Interaction of Human Cathepsin D with the Inhibitor Pepstatin

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1. Because of the proposed role of cathepsin D in a variety of biological and pathological processes, the characteristics of inhibition by the potentially useful agent, pepstatin, were determined. 2. The β and γ forms of human cathepsin D, separated by isoelectric focusing, have identical specific extinction coefficients and specific activity in the degradation of haemoglobin. 3. Cathepsin D showed tight binding of 1 mol of pepstatin per 43000 g of protein, indicating that titration with the inhibitor represents a useful method for determination of absolute concentrations of the enzyme. 4. The titration curves were used to determine apparent dissociation constants (K_D) for the binding of pepstatin and pepstatin methyl ester at pH 3.5; values of approx. 5×10^{-10} M were obtained. 5. Pepstatinyl-[3 H]glycine was synthesized and shown to have a K_D similar to that of pepstatin. Gel-chromatographic experiments showed that the binding of pepstatin and its derivatives is strongly pH-dependent. 6. The effect of pH on the K_D for pepstatinyl-glycine was determined by equilibrium dialysis. As the pH was raised from 5.0 to 6.4, K_D rose from 5×10^{-10} M to 2×10^{-6} M. 7. The catalytic activity of cathepsin D declines essentially to zero on going from pH 5.0 to pH 7.0, and we suggest that the binding site for substrate and pepstatin is abolished by a conformational change in the enzyme molecule. 8. The data indicate that, in biological experiments near neutral pH, large molar excesses of pepstatin over cathepsin D will be required for efficient inhibition.

Cathepsin D (EC 3.4.23.5) is a representative of the 'acid' or carboxyl-dependent class of endopeptidases, which also includes pepsin, chymosin and renin. These enzymes are inhibited by a variety of reagents that react covalently with carboxyl groups, e.g. diazoacetylornithine methyl ester in the presence of Cu^{2+} (Lundblad & Stein, 1969; Takahashi *et al.*, 1972) and 1,2-epoxy-3-(4-nitrophenoxy)propane (Tang, 1971), but few effective reversible inhibitors are known. Inouye & Fruton (1968) reported inhibition of pepsin by Phe-Phe-OMe (K_i 2.5×10^{-4} M). Two naturally occurring tight-binding inhibitors of carboxyl proteinases have been described, the pepsin inhibitor of *Ascaris lumbricoides* (Abu-Erreish & Peanasky, 1974), which is not effective against cathepsin D (Keilová & Tomašek, 1972), and pepstatin (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) (Morishima *et al.*, 1970). Pepstatin is obtained from the filtrates of cultures of *Streptomyces* (Umezawa, 1972), and inhibits pepsin with K_i approx. 10^{-10} M at pH 4.0 (Kunimoto *et al.*, 1974). It has been suggested that pepstatin is a general inhibitor of the carboxyl proteinases (Barrett & Dingle, 1972; Takahashi *et al.*, 1974).

There is evidence that cathepsin D may be respon-

sible for the degradation of proteins in such diverse processes as the intracellular digestion of proteins endocytosed by macrophages (Dingle *et al.*, 1973) and the breakdown of the extracellular matrix of cartilage (Dingle *et al.*, 1971). This evidence depends heavily on the use of inhibitory antisera, which have certain important disadvantages, including the high molecular weight of the antibody molecules (which restricts penetration of tissues), the fact that they are species-specific and the complex chemical nature of the inhibition. As a potent inhibitor of low molecular weight and toxicity, pepstatin clearly has potential importance as a new tool with which to examine the biological functions of cathepsin D.

In order that definite conclusions might be reached from the results of biological experiments with pepstatin it was necessary to establish the characteristics of the enzyme-inhibitor interaction at pH values approaching those in living tissues. It is the purpose of the present paper to report the results of experiments designed to measure the effect of pH on the dissociation constant of complexes of human cathepsin D with the naturally occurring anionic pepstatin, and also with neutral and cationic derivatives. It is also shown that pepstatin is a useful active-site titrant for cathepsin D.

Materials and Methods

Pepstatin was kindly given by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. All other reagents were obtained from commercial sources and were of analytical grade when available. Cathepsin D from human liver was prepared as described by Barrett (1970, 1973). For two experiments the enzyme was taken at the stage before separation of multiple forms by isoelectric focusing; this material is referred to as 'partially purified'. Except where otherwise stated experiments were made with the purified β -form. Silica-gel sheets (13179) for t.l.c. were from Kodak Ltd., Kirkby, Liverpool, U.K. Visking dialysis tubing (8/32) was from Scientific Instrument Centre Ltd., London W.C.1, U.K. Sephadex LH-20, G-50 and G-100 were from Pharmacia (Great Britain) Ltd., London W.5, U.K. AG1-X2 (Cl^- form, 200–400 mesh) and AG50W-X2 (H^+ form, 200–400 mesh) were from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K.

Triethylamine was distilled and the fraction of b.p. 88.5–92°C was redistilled from ninhydrin (1%, w/v). The fraction of b.p. 89–91°C was used.

