Dinitrophenyl-pepstatins as active-site-directed localization reagents for cathepsin D

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1. *N*-Pepstatinyl-*N'*-dinitrophenyl-1,6-diaminohexane, a potential active-site-directed localization reagent for cathepsin D, was found to bind non-specifically to immuno-precipitates containing cathepsin D. 2. Three new water-soluble localization reagents were synthesized, by using *NN'*-bis-(3-aminopropyl)piperazine, 3-oxa-1,5-diaminopentane or 3,6-dioxa-1,8-diamino-octane, as spacer arms between the pepstatin and dinitrophenyl moieties. 3. The hydrophilic dinitrophenyl-pepstatis were all tight-binding inhibitors of cathepsin D at pH 3.5, but showed little or no binding to immuno-precipitates containing the inactive enzyme at pH 7.4. 4. Gel-chromatographic experiments showed that, at pH 5.0, all the dinitrophenyl-pepstatis were bifunctional reagents able to bind cathepsin D and anti-dinitrophenyl antibody at the same time. Enzyme-inhibitor-antibody complexes were not formed at pH 7.4, thus confirming that the reagents were active-site-directed. 5. Cultured human synovial cells were fixed and incubated with the dinitrophenyl-pepstatis at pH 5.0 or pH 7.4. After washing briefly, the cells were incubated at the appropriate pH value with anti-dinitrophenyl antibody labelled with fluorescein. When examined by fluorescence microscopy the cells stained at pH 5.0 showed fluorescent perinuclear granules, which were not seen in the cells treated at pH 7.4. The distribution of cathepsin D, determined by indirect immunofluorescence at pH 7.4, closely resembled that revealed by the dinitrophenyl-pepstatis at pH 5.0. 7. *NN'*-(3-Pepstatinylaminopropyl-3'-dinitrophenylaminopropyl)piperazine gave the most intense lysosomal staining and showed no non-specific binding. We conclude that this reagent is suitable for the subcellular localization of the active conformation of cathepsin D.

We have proposed (Knight *et al.*, 1980) that tight-binding, or irreversible, active-site-directed inhibitors carrying reporter groups can be used to determine the subcellular location of proteinases that may be active under the conditions existing in living tissues. Briefly, the method employs an immunotrapping technique (Poole *et al.*, 1973, 1974) to immobilize the proteinase as insoluble immune complexes at the sites of release in the living tissue. These complexes are then treated *in situ* with the

labelled inhibitor. Since active-site-directed inhibitors require the native enzyme structure for efficient interaction (Knight, 1977), the binding of the inhibitor, assuming no non-specific interactions, demonstrates the presence of enzyme in the active conformation in the immune complex.

As a model system we studied the interactions between cathepsin D (EC 3.4.23.5) and derivatives of the tight-binding inhibitor pepstatin {*N*-[(3-methyl-1-oxobutyl)-*L*-valyl-*L*-valyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoyl-*L*-alanyl]-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid}. Pepstatin was modified to carry a dinitrophenyl (Dnp) group (Knight *et al.*, 1980), the photostable fluorophore bimane (Matthews *et al.*, 1981*a*) or a biotin group (Matthews *et al.*, 1981*b*). These pepstatin derivatives bound specifically to cathepsin D in the active conformation, and the bimane- and biotin-labelled inhibitors were used to demonstrate the subcellular location of cathepsin D

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

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in cultured human synovial cells (Matthews *et al.*, 1981a,b).

Because the cathepsin D–pepstatin interaction is stoichiometric (Knight & Barrett, 1976), it is probable that the subcellular location of the enzyme via the binding of a multiply labelled fluorescent protein will be a more sensitive method of detection than the direct localization of a fluorescent inhibitor. However, the pepstatin–biotin–avidin system (Matthews *et al.*, 1981b) may not always be suitable for the localization of cathepsin D, since avidin (pI 10.5) exhibits non-specific binding in cellular systems (Finn *et al.*, 1980) and endogenous avidin-binding activity has been observed in tissues rich in biotin (Wood & Warnke, 1981). In localization studies using labelled avidin, non-specific staining can be decreased, but not abolished, by chemical modification of the avidin lysine residues (Guesdon *et al.*, 1979; Finn *et al.*, 1980), or, alternatively, by pretreatment of the tissue with unlabelled avidin and biotin (Wood & Warnke, 1981).

