Interaction of dinitrophenyl-pepstatins with human cathepsin D and with anti-dinitrophenyl antibody

Development of potential reagents for the localization *in vivo* of active proteinases at sites of tissue injury

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Extracellular cathepsin D has been observed by various cytochemical methods at sites of tissue injury. However, the role of this enzyme in connective tissue matrix degradation is uncertain because there are no histochemical methods for determining whether or not the cathepsin D is active at such sites in living tissues. We considered that the combined use of a labelled tight-binding inhibitor with immunoprecipitation of the enzyme might overcome this problem. We have explored the application of derivatives of the inhibitor pepstatin, as only active cathepsin D binds pepstatin tightly. A series of *N*-pepstatinyl-*N'*-dinitrophenyl- α,ω -diaminoalkanes were synthesized with alkyl-chain lengths of two, four and six carbon atoms. These compounds were tight-binding inhibitors of human cathepsin D. In fluorescence-quenching titrations the dinitrophenyl groups were also fully available to bind high-affinity anti-dinitrophenyl antibody. It was shown by immunodiffusion in gels and by gel permeation chromatography that *N*-pepstatinyl-*N'*-dinitrophenyl-1,6-diaminohexane was a bi-functional inhibitor able to bind cathepsin D and anti-dinitrophenyl antibody at the same time.

Proteolytic enzymes are thought to play a crucial role in the destruction of articular and patellar cartilage that occurs in chronic joint inflammation (Dingle, 1978). Several tissue proteinases have the ability to degrade the proteoglycan and collagen components of the cartilage matrix (Dingle, 1976; Barrett, 1978), although isolated living cartilage, containing chondrocytes and no other cellular elements, contains only cathepsins B, D and F in measurable activity. However, the positive identification of the enzyme(s) responsible for the breakdown has not so far been possible because histochemical methods cannot be used to localize enzymes that may be active under the conditions naturally appertaining in living tissues.

We considered the possibility that labelled tight-binding, or irreversible, active site-directed enzyme

inhibitors might be useful for this purpose, since these compounds require the native, active enzyme structure for efficient interaction (Knight, 1977). Two obvious problems are associated with the use of proteinase-inhibitor interactions for localization: the reactions are stoichiometric and the reaction product remains freely diffusible. These objections may be overcome by using the enzyme-antibody capture technique developed by Poole *et al.* (1973, 1974) to bind the proteinase as insoluble immune complexes at the sites of release in the living tissue and then to treat these complexes with inhibitor *in vivo*. In the absence of non-specific interactions, the binding of the inhibitor should demonstrate the presence of active enzyme in the immune complex.

In the present paper we describe our initial studies using as a model cathepsin D (EC 3.4.23.5) and derivatives of the inhibitor pepstatin. Cathepsin D is secreted by human rheumatoid tissues (Poole *et al.*, 1976) and there has been much interest in determining its role in the degradation of the cartilage proteoglycans in arthritis (Dingle, 1976, 1978). Pepstatin (isovaleryl-L-valyl-L-valyl-4-amino-3-

Abbreviations used: Dnp, dinitrophenyl; IgG, immunoglobulin G.

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hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) is a naturally occurring tight-binding inhibitor of cathepsin D (Barrett & Dingle, 1972; Knight & Barrett, 1976), obtained from the filtrates of cultures of *Streptomyces* (Umezawa, 1972). Pepstatin itself cannot be used for the localization of cathepsin D, but requires chemical modification to carry some other moiety (a 'reporter' group) permitting the visualization of the inhibitor. We report here the synthesis of some Dnp-labelled pepstatin derivatives and describe their interactions with human cathepsin D and with a high-affinity antibody to the Dnp group raised in rabbits.

Materials and methods

Pepstatin and cathepsin D from human liver were kindly given by Dr. A. J. Barrett of this laboratory. Antisera to human cathepsin D and to rabbit IgG were raised in sheep as described previously (Weston, 1969). All other reagents and proteins were obtained from commercial sources and were of the purest grades available. Silica gel sheets (13181) for t.l.c. were from Kodak, Kirby, Liverpool, U.K. 1-Fluoro-2,4-dinitro[3,5-³H]benzene was from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex LH-20 and G-100 were from Pharmacia (Great Britain), Hounslow, Middx., U.K. AG50W-X2 resin (H⁺ form, 200–400 mesh) was from Bio-Rad Laboratories, Bromley, Kent, U.K.