Analytical methods

Melting points were determined with a Gallenkamp apparatus (MF-370) and are uncorrected. T.l.c. was performed in an Eastman Chromogram developing apparatus (13259) by using butan-1-ol/acetic acid/*n*-butyl acetate/water (4:1:4:1, by vol.) as solvent. Elemental analysis was performed by Dr. F. B. Strauss, Microanalytical Laboratory, Oxford OX2 7SA, U.K. Samples of *N*-pepstatinyl-[^3H]glycine for amino acid analysis (25 μg) were hydrolysed by 6M-HCl (0.2ml) containing 0.5% phenol at 110°C for 37 and 69h. After hydrolysis the solutions were evaporated over KOH pellets in an evacuated desiccator. The residues were dissolved in 0.2M-sodium citrate, pH2.2 (2.0ml), and a portion (1.0ml) was taken for amino acid analysis. Analyses were performed by R. H. Payne of LKB Instruments Ltd., South Croydon, Surrey, U.K.

Scintillation counting

Samples in ethanol, and aqueous samples up to 0.1ml in volume, were added to 10ml of toluene/2-ethoxyethanol (7:3, v/v), containing 4g of 2,5-diphenyloxazole/l (Hall & Cocking, 1965). Aqueous samples (1ml) were added to 10ml of toluene/Triton X-100 (2:1, v/v), containing 3.3g of 2,5-diphenyloxazole/l (Turner, 1969). Scintillation counting was performed in a Packard Tri-Carb liquid-scintillation spectrometer model 3375. Quenching corrections were applied by using a standard curve constructed by adding portions of chloroform (5–50 μl) to 10ml samples of toluene containing 4g of 2,5-diphenyloxazole/l and [^3H]n-hexadecane (3.82×10^6 d.p.m./l)

(standard for liquid-scintillation counting from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K.). Counting efficiency was between 31 and 34% (automatic external-standard ratio 0.64–0.69) in all cases. Data reported as c.p.m. were from samples counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer model 2111.

Specific activity of purified cathepsin D

The β and γ forms of human cathepsin D were separated by isoelectric focusing (Barrett, 1970); fractions containing each form were combined. A portion (5ml) of each pool was dialysed at 4°C against 0.05M-ammonium acetate, pH5.0 ($3 \times 200\text{ml}$). The dialysed enzyme solutions were clarified by filtration (Millipore 0.45 μm filter) and the absorbances measured at 280nm. The cathepsin D activity of each pool was assayed (Barrett, 1970), bovine haemoglobin being used as substrate. One unit of activity is defined as that quantity that would produce an increase in extinction (ΔE_{280}) of 1.0 in the assay during 60min under the specified conditions, a linear response being assumed. The enzyme concentrations were determined as follows. Duplicate portions (0.1ml) of the dialysed samples and dialysis buffer were pipetted into pre-weighed aluminium boats with a calibrated micropipette. These solutions were dried down overnight at 21°C over P_2O_5 and NaOH. The desiccator was then evacuated to 13Pa and the boats were dried to constant weight in an oven at 65°C. Weighings were made with a Cahn Electrobalance Model G (Cahn Instrument Co., Paramount, CA, U.S.A.).

Tryptophan content of purified cathepsin D

The ratio of tryptophan to tyrosine content in each form of the enzyme was determined spectrophotometrically by the method of Goodwin & Morton (1946). The number of tyrosine residues per molecule of γ -isoenzyme has been determined independently by amino acid analysis (Barrett, 1971).

Synthesis of pepstatin derivatives

Pepstatin methyl ester. Pepstatin methyl ester was prepared by treatment of pepstatin in methanol with diazomethane (Umezawa *et al.*, 1970). The product was recrystallized from methanol and had m.p. 248–249°C (cf. 249–251°C, Umezawa *et al.*, 1970).

***N*-Pepstatinyl-diaminoethane.** Pepstatin (55mg, 0.08mmol) was dissolved in dimethyl sulphoxide (1.0ml) at 80°C. Triethylamine (0.025ml, 0.18mmol) and chloroacetonitrile (0.025ml, 0.39mmol) were added and the mixture was stirred at 80°C for 4h. Dimethyl sulphoxide (1.0ml) and 1,2-diaminoethane (0.3ml, 4.5mmol) were then added and stirring was continued overnight at 21°C. The reaction mixture was diluted with ethanol (2ml) and insoluble material removed by centrifugation. The precipitate was

washed with ethanol (2×2 ml) by centrifugation, the combined supernatant and washings were evaporated at 80°C *in vacuo* and the residue was dissolved in ethanol. This solution (4.5 ml) was applied to a column (2 cm×32 cm) of Sephadex LH-20 and eluted with ethanol at a flow rate of 25 ml/h. Fractions (4 ml) were collected and 5 µl of each was spotted on to a silica-gel sheet and developed with cadmium/ninhydrin reagent. Fractions representing the first ninhydrin-positive peak eluted from the column were combined and evaporated to dryness (29 mg), redissolved in aq. 50% (v/v) ethanol (5 ml) and adjusted to pH 8.5 by a few drops of 1 M-triethylammonium bicarbonate, pH 8.5. The solution was applied to a column (1 cm×25 cm) of Bio-Rad AG50W-X2 (triethylammonium form) and washed with aq. 50% (v/v) ethanol (80 ml). *N*-Pepstatinyl-diaminoethane was eluted with ethanol/water/triethylamine (9:9:2, by vol.). The ninhydrin-positive fractions were evaporated to dryness at 35°C *in vacuo* and the residue (11 mg) was dissolved in ethanol (2 ml). The solution was applied to a column (1 cm×53 cm) of Sephadex LH-20 and eluted with ethanol. The absorbance of the effluent at 230 nm was monitored; fractions (1.1 ml) were collected and tested for ninhydrin-positive material. Fractions containing *N*-pepstatinyl-diaminoethane were combined and the ethanol was removed under reduced pressure, leaving a colourless solid m.p. 238–240°C (decomp.) (yield: 11 mg, 18%) (Found: C, 59.2; H, 9.2; N, 12.4. Calc. for C₃₆H₆₉N₇O₈·C₂H₅OH: C, 59.0; H, 9.8; N, 12.7%).