We have suggested (Knight *et al.*, 1980) that cathepsin D-bound Dnp-pepstatis can be located via the binding of high-affinity antibody to the Dnp group. In these antibody preparations most of the antibody species bind the hapten, *N*⁶-Dnp-lysine, with apparent dissociation constants less than 10^{-10} M (cf. Knight & Green, 1976). In contrast with avidin, such anti-Dnp antibody shows negligible non-specific binding to cultured human synovial cells. We report below the subcellular distribution of cathepsin D in these cells with Dnp-labelled pepstatis and a fluorescein-labelled antibody to Dnp.

Materials and methods

Pepstatin was from Protein Research Foundation, Osaka, Japan. *N*-Pepstatinyl-*N'*-2,4-dinitro[3,5-³H]phenyl-1,6-diaminohexane was made as described previously (Knight *et al.*, 1980). *NN'*-Bis(3-aminopropyl)piperazine and 3-oxa-1,5-diaminopentane dihydrochloride were from Aldrich Chemical Co., Gillingham, Dorset, U.K. 3,6-Dioxo-1,8-diamino-octane was kindly given by Professor J.-M. Lehn, Université Louis Pasteur, Strasbourg, France. Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (Castro *et al.*, 1976) was kindly given by Dr. G. Evin, INSERM Unité 36, Paris, France. 1-Fluoro-2,4-dinitro[3,5-³H]benzene was from Amersham International, Amersham, Bucks., U.K. Cathepsin D from human liver (specific activity 440 units/mg) was kindly given by Dr. A. J. Barrett of this laboratory. Concentrations of active enzyme were determined by titration with pepstatin (Knight & Barrett, 1976). Antisera to human cathepsin D raised in sheep were kindly given by Ms. R. M. Hembry of this

laboratory. All other reagents were obtained from commercial sources and were of the purest grades available. Sephadex LH-20 was from Pharmacia, Hounslow, Middx., U.K. Ultragel AcA 44 was from LKB Instruments, South Croydon, Surrey, U.K. AG50W-X2 resin (H⁺ form; 200–400 mesh) was from Bio-Rad Laboratories, Bromley, Kent, U.K. Silica-gel sheets (13181) for t.l.c. were from Kodak, Kirkby, Liverpool, U.K. Silica-gel plates for preparative-layer chromatography (Merck 5717) were from BDH Chemicals, Poole, Dorset, U.K.

Pepstatin [³H]methyl ester

Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide (Vogel, 1956) and ³H-labelled by exchange with ³H₂O at 0°C (Markey & Shaw, 1978). ³H₂O (0.2 ml, 37 GBq) was made alkaline with 5 M-NaOH (40 µl), and diazomethane in diethyl ether (2 ml, approx. 10 mg/ml) was added. After 25 min at 0°C the ether phase was added to pepstatin (10 mg, 14.5 µmol) in dimethyl sulphoxide (0.3 ml) and allowed to react for 24 h. The solvents were removed *in vacuo* and pepstatin [³H]methyl ester was isolated by chromatography on Sephadex LH-20 (Knight & Barrett, 1976). The product (10 mg) had m.p. 249–250°C (cf. 249–251°C; Umezawa *et al.*, 1970) and was radiochemically homogeneous on t.l.c. (*R*_F 0.5, chloroform/methanol/acetic acid, 95:4:1, by vol.). The specific radioactivity of pepstatin [³H]methyl ester was 1.1 MBq/µmol.