N-Pepstatinyl-diaminoalkanes. *N*-Pepstatinyl-1,2-diaminoethane was synthesized as described by Knight & Barrett (1976). *N*-Pepstatinyl-1,4-diaminobutane and *N*-pepstatinyl-1,6-diaminohexane were made by the same method. Briefly, pepstatin (20 mg, 0.03 mmol) was dissolved in dimethylsulphoxide (1 ml) at 80°C and triethylamine (0.03 ml, 0.21 mmol) and chloroacetonitrile (0.01 ml, 0.15 mmol) were added. After 4 h at 80°C, 1,4-diaminobutane (1.6 mmol), or 1,6-diaminohexane (1.6 mmol), in dimethylsulphoxide (1 ml) was added and the mixture was stirred for 16 h at 45°C. The solvent was removed at 40°C *in vacuo* and the *N*-pepstatinyl-diaminoalkane was purified on Sephadex LH-20 and on Bio-Rad AG50-X2 (Knight & Barrett, 1976). *N*-Pepstatinyl-1,4-diaminobutane (yield: 4.4 mg, 20%) and *N*-pepstatinyl-1,6-diaminohexane (yield: 6.6 mg, 28%) were both homogeneous by t.l.c. on silica gel with butan-1-ol/n-butyl acetate/acetic acid/water (4:4:1:1, by vol.) as solvent, and were used without further purification.

N-Pepstatinyl-N'-2,4-dinitrophenyl-diaminoalkanes. 1-Fluoro-2,4-dinitrobenzene (10 µl, 80 µmol) was added to *N*-pepstatinyl-1,2-diaminoethane (4 mg, 5.5 µmol) in dimethylformamide (1 ml). After being left in darkness at 21°C for 24 h the solvent was evaporated at 40°C *in vacuo*. The

residue was dissolved in ethanol (1 ml) and applied to a column (1 cm × 60 cm) of Sephadex LH-20 and eluted with ethanol (12 ml/h). Fractions (1 ml) representing the first yellow peak eluted from the column were combined and evaporated to dryness (yield: 4.8 mg, 98%). *N*-Pepstatinyl-*N'*-Dnp-1,2-diaminoethane was homogeneous on silica gel t.l.c. in butan-1-ol/n-butyl acetate/acetic acid/water (4:4:1:1, by vol.; *R_F* 0.7) and also in chloroform/methanol (9:1, v/v; *R_F* 0.6). *N*-Pepstatinyl-*N'*-Dnp-1,4-diaminobutane and *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane were made by the same method and purified on Sephadex LH-20. Each product was homogeneous on t.l.c.

N-Pepstatinyl-N'-2,4-dinitro[3,5-³H]phenyl-1,6-diaminohexane. 1-Fluoro-2,4-dinitro[3,5-³H]benzene of specific radioactivity 518 MBq/µmol was supplied as a solution (37 MBq/ml) in toluene. This solution was evaporated to dryness at 40°C *in vacuo* and the residue (0.07 µmol) was dissolved in dimethylsulphoxide (1 ml). *N*-Pepstatinyl-1,6-diaminohexane (3 mg, 3.8 µmol) in dimethylsulphoxide (1 ml) was allowed to react in darkness with unlabelled 1-fluoro-2,4-dinitrobenzene (0.18 µmol) for 16 h at 21°C before addition of the radioactive reagent. This mixture was left for 24 h at 21°C before addition of 1-fluoro-2,4-dinitrobenzene (5 µl, 40 µmol). After reaction overnight the solvent was removed at 40°C *in vacuo*. The residue was dissolved in ethanol (1 ml) and applied to a column (1 cm × 60 cm) of Sephadex LH-20 and eluted with ethanol (12 ml/h). A portion (1 µl) of each fraction (1 ml) was taken for scintillation counting. Fractions containing *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane were combined and evaporated to dryness (yield: 2.9 mg, 81%). The product showed one component on t.l.c. on silica gel (*R_F* 0.6; chloroform/methanol, 9:1, v/v) which contained 95% of the radioactivity. The specific radioactivity of this product was 20 MBq/µmol.