Pepstatin 4-nitrophenyl ester. Pepstatin (14 mg, 20 µmol) was dissolved in pyridine (1 ml) at 21°C. 4-Nitrophenol (14 mg, 100 µmol) and dicyclohexylcarbodi-imide (21 mg, 100 µmol) were added and the mixture was stirred at 21°C overnight. The reaction mixture was suspended in ethanol (2 ml), filtered and applied to a column (2 cm×31 cm) of Sephadex LH-20 and eluted with ethanol (25 ml/h). The absorbance of the column effluent was monitored at 230 nm, and fractions (2 ml) were collected. A portion (5 µl) of each fraction was spotted on a silica-gel sheet and treated with 0.1 M-NaHCO₃, pH 8.3. The appearance of a yellow colour indicated the release of 4-nitrophenol by hydrolysis of pepstatin 4-nitrophenyl ester. The concentration of the ester in these fractions was estimated spectrophotometrically by assuming $\epsilon_{267\text{nm}} = 9800$ in ethanol, the value determined with a standard solution of *N*-t-butyloxycarbonyl-L-alanine 4-nitrophenyl ester. The yield of active ester was 9.5 mg (58%). This material was used without further purification.

***N*-Pepstatinyl-[³H]glycine.** [2-³H]Glycine of specific radioactivity 2.33 Ci/mmol was supplied as an aqueous solution (1.0 mCi/ml) by The Radiochemical Centre. A portion (1 ml) of this solution was evaporated to dryness at 35°C *in vacuo*. The residue (0.43 µmol) was taken up in pyridine (0.2 ml) and

added to a solution of pepstatin 4-nitrophenyl ester (3.2 mg, 4 µmol) in ethanol (0.3 ml). The mixture was stirred in darkness at 21°C for 24 h. Pyridine (0.5 ml) and glycine (11.4 mg, 150 µmol) were added and the mixture was stirred for a further 24 h. Ethanol (1 ml) was added and the mixture filtered. The filtrate was evaporated to dryness at 40°C *in vacuo* and the residue dissolved in ethanol. This solution was applied to a column (1 cm×53 cm) of Sephadex LH-20 and eluted with ethanol. Fractions (1.6 ml) were collected and the absorbance of the effluent was monitored at 230 nm. A portion (5 µl) of each fraction was taken for scintillation counting. Fractions containing *N*-pepstatinyl-[³H]glycine were combined, evaporated to dryness (1.7 mg) and redissolved in ethanol (0.3 ml). Unchanged pepstatin 4-nitrophenyl ester was decomposed by addition of triethylamine (5 µl). After 4 h at 21°C, ethanol (0.2 ml) was added and the mixture rechromatographed on a column (1 cm×53 cm) of Sephadex LH-20 in ethanol. Fractions (1.6 ml) containing *N*-pepstatinyl-[³H]glycine were combined and evaporated to dryness (1.6 mg).

This material had 50% of the inhibitory capacity of pepstatin (see the Results section). An attempt at further purification by adsorption on Bio-Rad AG1-X2 anion-exchange resin and elution with 1 M-HCl was not successful. The specific radioactivity of this product was 70 d.p.m./pmol.

Chromatography of N-pepstatinyl-[³H]glycine-cathepsin D complexes on Sephadex G-100

Bovine serum albumin (10 mg) and cytochrome *c* (5.5 mg) were dissolved in 0.5 ml of 0.05 M-sodium acetate, pH 5.5, containing 0.1% Brij 35. To this solution was added 0.1 ml (54 units) of partially purified cathepsin D solution and 1 µl of *N*-pepstatinyl-[³H]glycine solution (0.95 µg, 18500 c.p.m.). The mixture was run on a column (1.5 cm×27 cm) of Sephadex G-100 at 4°C eluted with the same buffer (30 ml/h); fractions (1.1 ml) were collected. The absorbance of the column effluent was monitored at 280 nm. A portion (0.1 ml) of each fraction was taken for scintillation counting. A further portion (0.5 ml) was taken for assay of cathepsin D activity. An experiment was performed under identical conditions in 0.05 M-Tris/HCl, pH 7.8, containing 0.1 M-NaCl, except that cytochrome *c* was omitted.

Chromatography of pepstatin-cathepsin D complexes on Sephadex G-50

A column (1.5 cm×27 cm) of Sephadex G-50 (fine grade) was washed with the appropriate buffer at 4°C. All buffers contained 0.1 M-NaCl and 0.1% Brij 35. The buffering components were 0.05 M-sodium acetate/acetic acid in the range pH 4.00–5.50, 0.05 M-*N*-(2-acetamido)iminodiacetic acid/NaOH in the range pH 5.50–7.00 and 0.05 M-Tris/HCl in the range pH 7.00–7.30. A portion (0.45 ml) of buffer was mixed

with cathepsin D (0.05ml, 24 units) and pepstatin (2 μ l, 2 μ g). After standing for 1–2h at 21°C the sample was applied to the column and eluted with buffer (50ml/h), 2ml fractions being collected in pre-weighed tubes. A portion (0.5ml) of each fraction was taken for assay of cathepsin D activity. Similar experiments were performed with enzyme inhibited by pepstatin methyl ester or *N*-pepstatinyldiaminoethane.