NN'-(3-Pepstatinylaminopropyl-3'-aminopropyl)piperazine

Pepstatin (10 mg, 14.5 µmol) was dissolved in dimethyl sulphoxide (0.3 ml) and dimethylformamide (0.25 ml) at 0°C. Triethylamine (2 µl, 14.4 µmol) and isobutyl chloroformate (2 µl, 15.4 µmol) were added. After 25 min at 0°C, *NN'*-bis(3-aminopropyl)piperazine (15 µl, 73 µmol) was added and the reaction mixture was allowed to warm to 21°C overnight. The solvents were removed *in vacuo* and *NN'*-(3-pepstatinylaminopropyl-3'-aminopropyl)piperazine was purified on Sephadex LH-20 and Bio-Rad AG50W-X2 resin (Knight & Barrett, 1976). The product (yield: 2.3 mg, 18%) was homogeneous by t.l.c. on silica gel (*R*_F 0.2; methanol/chloroform, 19:1, v/v).

NN'-(3-Pepstatinylaminopropyl-3'-dinitro[3,5-³H]-phenylaminopropyl)piperazine

NN'-(3-Pepstatinylaminopropyl-3'-aminopropyl)piperazine (2.3 mg, 2.6 µmol) in dimethyl sulphoxide (0.3 ml) was allowed to react in darkness with 1-fluoro-2,4-dinitro[3,5-³H]benzene (37 MBq, 0.042 µmol) for 24 h at 21°C before addition of the unlabelled reagent (5 µl, 40 µmol). After 24 h the solvent was removed at 40°C *in vacuo*. The residue

was dissolved in ethanol (1 ml) and applied to a column (1 cm $\times$ 60 cm) of Sephadex LH-20 and eluted with ethanol (12 ml/h). Fractions (1 ml) corresponding to the first yellow band eluted from the column were combined (8 ml) and evaporated. The residue was dissolved in ethanol (1 ml) and rechromatographed on Sephadex LH-20. The product from the second column was dissolved in methanol (0.25 ml) and applied to a silica-gel preparative-layer chromatography plate, which was developed with chloroform/methanol (9:1, v/v). The major yellow band was scraped off and eluted with methanol (2 ml). After evaporation the product was freed from silica by dissolving in chloroform. *NN'*-(3-Pepstatinylaminopropyl-3'-[^3H]Dnp-aminopropyl)-piperazine (yield: 0.42 mg, 15%) showed one component on t.l.c. on silica gel (R_F 0.7; chloroform/methanol, 4:1, v/v), which contained 97% of the radioactivity. The specific radioactivity of this product was 6.0 MBq/ μmol .

N-Pepstatinyl-N'-dinitro[3,5- ^3H]phenyl-3-oxa-1,5-diaminopentane

The methods described above were used to prepare *N*-pepstatinyl-*N'*-[^3H]Dnp-3-oxa-1,5-diaminopentane. Briefly, *N*-pepstatinyl-3-oxa-1,5-diaminopentane was made by the mixed-anhydride procedure and purified on Sephadex LH-20 and Bio-Rad AG50W-X2 resin. This compound was treated with 1-fluoro-2,4-dinitro[3,5- ^3H]benzene, followed by an excess of unlabelled reagent. The product was isolated by two chromatographic runs on Sephadex LH-20 and preparative-layer chromatography on silica gel. *N*-Pepstatinyl-*N'*-[^3H]Dnp-3-oxa-1,5-diaminopentane showed one component on silica gel (R_F 0.7, chloroform/methanol, 9:1, v/v), which contained 98% of the radioactivity. The specific radioactivity of this product was 6.0 MBq/ μmol .