Anti-Dnp antibody

Anti-Dnp antibody was produced in rabbits (Eisen, 1964) and purified as described by Eisen *et al.* (1967). A univalent (Fab) fragment preparation (Knight & Green, 1976) gave only one protein band on slab-gel electrophoresis (Davies *et al.*, 1978), corresponding to mol.wt. 45 000. The fragments were labelled with fluorescein isothiocyanate (The & Feltkamp, 1970) giving 2.0 fluorescein groups/molecule (Goldman, 1968).

Scintillation counting

Packard Instruments Co., Cavenham, Berks., U.K. supplied Pico-Fluor 30 and a toluene solution of 2,5-phenyloxazole (5 g/l) and 1,4-bis(5-phenyloxazol-2-yl)benzene (0.1 g/l). Scintillation fluid was prepared by mixing these solutions (1:1, v/v).

Aqueous samples up to 0.1 ml in volume were added to 4 ml of fluid. Scintillation counting was performed in a Packard PDL liquid-scintillation spectrometer and quenching corrections were applied. Data reported as c.p.m. were from samples counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer model 2111.

Inhibitor titrations with cathepsin D

Experiments were made as described by Knight & Barrett (1976) with the purified γ -form of human cathepsin D, separated by isoelectric focusing (Barrett, 1970). The specific activity was 840 units/mg, close to the value previously reported (870 units/mg; Knight & Barrett, 1976). One unit of activity is defined as that producing an increase in absorbance (ΔA_{280}) of 1.0 in the assay during 60 min under the specific conditions, a linear response being assumed.

Fluorescence-quenching titrations

Titration (Velick *et al.*, 1960) were made at 21°C in a Farrand mark 1 spectrofluorimeter. The excitation wavelength was 285 nm and fluorescence emission was measured at 345 nm. The antibody concentration was approx. 0.2 μ M (with respect to binding sites) in 0.05 M-sodium phosphate buffer, pH 7.45, and the concentration of Dnp compound was 30–100 μ M in Dnp groups. Corrections were made for dilution and for internal quenching during the titration (Knight & Green, 1979).

Double immunodiffusion

Double immunodiffusion in gels was done in Ouchterlony (1958) plates prepared with 1% agarose containing 0.05 M-acetate buffer (pH 5.0)/0.10 M-NaCl/0.1% Brij 35. Human cathepsin D (0.1 ml, 50 units/ml) was mixed with *N*-pepstatiny-*N'*-Dnp-1,2-diaminoethane (0.02 ml, 10 μ M). After 15 min at 21°C rabbit anti-Dnp antibody (0.075 ml, 0.36 mg/ml) was added and the volume was brought to 0.45 ml by the addition of the pH 5.0 buffer. After 30 min at 21°C this mixture (40 μ l) was placed in the centre well of the Ouchterlony plate. A sheep antiserum to human cathepsin D (40 μ l) was placed

in a peripheral well and the flanking wells were filled with a sheep antiserum to rabbit IgG (40 μ l). After diffusion for 48 h at 21°C in a moist atmosphere the plate was washed for 24 h. Double-diffusion experiments were also made with the same concentrations of cathepsin D, anti-Dnp antibody and either *N*-pepstatiny-*N'*-Dnp-1,4-diaminobutane or *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane. In a control experiment the human cathepsin D and rabbit anti-Dnp antibody were diffused against the sheep antisera without adding Dnp-pepstatin.

Gel filtration of complexes on Sephadex G-100

Gel filtration experiments were made by using a column (1.5 cm $\times$ 26 cm, 46 ml) of Sephadex G-100 eluted at 4°C with 0.05 M-sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl and 0.1% Brij 35. The flow rate was 15 ml/h and fractions (0.85 ml) were collected. The void volume of the column (13.1 ml) was measured with Blue Dextran 2000 (Fischer, 1969). The elution characteristics of the column were monitored every three or four runs by measuring the partition coefficients of bovine serum albumin (α 0.16), cytochrome *c* (α 0.51) and *N*⁶-Dnp-lysine (α 0.98). Various mixtures of cathepsin D, *N*-pepstatiny-*N'*-[³H]Dnp-1,6-diaminohexane and fluorescein-labelled fragments of anti-Dnp antibody, as described in the Results section, were prepared in elution buffer (approx. 1 ml) and left at 21°C for at least 30 min before being applied to the column. The absorbance of each fraction was measured at 280 nm and a portion (0.1 ml) was taken for the assay of cathepsin D activity. Further portions were taken, if necessary, for scintillation counting (0.1 ml) and for the measurement of fluorescein fluorescence (0.5 ml).