Determination of dissociation constants

(a) *Enzymic titration method.* Cathepsin D (approx. 1 unit/tube) was assayed (Barrett, 1970) in triplicate in the presence of pepstatin (0–62.5 μ g/tube). The reactions were usually started by introduction of the enzyme, but for some experiments the enzyme was preincubated with pepstatin and the assay started by the addition of haemoglobin and assay buffer.

(b) *Equilibrium dialysis.* Dialysis tubing (Visking 8/32) was simmered in disodium EDTA (1%, w/v) and NaHCO₃ (1%, w/v) for 2h and thoroughly washed in water. The clean tubing was stored at 4°C in aq. 1% (w/v) Brij 35 containing 1% (v/v) butan-1-ol, and rinsed with water before use. The procedure followed at pH5 is described below in detail.

Cathepsin D solution (25 μ l, 0.41 nmol) was diluted with 25ml of 0.05M-sodium acetate/acetic acid, pH5, containing 0.1M-NaCl and 0.1% (w/v) Brij 35. To avoid denaturation of the enzyme this solution was kept at 0°C and portions were transferred as required by polypropylene pipettes into polystyrene tubes. The time needed to reach dialysis equilibrium was determined as follows. *N*-Pepstatinyl-[³H]glycine solution (0.4ml, 0.2 μ g) was added to enzyme solution (8.0ml) and 1.0ml portions were placed in dialysis bags. Each bag was placed in a polystyrene tube (9ml capacity) and buffer (5.0ml) added. The tubes were capped and placed in a roller-rack at 4°C. Duplicates were removed after 1, 2, 5 and 7 days.

Since pepstatin and cathepsin D form an equimolar complex (Barrett & Dingle, 1972) the apparent dissociation constant, K_D , was calculated from the equation of Johnson & Knowles (1966):

$$K_D = i_0(e - \Delta i) / \Delta i$$

where i_0 is the molar concentration of inhibitor outside the dialysis bag, e is the total enzyme concentration and Δi is the difference between the concentrations of inhibitor inside and outside the dialysis bag. Since the volume of enzyme solution inside the bag changed during dialysis, and quantitative transfer of the bag contents to scintillation fluid was not possible, the following procedure was adopted. The dialysis bag was carefully blotted free of external solution and weighed in a sealed container. The contents were transferred to a weighed scintillation vial and the vial was reweighed. Both knots were cut from the dialysis membrane and the pieces were weighed after care-

fully squeezing out remaining fluid with absorbant paper. The liquid in the vial was made up to 1.0ml with water, and 10ml of scintillant added. A portion (1.0ml) of the external solution was also counted for radioactivity. The equation for the apparent dissociation constant becomes, in this system:

$$K_D = i_0^* \left(\frac{e}{w_1} - \frac{\Delta i^*}{k} \right) / \Delta i^*$$

where i_0^* is the radioactivity (d.p.m./g) of the solution outside the bag, e is the original enzyme concentration inside the bag, w_1 is the final weight of the bag contents, Δi^* is the difference between the radioactivity of the solutions inside and outside the dialysis bag (d.p.m./g) and k is the specific radioactivity (d.p.m./mol) of the *N*-pepstatinyl-[³H]glycine.

The apparent dissociation constant at pH5 was determined as follows. Three portions (each 3.0ml) of diluted enzyme solution were pipetted into tubes in ice. These solutions were mixed with portions (0.075ml, 0.15ml and 0.30ml) of *N*-pepstatinyl-[³H]glycine solution (0.5 μ g/ml), and 1.0ml portions were dialysed at 4°C as described above. Apparent dissociation constants were determined after 6 or 7 days.

A similar procedure was followed at the other pH values, but since the values of the apparent dissociation constant increased with pH, it was necessary to use higher concentrations of enzyme and inhibitor to give a measurable concentration of complex. The proportion of the total enzyme concentration converted into complex varied between approx. 45 and 80% over the range of inhibitor concentrations used at pH5, and between approx. 1 and 5% at pH6.4. The formation of 1% complex at pH6.4 resulted in an approx. 10% increase in the radioactivity (d.p.m./ml) inside the dialysis bag over the value for the external solution (approx. 1800d.p.m./ml).

Results

Properties of the purified forms of human cathepsin D

The β - and γ -forms of human cathepsin D have many properties in common (Barrett, 1970; Dingle *et al.*, 1971). In the course of the present work we have re-examined them with regard to specific extinction coefficient at 280nm, and specific activity against haemoglobin. The two forms appear identical in both respects. The specific extinction coefficient ($E_{280}^{1\%} = 10.5$) was close to the value of 10.0 found by Barrett (1970) on the basis of protein determinations by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. We have also determined the tryptophan content of each form by the method of Goodwin & Morton (1946) by taking the content of tyrosine, from the data of Barrett (1971), as 4 (found 3.8) residues/molecule (mol.wt.

Fig. 1. Inhibition of cathepsin D by pepstatin

Standard assay conditions were used as described in the text. The assays were started by the addition of cathepsin D (1.06 μg) to the mixture of haemoglobin and pepstatin at 45°C. The trichloroacetic acid-soluble products of digestion of haemoglobin were measured as the change in absorbance at 280 nm (ΔE_{280}). Each point represents the mean of three determinations.