N-Pepstatinyl-3,6-dioxa-1,8-diamino-octane

3,6-Dioxa-1,8-diamino-octane (5 μl , 43 μmol) and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (19 mg, 43 μmol) were dissolved in dimethylformamide (1 ml) at 21°C. Pepstatin (10 mg, 14.5 μmol) and triethylamine (8 μl , 57 μmol) were added and the mixture was stirred for 72 h. After treatment with water (0.1 ml) the solvent was filtered and evaporated to dryness. *N*-Pepstatinyl-3,6-dioxa-1,8-diamino-octane was purified on Sephadex LH-20 and Bio-Rad AG50W-X2 resin (Knight & Barrett, 1976). The product (yield: 3.5 mg, 29%) was homogeneous by t.l.c. on silica gel (R_F 0.5, methanol).

N-Pepstatinyl-N'-dinitro[3,5- ^3H]phenyl-3,6-dioxa-1,8-diamino-octane

1-Fluoro-2,4-dinitro[3,5- ^3H]benzene (37 MBq, 0.042 μmol) in benzene (1 ml) was made to react

with *N*-pepstatinyl-3,6-dioxa-1,8-diamino-octane (1 mg, 1.22 μmol) in dimethyl sulphoxide (0.1 ml) for 24 h in darkness. 1-Fluoro-2,4-dinitrobenzene (5 μl , 40 μmol) was added and the mixture was left for a further 24 h. The solvents were removed *in vacuo* and the residue was dissolved in ethanol (0.5 ml) and applied to a column (1 cm $\times$ 60 cm) of Sephadex LH-20 that was eluted with ethanol (12 ml/h). A portion (1 μl) of each fraction (1 ml) was taken for the counting of radioactivity and a further portion (1 μl) for the assay of cathepsin D inhibition (Knight & Barrett, 1976). The leading inhibitory fractions that contained radioactivity were pooled (6 ml) and evaporated. The residue was dissolved in ethanol (0.5 ml) and rechromatographed on Sephadex LH-20. The leading fractions containing the radioactive inhibitor were combined (4 ml) and evaporated. *N*-Pepstatinyl-*N'*-[^3H]Dnp-3,6-dioxa-1,8-diamino-octane (yield: 20 μg , 0.2%) showed one component on t.l.c. on silica gel (R_F 0.85; chloroform/methanol, 9:1, v/v), which contained 96% of the radioactivity. The specific radioactivity of this product was 4.1 MBq/ μmol .

Anti-Dnp antibody

Fluorescein-labelled univalent (Fab) fragments of rabbit antibody to Dnp were prepared as described previously (Knight *et al.*, 1980). Bimane-labelled Fab fragments were also made (Matthews, 1982) and were shown by absorbance measurements at 280 nm (McGuigan & Eisen, 1968) and 385 nm (Kosower *et al.*, 1979) to carry 2.9 bimane groups/molecule.

Binding of pepstatin derivatives to immunoprecipitates containing cathepsin D

Cathepsin D (0.2 ml, 0.054 nmol) was mixed with sheep antiserum to human cathepsin D (0.2 ml) in immunoprecipitin tubes. The equivalent volumes of antigen and antibody solutions had been determined by double immunodiffusion (Ouchterlony, 1958). The tubes were left for 30 min at 21°C and then for 24 h at 4°C. After centrifugation at 10 000 *g* for 3 min the immunoprecipitates were washed twice by resuspension in the appropriate buffer (0.2 ml) and re-centrifugation. Experiments were done in duplicate in 0.05 M-sodium acetate/acetic acid, pH 5.0, and in 0.05 M- $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 7.4. Both buffers contained 0.1 M-NaCl and 0.1% Brij 35. The ^3H -labelled pepstatin derivative (0.2 ml, approx. 1 μM) was added to the immunoprecipitate and, after vigorous shaking, the mixture was left at 4°C for 24 h with occasional shaking. After centrifugation at 10 000 *g* for 3 min the precipitate was washed three times by resuspension in buffer (0.2 ml) for approx. 1 min and centrifugation. The final precipitate was dissolved in 30% (v/v) acetic acid (0.2 ml) and a

portion (0.1 ml) was taken for scintillation counting (Knight *et al.*, 1980).