Results

Inhibition of cathepsin D by Dnp-pepstatins

N-Pepstatiny-*N'*-Dnp-diaminoalkanes behaved like pepstatin and bound tightly and stoichiometrically to human cathepsin D at pH 3.5 (Table 1). A titration curve obtained with *N*-pepstatiny-*N'*-[³H]Dnp-1,6-diaminohexane is shown in Fig. 1.

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 by Knight & Barrett (1976). The results for duplicate experiments are given.

Expt.	10 ¹⁰ $\times$ Apparent dissociation constant (M) for:			
	Pepstatin	<i>N</i> -Pepstatiny- <i>N'</i> -Dnp-1,2-diaminoethane	<i>N</i> -Pepstatiny- <i>N'</i> -Dnp-1,4-diaminobutane	<i>N</i> -Pepstatiny- <i>N'</i> -Dnp-1,6-diaminohexane
1	4.3	5.2	2.3	7.1
2	3.2	3.7	3.6	4.5*

* ³H-labelled derivative.

Fig. 1. Inhibition of cathepsin D by pepstatin and by *N*-pepstatinyl-*N'*-[^3H]Dnp-1,6-diaminohexane

Standard assay conditions were used as described by Barrett (1970). The assays were started by the addition of buffered haemoglobin to the mixture of cathepsin D (1.35 units) and pepstatin (●) or *N*-pepstatinyl-*N'*-[^3H]Dnp-1,6-diaminohexane (■) at 45°C. The trichloroacetic acid-soluble products of digestion of haemoglobin were measured by their A_{280} .

The shape of the titration curve enabled the calculation of the apparent dissociation constant, K_D , from the deviation from stoichiometric inhibition in the region of the equivalence point (Green & Work, 1953; Knight & Barrett, 1976). The inhibitor concentration was assumed to be equal to that of the Dnp group and was calculated from the value of A_{344} in ethanol by using ϵ_{344} 17 300 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (the value for *N*⁶-Dnp-lysine hydantoin). This assumption was tested by titrating the same concentration of enzyme with pepstatin (Fig. 1). It was found that the two titration curves had the same equivalence point. Thus the preparation of *N*-pepstatinyl-*N'*-[^3H]Dnp-1,6-diaminohexane contained equimolar amounts of Dnp group and pepstatin. Similar results were obtained with the unlabelled *N*-pepstatinyl-*N'*-Dnp-diaminoalkanes.

Characterization of anti-Dnp antibody

The fluorescence-quenching titration curves of anti-Dnp antibody with *N*⁶-Dnp-lysine (Fig. 2)

Fig. 2. Fluorescence quenching of anti-Dnp antibody by *N*⁶-Dnp-lysine and by *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane

Titration were made at 21°C in 0.05 M-sodium phosphate buffer, pH 7.45. *N*⁶-Dnp-lysine (●), or *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane (■), was added to anti-Dnp antibody (2 ml, 0.22 μM in Dnp-binding sites). Corrections were made for dilution and for internal quenching as described by Knight & Green (1979). The titration curve for *N*⁶-Dnp-lysine has been drawn.

showed an extensive linear portion, followed by curvature in the neighbourhood of the equivalence point. Values of the apparent dissociation constant, K_{app} , were calculated as described by Knight & Green (1976). We found K_{app} 4.3×10^{-9} M at 0.20 μM-antibody [determined spectrophotometrically (McGuigan & Eisen, 1968)] and K_{app} 0.1×10^{-9} M at 0.04 μM-antibody, indicating that most of the antibody species in the preparation bound the hapten with $K_{app} < 10^{-10}$ M (cf. Knight & Green, 1976). The maximum observed degree of fluorescence quenching in the presence of excess of antigen, Q_{max} , was 0.55. A comparison of antibody concentrations determined spectrophotometrically and by fluorescence-quenching titrations showed the preparation to have about 0.68 mol of Dnp-binding sites/mol. The low activity was a consequence of firmly bound dinitrophenol, often present in preparations of high-affinity antibody to Dnp (Stevenson *et al.*, 1970).