Fig. 2. Inhibition of cathepsin D by *N*-pepstatinyl-diaminoethane

Assay conditions were as in Fig. 1. Haemoglobin was incubated with *N*-pepstatinyl-diaminoethane and the assay started by the addition of cathepsin D to give a final concentration of 25 nm. Each point represents the mean of three determinations. The lines represent the decreases in ΔE_{280} for this enzyme concentration, calculated on the basis of an apparent dissociation constant, K_D , of 1 nm (line 'a'), 5 nm (line 'b') and 10 nm (line 'c').

43000). The specific activity was found to be 870 unit/mg, 45% greater than the value of 600 reported by Barrett (1970). It is possible that the discrepancy may be due in part to the use of haemoglobin as substrate. Although assays made with a single batch of this substrate show excellent reproducibility, it is conceivable that different batches could show some variation in susceptibility to digestion. Clearly, protein substrates are not ideal, but no satisfactory synthetic substrate for cathepsin D is available.

Molecular-weight determination by titration with pepstatin

The property of pepstatin of binding tightly and stoichiometrically to human cathepsin D (Barrett & Dingle, 1972) allowed the concentration of active enzyme to be determined by titration with the inhibitor. A representative titration is shown in Fig. 1. The enzyme concentration was 1.06 $\mu\text{g}/\text{ml}$, and when extrapolated, the initial linear portion of the curve intersected the abscissa at a pepstatin concentration of 24.6 nm. On the assumption that both enzyme and inhibitor were pure, the mol.wt. of human cathepsin D was calculated to be 43000.

Apparent dissociation constant of the pepstatin-cathepsin D complex

Barrett & Dingle (1972) had estimated the dissociation constant for human cathepsin D and pepstatin to be less than 10^{-8}M in experiments done at pH 3.5. The shape of the titration curve (Fig. 1) enabled the calculation of the apparent dissociation constants from the deviations from stoichiometric inhibition in the region of the equivalence point (Green & Work, 1953). These calculations are valid only if the system has reached equilibrium (Green, 1957), and it has been reported that pepstatin combines relatively slowly with pepsin (Kunimoto *et al.*, 1974). That this was not the case for pepstatin and human cathepsin D was shown by our finding that titration curves obtained for enzyme preincubated with inhibitor were superimposable on those obtained without preincubation. Pepstatin methyl ester gave titration curves similar to that for pepstatin shown in Fig. 1. The initial slopes and baselines of these curves were

Table 1. Apparent dissociation constants for complexes between pepstatin or its derivatives and human cathepsin D

The dissociation constants were determined at 45°C in 0.25 M-sodium formate buffer, pH 3.5, as described in the text. The results for duplicate experiments are given.

	Inhibitor			
	Pepstatin	Pepstatin methyl ester	Pepstatinyl-diaminoethane	Pepstatinyl-[^3H]glycine
Expt. 1	$3.6 \times 10^{-10}\text{M}$	$5.6 \times 10^{-10}\text{M}$	$5.1 \times 10^{-9}\text{M}$	$5.0 \times 10^{-10}\text{M}$
Expt. 2	$4.1 \times 10^{-10}\text{M}$	$8.0 \times 10^{-10}\text{M}$	$5.7 \times 10^{-9}\text{M}$	$4.5 \times 10^{-10}\text{M}$

well defined and allowed precise determinations of the equivalence points. However, the cationic derivative *N*-pepstatinyl-diaminoethane behaved very differently. As shown in Fig. 2, the degree of dissociation did not permit an accurate determination of the equivalence point; the dissociation constant was determined by curve-fitting, the enzyme concentration was defined by a previous titration with pepstatin. The values of the apparent dissociation constants for the three enzyme-inhibitor complexes are given in Table 1.

Preparation and properties of pepstatinyl-[^3H]glycine

For the study of the binding over a wide range of pH values, it was necessary to prepare a radioactively labelled ligand. The free carboxyl group of pepstatin seemed not to be essential for the interaction of pepstatin with pepsin (Aoyagi *et al.*, 1971). We therefore decided to attach a [^3H]glycine residue to this carboxyl group to obtain a product of the specific radioactivity required to enable the binding to be studied by equilibrium dialysis. Amino acid analysis of the product showed a glycine/alanine ratio of 0.3. The product was therefore a mixture of pepstatinyl-glycine and pepstatin, but is referred to in this paper as pepstatinyl-[^3H]glycine.

When human cathepsin D was titrated with pepstatin and pepstatinyl-[^3H]glycine at the same apparent inhibitor concentration, it was observed that the equivalent concentration of pepstatinyl-[^3H]glycine was much greater than that of pepstatin (Fig. 3). The shape of the inhibition curves was not consistent with the pepstatinyl-[^3H]glycine forming a much weaker complex with cathepsin D (Bieth, 1974), but rather

Fig. 3. Inhibition of cathepsin D by pepstatin or *N*-pepstatinyl-[^3H]glycine

Assay conditions were as in Fig. 1. Haemoglobin was incubated with pepstatin (○) or *N*-pepstatinyl-[^3H]glycine (△) at the same apparent inhibitor concentration, and the assay started by the addition of cathepsin D to give a final concentration of 30 nM. The values obtained for *N*-pepstatinyl-[^3H]glycine were corrected on the assumption that the true concentration of this inhibitor was 50% of the apparent concentration. The corrected points (□) lie on the curve for pepstatin.