Inhibition titrations

Cathepsin D activity was measured at 45°C in 0.25 M-sodium formate buffer, pH 3.5, as described by Barrett (1970). 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 specified conditions, a linear response being assumed. The assays were made in duplicate and were started by the addition of buffered haemoglobin to a mixture of cathepsin D (approx. 1 unit, 0.027 nmol/tube) and Dnp-pepstatin (0–0.04 nmol/tube). After 20 min the trichloroacetic acid-soluble products of digestion of haemoglobin were measured by their A_{280} .

Gel filtration of complexes on Ultragel Aca 44

Gel-filtration experiments were performed at pH 5.0 and pH 7.4 with a column (1.5 cm × 78 cm, 138 ml) of Ultragel Aca 44 eluted with the appropriate buffer at 4°C. The buffers were 0.05 M-acetate (pH 5.0)/0.1 M-NaCl/0.1% Brij 35 and 0.05 M-phosphate (pH 7.4)/0.1 M-NaCl/0.1% Brij 35. The flow rate was 12 ml/h and fractions (0.9 ml) were collected. The elution characteristics of the column were determined with the following standards: Blue Dextran 2000; rabbit IgG (mol.wt. 150 000); avidin (68 000); cytochrome *c* (11 700); *N*⁶-Dnp-lysine hydrochloride (349). Various mixtures of cathepsin D (50 units, 1.34 nmol), pepstatin [³H]Dnp derivative (0.35 nmol) and bimanane-labelled fragments of anti-Dnp antibody (2.5 nmol), as described in the Results section, were prepared in elution buffer (approx. 0.9 ml) and left at 21°C for at least 30 min before being applied to the column. Portions (0.1 ml) of each fraction were taken for the assay of cathepsin D activity and for scintillation counting. The remainder was diluted with water to 3 ml and bimanane fluorescence was measured with a Farrand mark 1 spectrofluorimeter (excitation wavelength, 380 nm; emission wavelength, 460 nm).

Human synovial-cell cultures

Confluent cultures of human synovial cells were grown on glass coverslips as previously described (Matthews *et al.*, 1981a).

Staining of cathepsin D with Dnp-pepstatins

Human synovial-cell cultures were rinsed three times in serum-free Dulbecco's modified Eagle's medium. The cultures were divided into two groups. Fixation and staining procedures were made with the first group at pH 5.0 in 0.05 M-acetate buffer and with the second group at pH 7.4 in 0.05 M-phosphate buffer. Both buffers contained 0.1 M-NaCl and 0.1% Brij 35. The cells were fixed for 3 min at 21°C in paraformaldehyde (4%, w/v) and then washed for

30 min in three changes of buffer at the same pH value.

After rinsing, the coverslips were immersed in Dnp-pepstatin (1 μ M) at either pH 5.0 or pH 7.4 and incubated in a moist atmosphere for 30 min at 21°C. The cells were briefly rinsed and then immersed in fluorescein-labelled fragments of antibody to Dnp (0.2 mg/ml) at the appropriate pH value. After 30 min at 21°C the cells were quickly rinsed in the buffer alone and mounted in glycerol.

The subcellular location of cathepsin D in human synovial cells was also determined by the immunohistochemical method described by Matthews *et al.* (1981a).

Fluorescence microscopy

The cells were examined in a Zeiss fluorescence microscope equipped with fluorescein optics. All observations were recorded on Kodak Tri-X, or on Ektachrome 400 film that was uprated at ASA 1600.

Results

Binding of [³H]Dnp-pepstatins to immunoprecipitated cathepsin D

Minimal non-specific binding is a necessary prerequisite for the application of the Dnp-pepstatins to the subcellular location of cathepsin D. Since we have proposed the application of these reagents to the detection of cathepsin D in extracellular immunoprecipitates (Knight *et al.*, 1980), we measured the binding of [³H]Dnp-pepstatins to immune complexes containing cathepsin D. Experiments were made *in vitro* at pH 5.0 and at pH 7.4. At the higher pH value, cathepsin D is inactive and no longer binds pepstatin derivatives tightly (Knight & Barrett, 1976; Matthews *et al.*, 1981a).