Titration of antibody with Dnp-pepstatis

When the Dnp-labelled pepstatins were added to antibody the protein fluorescence was quenched. A

titration curve obtained with *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane at pH 7.45 is also shown in Fig. 2. It can be seen that the pepstatin-bound Dnp group had the same fluorescence-quenching efficiency as that of the hapten and a similar apparent dissociation constant. *N*-Pepstatiny-*N'*-Dnp-1,2-diaminoethane behaved similarly (Table 2).

Binding of Dnp-pepstatins by cathepsin D in the presence of antibody

The inhibition experiments and fluorescence-quenching titrations demonstrated that all the Dnp-pepstatins bound tightly to both human cathepsin D and to anti-Dnp antibody. However, our proposed application of the Dnp-pepstatins to the localization of cathepsin D requires the Dnp-labelled inhibitors to behave in a bifunctional manner, that is, to bind the proteinase and the anti-Dnp antibody simultaneously.

In studies of antibody binding to bis-Dnp compounds (Green, 1969; Wilder *et al.*, 1975), bi-functional behaviour only occurred when the ligands were separated by more than a minimum distance. At shorter distances, protein-protein interactions weakened the binding of the second protein molecule. The Dnp-pepstatins showed similar behaviour. In the presence of an excess of anti-Dnp antibody, which showed no change in K_{app} on lowering the pH to 3.5 (Velick *et al.*, 1960; Knight & Green, 1979), *N*-pepstatiny-*N'*-Dnp-1,2-diaminoethane was bound less tightly by cathepsin D (Fig. 3).

When a similar experiment was made with *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane, the titration curve obtained when anti-Dnp antibody was present was identical with that observed in the absence of antibody (Fig. 4), and gave K_D 6.4×10^{-10} M. These results implied that *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane acted as a bifunctional reagent.

Immunochemical studies of complex formation

We sought confirmation of the apparent bi-functional properties of *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane by the direct demonstration of

anti-Dnp antibody-Dnp-pepstatin-cathepsin D complexes. The application of double-immunodiffusion in agarose gels (Ouchterlony, 1958) was suggested by Dr. A. J. Barrett. Sheep antisera to human cathepsin D and to rabbit IgG were placed in adjacent wells, and a mixture of human cathepsin D, rabbit antibody to Dnp and a Dnp-pepstatin was placed in the central well. The patterns obtained after diffusion for 48 h at pH 5.0 are shown in Fig. 5. When *N*-pepstatiny-*N'*-Dnp 1,6-diaminohexane was present the precipitin lines formed against the

Fig. 3. Effect of antibody to Dnp on the inhibition of cathepsin D by *N*-pepstatiny-*N'*-Dnp-1,2-diaminoethane. Cathepsin D (16 nM) was titrated with *N*-pepstatiny-*N'*-Dnp-1,2-diaminoethane (■) as described in the legend to Fig. 1. Titrations were also done in the presence of antibody to Dnp at the following concentrations of Dnp-binding sites: ▲, 0.12 μ M; ●, 0.48 μ M. Apparent dissociation constants, K_D , were calculated from the enzyme activity remaining at the equivalence point (Green & Work, 1953; Bieth, 1974). The values obtained were: ■, 0.26 nM; ▲, 4 nM; ●, 8 nM.

Table 2. Apparent dissociation constants for complexes between *N*⁶-Dnp-lysine or Dnp-pepstatins and rabbit antibody to Dnp

The dissociation constants were measured by fluorescence-quenching titrations made at 21°C as described in the legend to Fig. 2. Experiments were made (a) with whole antibody or (b) with univalent (Fab) fragments of antibody prepared by digestion with papain. In each case the antibody concentration, determined spectrophotometrically (McGuigan & Eisen, 1968), was approx. 0.2 μ M. Corrections for internal quenching and for dilution were applied.

Expt.	Apparent dissociation constant (nM) for:		
	<i>N</i> ⁶ -Dnp-lysine	<i>N</i> -Pepstatiny- <i>N'</i> -Dnp-1,2-diaminoethane	<i>N</i> -Pepstatiny- <i>N'</i> -Dnp-1,6-diaminohexane
(a)	4.3	4.9	2.9
(b)	3.3	8.1	8.3

Fig. 4. Effect of antibody to Dnp on the inhibition of cathepsin D by *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane

Cathepsin D (33 nM) was titrated with *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane (■) as described in the legend to Fig. 1. A titration was also done (●) in the presence of antibody to Dnp (0.15 μM in Dnp-binding sites).