Fig. 4. Chromatography on Sephadex G-100 of cathepsin D-pepstatinyl-[^3H]glycine mixtures at (a) pH 5.5 and (b) pH 7.8

A mixture of partially purified cathepsin D (54 units) and *N*-pepstatinyl-[^3H]glycine (0.95 μg , 18500 c.p.m.) was applied to a column (1.5 cm $\times$ 27 cm; 48 ml) of Sephadex G-100 at 4°C and eluted with (a) 0.05 M-sodium acetate/acetic acid, pH 5.5, containing 0.1% Brij 35, or (b) 0.05 M-Tris/HCl, pH 7.8. The flow rate in each case was 30 ml/h. A portion (0.5 ml) of each fraction (1.1 ml) was taken for assay of cathepsin D activity (----) and another portion (0.1 ml) was taken for scintillation counting (—). The elution volumes of the molecular-weight markers, bovine serum albumin (BSA; mol.wt. 66500) and cytochrome c (Cyt c; mol.wt. 11700), are shown by the arrows.

suggested that the pepstatinyl-[^3H]glycine preparation contained 50% of a contaminant, which did not bind appreciably to cathepsin D. When the inhibition curve for pepstatinyl-[^3H]glycine was corrected on this basis the curve became superimposable on that of pepstatin (Fig. 3). Dissociation constants were calculated from the equivalence points after determination of the enzyme concentration by a prior titration with pepstatin. The values are given in Table 1 and are very similar to those obtained with pepstatin.

The presence of an impurity in the pepstatinyl-[^3H]glycine preparation would not invalidate the use of this material to study pepstatin-cathepsin D binding if it were shown that all the radioactivity was bound by the enzyme. The extent of binding was explored by gel chromatography.

When a twofold excess of cathepsin D was added to pepstatinyl-[^3H]glycine at pH 5.5 and the mixture

chromatographed on Sephadex G-100, the enzyme was eluted at the expected position (Barrett, 1970) between serum albumin and cytochrome *c* (Fig. 4a). All the radioactivity was recovered from the column, about 85% being eluted in the fractions that also contained enzyme. These results suggested that all the ^3H label in the pepstatinyl- ^3H glycine preparation was bound to the enzyme and were consistent with the finding that the material was radiochemically homogeneous on t.l.c.

In a similar experiment at pH 7.8 the relative elution positions of cathepsin D and pepstatinyl- ^3H glycine were radically changed. As is shown in Fig. 4(b) the enzyme was eluted at the same position, but had no associated radioactive label. The peak of pepstatinyl- ^3H glycine was eluted at approx. 90% of the column volume.

The recovery of haemoglobin-degrading activity from the column showed that the inhibitor had not been released through denaturation of the enzyme, but rather, suggested that the binding of pepstatin to cathepsin D was strongly pH-dependent.

Effect of pH on the stability of the pepstatin–cathepsin D complex

To ensure that the results obtained with pepstatinyl- ^3H glycine were not attributable to the additional glycine residue, the following experiments were done. Cathepsin D was mixed with an excess of pepstatin at pH values in the range 4.0–7.3 and the mixtures were chromatographed on Sephadex G-50. The range of molecular-weight fractionation by Sephadex G-50 is reported by the manufacturers to be 1500–30000, and complete separation of cathepsin D from excess of pepstatin was expected. This was indeed the case, for when cathepsin D and an excess of pepstatinyl- ^3H glycine was chromatographed at pH 4.0, the enzyme-bound and free inhibitor were eluted in non-overlapping peaks.

The recovery of enzyme activity from the column will depend on the proportion of enzyme originally bound to the inhibitor, and also on the rate at which the complex dissociates, for the enzyme and inhibitor are separated during chromatography and cannot reassociate freely. Under the conditions of these experiments 98% of human cathepsin D was bound to pepstatin in the original sample if the dissociation constant was not greater than 10^{-7}M , but this proportion fell to 35% when the dissociation constant was 10^{-5}M .

The recovery of enzymic activity from the Sephadex G-50 column was approx. 10% between pH 4 and pH 5, but thereafter increased rapidly as the pH was raised, until all the activity was recovered at pH 7.3 (Fig. 5). Pepstatin methyl ester gave results similar to those observed with pepstatin, but with *N*-pepstatinyl-diaminoethane the curve was displaced by approx. 0.3pH unit towards lower pH values (Fig. 5).

Fig. 5. Chromatography on Sephadex G-50 of complexes of cathepsin D and pepstatin, pepstatin methyl ester or *N*-pepstatinyl-diaminoethane in the range pH 4.0–7.3

A mixture of cathepsin D (24 units) and inhibitor ($2\mu\text{g}$) [pepstatin (Δ); pepstatin methyl ester ($\circ$); *N*-pepstatinyl-diaminoethane ($\square$)], in the appropriate buffer (see the Materials and Methods section), was applied to a column (1.5cm $\times$ 27cm; 48 ml) of Sephadex G-50 at 4°C. The column was eluted with the same buffer (flow rate 50 ml/h), and fractions (2 ml) were collected. A portion (0.5 ml) of each fraction was taken for assay, and the number of units of cathepsin D activity eluted from the column was determined. Control experiments were done in the absence of inhibitor ($\blacktriangle$). Two curves have been drawn, one through the points for pepstatin and pepstatin methyl ester, the other through the points for *N*-pepstatinyl-diaminoethane.

These results suggested that the dissociation constant for the complex between pepstatin and human cathepsin D increased considerably between pH 5.0 and 7.3. Dissociation constants were therefore determined directly by equilibrium dialysis with pepstatinyl- ^3H glycine.

