovaleryl-pepstatin by degradation with pepstatin hydrolase from *Bacillus sphaericus* as described previously (Tone *et al.*, 1975a,b). DL-Lactic acid (0.08 ml) in anhydrous *NN*-dimethylformamide (5 ml) was added to *NN'*-carbonyldi-imidazole (162 mg) and the reaction was allowed to proceed for 1.5 h at room temperature. Then a solution of Val-statyl-Ala-statine (502 mg) and triethylamine (0.14 ml) in anhydrous dimethylformamide was added and the coupling was allowed to proceed for a further 24 h at room temperature. The mixture was chromatographed on columns of Dowex 50X8 and silica gel successively and a white powder of lactoyl-pepstatin {246 mg, m.p. 113–115°C, $[\alpha]_D^{25}$ –96° (c 0.5 in water)} was obtained in a yield of 40%. The structure of the product was confirmed from the mass spectrum of its methyl ester derivative ($M^+ + 1$, m/z 589).

Lactoyl-pepstatin was dissolved in water and diluted as appropriate in water before use. Isovaleryl- and acetyl-pepstatis were dissolved in dimethyl sulphoxide at a concentration of approx. 20 mg/ml, diluted to appropriate concentrations by serial dilutions in a dimethyl sulphoxide/water mixture (1:9, v/v) and passed through a millipore filter before use.

A shortened derivative (residues 1–12) of the naturally occurring 16-residue pepsin inhibitor peptide was synthesized as described by Dunn & Deyrup (1979) and Dunn & Lewitt (1980). It has been shown previously that residues 13, 14, 15 and 16 in the naturally occurring peptide are not required for the pepsin inhibitory activity (Dunn *et al.*, 1978). The absolute concentrations of the pepstatis and pepsin inhibitor peptide were determined by amino acid analysis after hydrolysis for 72 h in 6 M-HCl at 105°C in sealed, evacuated tubes (norleucine and nitrotyrosine were added as internal standards).

Cathepsin D was purified to homogeneity from pig spleen by the two-step procedure of Afting & Becker (1981). Its concentration was determined by active-site titration with the very tightly binding inhibitor isovaleryl-pepstatin, as described by Knight & Barrett (1976). The enzyme was assayed at pH 3.5 and at pH 5.0, with 2.5% haemoglobin as substrate, by the method of Smith & Turk (1974). When inhibitors to be tested were introduced, all samples (test and controls) were preincubated in 0.02 M-acetate buffer, pH 3.5 or 5.0, at 37°C for 2 min (with the pepstatis) and for 10–15 min (with the pepsin inhibitor peptide) before addition of the haemoglobin substrate in the same buffers (final vol. 0.6 ml).

Results and discussion

Isovaleryl-pepstatin binds to cathepsin D so tightly (K_i between 10^{-9} and 10^{-10} M) at pH values

below 5 that it has been used as an active-site titrant for determination of the concentration of the enzyme (Knight & Barrett, 1976). In the present study the concentration of active cathepsin D was thus determined by titration with isovaleryl-pepstatin over the range 0–1.5 nmol of inhibitor/nmol of cathepsin D. Inclusion of isovaleryl-pepstatin at a molar ratio of 2:1 (isovaleryl-pepstatin/cathepsin D) resulted in complete inactivation of the enzyme at pH 3.5. Acetyl-pepstatin was equally effective in that it removed the activity of cathepsin D totally when introduced at a molar ratio of 2:1.

By contrast, inclusion of the water-soluble lactoyl-pepstatin at a molar ratio of 2:1 decreased the activity of cathepsin D at pH 3.5 only to 66% of that measured in the absence of the inhibitor. This suggests a less tight binding of this derivative to cathepsin D and this was confirmed, since residual cathepsin D activity (12%) could still be measured even in the presence of a 50-fold molar excess of lactoyl-pepstatin. Analysis of the data by the method of Easson & Stedman (1936) and Strauss & Goldstein (1943) gave an apparent K_i value of 3×10^{-7} M for lactoyl-pepstatin at pH 3.5. At pH 5.0, the inhibitory potency of lactoyl-pepstatin was even less than at pH 3.5 (for example, 50% residual cathepsin D activity was measured in the presence of a 22-fold molar excess of lactoyl-pepstatin). This is in contrast with the situation observed for isovaleryl-pepstatin (Knight & Barrett, 1976), where the K_i value does not appear to change significantly over the pH range 3.5–5.0.

Thus the tetrapeptide with the hydrophilic lactoyl substituent as the acylating group appears to be about two to three orders of magnitude less effective as an inhibitor of cathepsin D than the more common pentapeptide derivatives with hydrophobic fatty acyl blocking groups. In contrast with modifications at the C-terminal end of the inhibitor, which appear to be 'acceptable' in that they do not change in inhibitory potency significantly [such as, for example, the use of pepstatin-aminoethyl-Sepharose as an affinity absorbent (Afting & Becker, 1981) or the coupling of a fluorescent reporter group (Matthews *et al.*, 1981) to permit subcellular-location studies], shortening of pepstatin by one residue together with the introduction of a hydrophilic blocking group at the N-terminal end of the polypeptide chain seems to result in a diminution of the strength of the inhibitor's interaction with the enzyme. Thus, despite the attractions of its markedly enhanced aqueous solubility, lactoyl-pepstatin does not appear to interact with cathepsin D tightly enough to be of much value as a tool for cell-biology research or as a potential therapeutic agent in inflammatory and neoplastic diseases.

The effect of the pepsin inhibitor peptide was tested at pH 3.5 by using a peptide/cathepsin D

molar ratio of 100:1. The peptide did not alter the activity of cathepsin D at this pH. This is in keeping with previous findings for pig and cow pepsins (Herriott, 1938): the peptide binds to, and inhibits, pig and cow pepsins only at pH values above about 4.5. However, addition of the peptide at molar ratios as high as 400:1 had no effect whatsoever on the activity of cathepsin D at pH 5.0.

Thus, if the activation segment of cathepsin D is indeed similar to that of pepsinogen, as suggested by Puizdar & Turk (1981), it would appear that the inhibition of cathepsin D by the activation peptides from cathepsinogen D (Puizdar & Turk, 1981) cannot be due to a homologous peptide derived from the *N*-terminal end of the activation segment of the precursor. Indeed, it has been suggested previously (Wang & Edelman, 1971; Dunn *et al.*, 1978) that, in addition to the well-characterized *N*-terminal (residues 1–12 or 16) inhibitor peptide, pig pepsinogen also contains an additional inhibitory activity within residues 25–41 of the activation segment. Thus the inhibitory peptide from cathepsinogen D described by Puizdar & Turk (1981) may correspond to this region of pig pepsinogen.

Pig (and cow) pepsin(s) are inhibited strongly by the pepsin inhibitor peptide (Kay & Dykes, 1977; Dunn *et al.*, 1978) and lactoyl-pepstatin inhibits pig pepsin as strongly as does isovaleryl-pepstatin (Kay & Valler, 1981). On the other hand, chicken pepsin and calf chymosin are not affected by the inhibitor peptide and lactoyl-pepstatin binds only weakly (Kay & Dykes, 1977; Keilova *et al.*, 1977; Kay & Valler, 1981). Thus these enzymes (all aspartic proteinases) appear to respond differently to the two (potential) inhibitor(s) and cathepsin D seems to resemble chicken pepsin/chymosin in its response rather than pig/cow pepsins. Clearly, subtle distinctions must exist in the active-site regions of these aspartic proteinases.

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