antiserum to rabbit IgG were both continuous with that formed against the antiserum to human cathepsin D. This pattern of identity demonstrated that human cathepsin D and rabbit anti-Dnp antibody migrated through the gel as a single molecular species. When the alkyl chain of the Dnp-pepstatin was decreased by two methylene groups the reaction of identity was much less well-defined, suggesting that many of the anti-Dnp antibody and human cathepsin D molecules diffused independently. A further decrease in chain length, to *N*-pepstatinyl-*N'*-Dnp-1,2-diaminoethane, resulted in a pattern very similar to that seen when Dnp-pepstatin was omitted (Fig. 5).

Gel chromatography on Sephadex G-100 at pH 5.0

The formation of an enzyme-inhibitor-antibody complex with the 1,6-diaminohexyl chain was demonstrated directly by gel chromatography. For these experiments the anti-Dnp antibody was digested with papain to give univalent fragments (mol.wt. 45 000), which were then labelled with fluorescein. *N*-Pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane was used. Human cathepsin D has mol.wt. 43 000 (Knight & Barrett, 1976) and so an enzyme-inhibitor-antibody complex will have mol.wt. 89 000 with associated radioactivity and fluorescence. The range of molecular-weight fractionation by Sephadex G-100 is reported by the manufacturers to be 4000–150 000 and separation of the complex from excess of cathepsin D and antibody fragments was expected.

Fig. 5. Ouchterlony gel diffusion of mixtures of cathepsin D, Dnp-pepstatin and antibody to Dnp

The Ouchterlony plate was loaded and developed as described in the Materials and methods section. Each central well contained human cathepsin D and rabbit antibody to Dnp. These wells also contained either *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane (well 6), *N*-pepstatinyl-*N'*-Dnp-1,4-diaminobutane (well 4), *N*-pepstatinyl-*N'*-Dnp-1,2-diaminoethane (well 2) or no added Dnp-pepstatin (well C). Wells labelled a contained a sheep antiserum to rabbit IgG and wells labelled b contained a sheep antiserum to human cathepsin D. The precipitin line patterns are discussed in the text.

However, when *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane was chromatographed on Sephadex G-100, approx. 95% of the radioactivity was eluted from the column at the position of serum albumin. The eluting buffer contained 0.1% Brij 35 to ensure recovery of the cathepsin D activity from the column (Barrett, 1970) and we concluded that the inhibitor was binding to detergent micelles. When an excess of cathepsin D was present, binding to micelles did not occur and the radioactivity was eluted in the fractions that also contained enzyme (Fig. 6a). Similarly, when the inhibitor was chromatographed with an excess of fluorescein-labelled antibody fragments, the radioactivity was eluted in the fluorescent fractions (Fig. 6b). As might have been expected, cathepsin D and fragments of anti-Dnp antibody, and their binary complexes with *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane, had similar partition coefficients (α 0.30). In the presence of both cathepsin D and antibody fragments the relative elution positions of the proteins and *N*-

Fig. 6. Chromatography on Sephadex G-100 of *N*-pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane with (a) cathepsin D and (b) fragments of antibody to Dnp. A column (1.5 cm $\times$ 26 cm, 46 ml) of Sephadex G-100 at 4°C was eluted with 0.05 M-sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl and 0.1% Brij 35. The flow rate was 15 ml/h and fractions (0.85 ml) were collected. In (a) *N*-pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane (0.138 nmol, 68 600 c.p.m.) and cathepsin D (50 units, 1.34 nmol) were mixed in elution buffer and left at 21°C for 30 min before being applied to the column. Portions (0.1 ml) of each fraction were taken for the assay cathepsin D activity ($\square$) and for the counting of radioactivity ($\bullet$). In (b) a mixture of *N*-pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane (0.025 nmol, 12 900 c.p.m.) and fluorescein-labelled fragments of antibody to Dnp (0.36 nmol) was applied to the column. A portion (0.5 ml) of each fraction was taken for the measurement of fluorescein fluorescence ($\circ$) and a further portion (0.1 ml) for scintillation counting ($\bullet$). The arrows show the elution volumes of the following markers: 1, Blue Dextran 2000; 2, bovine serum albumin (mol.wt. 66 500); 3, cytochrome *c* (mol.wt. 11 700); 4, *N*⁶-Dnp-lysine hydrochloride (mol.wt. 349).