Binding of human cathepsin D and pepstatinyl- ^3H glycine measured by equilibrium dialysis

Dialysis was performed at 4°C in the range pH 4.5–6.4. In experiments at pH 5.0 the apparent dissociation constant, K_D , remained constant after 5 days, and in the experiments reported here 6 or 7 days were allowed for equilibration. The enzyme retained full activity during storage for this period under the conditions used.

The effect of pH on the apparent dissociation constant at 4°C is shown in Fig. 6, which also contains data obtained at pH 3.5 and 45°C by the enzymic

Fig. 6. Effect of pH on the apparent dissociation constant (K_D) of the complex between cathepsin D and N-pepstatinyl- $[^3\text{H}]$ glycine

Equilibrium-dialysis experiments were made at 4°C in the range pH 4.5–6.4 as described in the Materials and Methods section. At each pH value the cathepsin D concentration was kept constant and the inhibitor concentration varied over a fourfold range. The results are shown as the logarithms of the mean values of $K_D \pm \text{s.e.m.}$ (9) (M). The horizontal line has been drawn through the mean value of the results obtained at pH 4.5 and 5.0. A least-squares linear regression line has been drawn through the results obtained at pH 5.5, 6.0 and 6.4. The mean of results obtained by the enzymic titration method at 45°C and pH 3.5 is also shown (●).

titration method. The apparent dissociation constant did not change between pH 3.5 and 5.0, but between pH 5.0 and 6.4 there was a marked decrease in the strength of binding, the apparent dissociation constant increasing from $5 \times 10^{-10} \text{ M}$ at pH 5.0 to $2 \times 10^{-6} \text{ M}$ at pH 6.4.

Discussion

It was shown previously (Barrett & Dingle, 1972) that pepstatin is a tight-binding inhibitor of human cathepsin D, and the dissociation constant was estimated to be less than 10^{-8} M . In the present work the characteristics of inhibition have been studied further. Plots of the loss of activity of cathepsin D during titration with pepstatin proved suitable for definition of the molarity of the enzyme solution and also permitted evaluation of the dissociation constant.

The equivalence points of the titrations were consistent with the binding of 1 mol of pepstatin per 43000 g of cathepsin D. Barrett (1970) reported that the elution volume of cathepsin D in gel chromatography is indistinguishable from that of ovalbumin (mol.wt. 44600; Castellino & Barker, 1968). Molecular weights of 40000–43000 have been obtained

for bovine cathepsin D (Sapolsky & Woessner, 1972; Ferguson *et al.*, 1973). The good agreement between values obtained by titration with pepstatin and by other methods indicated that pepstatin provides a method for defining the operational molarity of solutions of cathepsin D, and there are reasons for thinking that the method may be applicable to carboxyl proteinases in general (Barrett & Dingle, 1972; Kunimoto *et al.*, 1974; McKown *et al.*, 1974).

Kunimoto *et al.* (1974) reported that the interaction of pepstatin with pepsin was time-dependent, whereas we have found that the degree of inhibition of cathepsin D was unaffected by preincubation. The data are therefore suitable for treatment by the method of Green & Work (1953) to obtain a dissociation constant. A value of $4 \times 10^{-10} \text{ M}$ was obtained at pH 3.5 for pepstatin itself, whereas the methyl ester of pepstatin gave a value of $7 \times 10^{-10} \text{ M}$ and pepstatinyl-diaminoethane a value of $5 \times 10^{-9} \text{ M}$. Aoyagi *et al.* (1971) have shown that various esters of pepstatin retain the full inhibitory activity of pepstatin for pig cathepsin D, but from our work it seems that introduction of a positive charge in this part of molecule significantly weakens binding. K_i for a preparation of pepstatinyl-glycine was $5 \times 10^{-10} \text{ M}$.

Gel chromatography, used as a criterion of the purity of the pepstatinyl- $[^3\text{H}]$ glycine, showed a major effect of pH on the enzyme-inhibitor interaction. There was a large increase in the efficiency of separation of the enzyme from inhibitor as the pH of the buffer was raised. For pepstatin, its methyl ester and the diaminoethane derivative the increased separation became apparent above pH 5; the increase was steeper for the amine than for the acidic and neutral compounds, which behaved in an identical manner. It was not possible to decide from these experiments to what extent the change was due to an increase in K_i with pH or an increase in the rate constant for dissociation of the complex after removal of the free inhibitor.

The effect of pH on the dissociation constant of the pepstatinyl- $[^3\text{H}]$ glycine–cathepsin D complex was investigated directly by equilibrium dialysis; the dissociation constant increased from $5 \times 10^{-10} \text{ M}$ at pH 5.0 to 2×10^{-6} at pH 6.4. Above pH 6.4, binding was too weak to be measured at attainable concentrations of enzyme and inhibitor. Since a variety of peptides with C-terminal glycine have $\text{p}K_a$ values below pH 3.3 (Perrin, 1965), the changes in binding affinity observed above pH 5 could be attributed only to a change in the ionization state of the enzyme. In the range pH 5.5–6.4 the slope of the plot of $\log K_D$ against pH was $-3.0 (\pm 0.1, \text{s.e.m.})$, indicating that the increase in dissociation constant is accompanied by the loss of three protons (Dixon, 1953). We would suggest that the loss of the binding site for pepstatin accompanies a change in the tertiary structure of the enzyme. It seems significant that in the same pH

range the catalytic activity of cathepsin D falls essentially to zero (Barrett, 1971). It is conceivable that part of the change in affinity for pepstatin is due to ionization of the catalytically active un-ionized carboxyl group, since this could (by analogy with pepsin; Marcinişzyn *et al.*, 1975) prevent the formation of a tetrahedral intermediate. On the basis of a dialysis experiment, Woessner (1972) concluded that the affinity of bovine cathepsin D for pepstatin was as great at pH 7.2 as at acid pH. One possible explanation for the difference between this finding and our own would be that equilibrium had not been reached in Woessner's (1972) experiment.