Fig. 7. Chromatography on Sephadex G-100 of *N*-pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane with both cathepsin D and fragments of antibody to Dnp.

Chromatography was carried out on a calibrated column of Sephadex G-100 as described in the legend to Fig. 6. In (a) cathepsin D (50 units, 1.34 nmol) and *N*-pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane (0.069 nmol, 34 300 c.p.m.) were mixed at 21°C. After 30 min, fluorescein-labelled fragments of antibody to Dnp (0.27 nmol) were added and the mixture was left for 30 min before being applied to the column. In (b) the antibody fragments were mixed with *N*⁶-Dnp-lysine (10 nmol) before addition to the cathepsin D and *N*-pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane. In each experiment the fractions were assayed for radioactivity ($\bullet$), cathepsin D activity ($\square$) and fluorescence ($\circ$), as described in the legend to Fig. 6.

ence was eluted in the same fractions, showing that the high-molecular-weight species was the ternary complex. When an excess of *N*⁶-Dnp-lysine was added to the mixture of cathepsin D, ^3H -labelled inhibitor and antibody fragments the radioactivity was eluted in the fractions containing enzyme (Fig. 7b) and no radioactive species of higher molecular weight was observed. A similar result was obtained (results not shown) if, instead of *N*⁶-Dnp-lysine, an excess of pepstatin was added to the mixture. In this case, very little cathepsin D activity was recovered from the column (cf. Knight & Barrett, 1976). A single peak of radioactivity was eluted from the

pepstatiny-*N'*-[^3H]Dnp-1,6-diaminohexane were radically changed (Fig. 7a) and the peak of radioactivity (α 0.14) was eluted before the cathepsin D activity. Moreover, a small peak of fluores-

column in the fractions that also contained fluorescein-labelled antibody fragments.

Discussion

The present study has shown that Dnp-amino-alkyl derivatives of pepstatin retain the full inhibitory activity of pepstatin for human cathepsin D (Table 1). Moreover, it was possible to bind a globular antibody molecule to the Dnp group of *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane without dissociation of the cathepsin D-pepstatin complex (Fig. 4). This bifunctional behaviour was dependent, however, on the length of the alkyl chain between the pepstatin and Dnp moieties (cf. Green, 1969; Wilder *et al.*, 1975). When the chain was shorter than six carbon atoms, addition of the antibody led to increased dissociation of the enzyme-inhibitor complex (Fig. 3).

The influence of alkyl chain length upon the formation of enzyme-inhibitor-antibody complexes was demonstrated directly by double immunodiffusion in agarose gels (Fig. 5). The results confirmed the conclusion drawn from the titration experiments, that it was necessary to separate the pepstatin and Dnp moieties by a chain of six carbon atoms to obtain a bifunctional inhibitor.

Further evidence for the formation of a ternary complex was obtained by gel permeation chromatography on Sephadex G-100. It was anticipated that *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane would behave chromatographically like *N*-pepstatinyl-[³H]glycine (Knight & Barrett, 1976). However, the radioactivity did not emerge from the column in the expected position, but migrated with a partition coefficient similar to that of bovine serum albumin. It is probable that this behaviour was due to the association of the hydrophobic inhibitor with detergent micelles, since we also observed that the ³H-labelled inhibitor did not equilibrate across a dialysis membrane in the presence of the detergent (W. Hornebeck, unpublished work). This partitioning artefact did not occur in the presence of excess quantities of cathepsin D or of anti-Dnp antibody fragments (Fig. 6). When all three components were present, the radioactivity was eluted with a partition coefficient similar to that of the detergent-bound inhibitor, but the control experiment with Dnp-lysine (Fig. 7*b*), and with pepstatin, showed that an antibody-Dnp-pepstatin-cathepsin D complex had been formed.

The present study has defined the structural requirements necessary to obtain a bifunctional Dnp-labelled pepstatin. We have shown that *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane binds tightly to cathepsin D and that the Dnp group of the enzyme-inhibitor complex binds fluorescein-labelled fragments of anti-Dnp antibody. The application of

this reagent to the localization of cathepsin D in immunoprecipitates and in tissues remains to be worked out. However, should any structural changes be necessary, for example, to minimize any non-specific interactions, it is clearly essential to retain the separation between the pepstatin and Dnp moieties.

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