We conclude that the effect of pH on the interaction of pepstatin with cathepsin D should be taken into account in the interpretation of experiments with pepstatin in biological systems. Whereas, under the conditions of the usual biochemical assays, pepstatin interacts essentially stoichiometrically with cathepsin D, large molar excesses of the inhibitor will be required for efficient inhibition near neutral pH. From our data it is possible to calculate minimal concentrations of pepstatin to produce 95% inhibition of cathepsin D. If one assumes a concentration of enzyme of 1 unit/ml (which may be compared with about 100 units/g in human liver; Barrett, 1973) 0.024 µg of pepstatin/ml would be adequate in the range pH 3.5–5.0. In contrast, 25 µg/ml would be required at pH 6.4 and still larger amounts at higher pH. For other concentrations of enzyme and pH values, the concentrations of pepstatin giving a particular degree of inhibition may be calculated from our data as described by Bieth (1974).

A further important consideration in the use of pepstatin in biological experiments is the degree to which the inhibitor enters tissues and cells. There are already indications that cells of various types efficiently exclude pepstatin, and penetration of some tissues may also be restricted. Radiochemically labelled forms of acidic, neutral and basic derivatives of pepstatin could be used to determine these effects.

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References

- Abu-Erreish, G. M. & Peanasky, R. J. (1974) *J. Biol. Chem.* **249**, 1558–1566
- Aoyagi, T., Kunimoto, S., Morishima, H., Takeuchi, T. & Umezawa, H. (1971) *J. Antibiot.* **24**, 687–694
- Barrett, A. J. (1970) *Biochem. J.* **117**, 601–607
- Barrett, A. J. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 109–133, North-Holland, Amsterdam
- Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822
- Barrett, A. J. & Dingle, J. T. (1972) *Biochem. J.* **127**, 439–441
- Bieth, J. (1974) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J. & Truscheit, E., eds.), pp. 463–469, Springer-Verlag, Berlin, Heidelberg and New York
- Castellino, F. J. & Barker, R. (1968) *Biochemistry* **7**, 2207–2217
- Dingle, J. T., Barrett, A. J. & Weston, P. D. (1971) *Biochem. J.* **123**, 1–13
- Dingle, J. T., Poole, A. R., Lazarus, G. S. & Barrett, A. J. (1973) *J. Exp. Med.* **137**, 1124–1141
- Dixon, M. (1953) *Biochem. J.* **55**, 161–170
- Ferguson, J. B., Andrews, J. R., Voynick, I. M. & Fruton, J. S. (1973) *J. Biol. Chem.* **248**, 6701–6708
- Goodwin, T. W. & Morton, R. A. (1946) *Biochem. J.* **40**, 628–632
- Green, N. M. (1957) *Biochem. J.* **66**, 407–415
- Green, N. M. & Work, E. (1953) *Biochem. J.* **54**, 347–352
- Hall, T. C. & Cocking, E. C. (1965) *Biochem. J.* **96**, 625–633
- Inouye, K. & Fruton, J. S. (1968) *Biochemistry* **7**, 1611–1615
- Johnson, C. H. & Knowles, J. R. (1966) *Biochem. J.* **101**, 56–62
- Keilová, H. & Tomašek, V. (1972) *Biochim. Biophys. Acta* **284**, 461–464
- Kunimoto, S., Aoyagi, T., Nishizawa, R., Komai, T., Takeuchi, T. & Umezawa, H. (1974) *J. Antibiot.* **27**, 413–418
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lundblad, R. L. & Stein, W. H. (1969) *J. Biol. Chem.* **244**, 154–160
- Marcinişzyn, J. P., Hartsuck, J. A. & Tang, J. J. N. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 484
- McKown, M. M., Workman, R. J. & Gregerman, R. I. (1974) *J. Biol. Chem.* **249**, 7770–7774
- Morishima, H., Takita, T., Aoyagi, T., Takeuchi, T. & Umezawa, H. (1970) *J. Antibiot.* **23**, 263–265
- Perrin, D. D. (1965) *Dissociation Constants of Organic Bases in Aqueous Solution*, Butterworths, London
- Sapolsky, A. I. & Woessner, J. F., Jr. (1972) *J. Biol. Chem.* **247**, 2069–2076
- Takahashi, K., Mizobe, F. & Chang, W. J. (1972) *J. Biochem. (Tokyo)* **71**, 161–164
- Takahashi, K., Chang, W. J. & Ko, J. S. (1974) *J. Biochem. (Tokyo)* **76**, 897–899
- Tang, J. (1971) *J. Biol. Chem.* **246**, 4510–4517
- Turner, J. C. (1969) *Int. J. Appl. Radiat. Isot.* **20**, 499–505
- Umezawa, H. (1972) *Enzyme Inhibitors of Microbial Origin*, pp. 34–50, University Park Press, Baltimore, London and Tokyo
- Umezawa, H., Aoyagi, T., Morishita, H., Matsuzaki, M., Hamada, M. & Takeuchi, T. (1970) *J. Antibiot.* **23**, 259–262
- Woessner, J. F. (1972) *Biochem. Biophys. Res. Commun.* **47**, 965–970