When immunoprecipitates formed with human cathepsin D (2 units, 54 pmol) and a sheep antiserum to human cathepsin D were treated with an excess of pepstatin [³H]methyl ester (0.2 ml, 14 μ M) at pH 5.0, some ester (23 pmol) remained bound to the precipitate after three washes with buffer. In similar experiments at pH 7.4, less than 1 pmol of inhibitor remained in the precipitate after washing. When binding experiments were made with *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane (0.2 ml, 1.2 μ M), 32 pmol of inhibitor remained in the immunoprecipitates at pH 5.0 and 21 pmol at pH 7.4. We concluded that there was a strong non-specific interaction of *N*-pepstatinyl-*N'*-[³H]Dnp-1,6-diaminohexane with the immunoprecipitates, probably due to the hydrophobic nature of the inhibitor (cf. Knight *et al.*, 1980).

More hydrophilic [³H]Dnp-pepstatins were synthesized by using *NN'*-bis-(3-aminopropyl)piperazine, 3-oxa-1,5-diaminopentane or 3,6-dioxo-1,8-di-

amino-octane as spacer arms between the inhibitor and Dnp moieties. In binding experiments with immunoprecipitates these derivatives had greatly decreased non-specific binding. For example, *NN'*-(3-pepstatiny laminopropyl-3'-[³H]Dnp-aminopropyl)piperazine at pH 7.4 displayed approx. 5% of the binding seen at pH 5.0, and the binding of *N*-pepstatiny-*N'*-[³H]Dnp-3-oxa-1,5-diaminopentane could not be demonstrated at the higher pH value.

We observed previously (Knight *et al.*, 1980) that *N*-pepstatiny-*N'*-[³H]Dnp-1,6-diaminohexane behaved anomalously during chromatography on Sephadex G-100 at pH 5.0, the radioactivity being eluted from the column at the position of serum albumin. The eluting buffer contained 0.1% Brij 35 to ensure recovery of cathepsin D activity from the column (Barrett, 1970), and we concluded that the inhibitor was binding to detergent micelles. When a similar experiment was performed with *N*-pepstatiny-*N'*-[³H]Dnp-3-oxa-1,5-diaminopentane, a rather broad band of radioactivity was eluted from the column, but the most radioactive fractions were found at the elution position of *N*⁶-Dnp-lysine hydrochloride. This experiment confirmed that non-specific hydrophobic interactions were decreased by the use of a hydrophilic spacer arm.

Inhibition of cathepsin D by hydrophilic Dnp-pepstatins

The hydrophilic Dnp-pepstatins behaved like pepstatin and bound tightly and stoichiometrically to human cathepsin D at pH 3.5. The apparent dissociation constants, K_D , were calculated from the deviation from stoichiometric inhibition in the region of the equivalence points (Green & Work, 1953; Knight & Barrett, 1976). In each case 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·mol⁻¹·cm⁻¹ (the values for *N*⁶-Dnp-lysine hydantoin; cf. Knight *et al.*, 1980). The Dnp-pepstatins with *NN'*-bis-(3-aminopropyl)piperazine, 3-oxa-1,5-diaminopentane and 3,6-dioxa-1,8-diamino-octane spacer arms had K_D values of 6.3×10^{-10} M, 3.2×10^{-10} M and 3.0×10^{-10} M respectively.

Gel chromatography of enzyme-inhibitor-antibody complexes on Ultragel Aca 44

Fluorescence-quenching studies (Knight *et al.*, 1980) showed that, as expected for a low-molecular-weight Dnp compound (Velick *et al.*, 1960), the Dnp group of *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane was freely available to bind high-affinity antibody to Dnp. We have assumed that the hydrophilic Dnp-pepstatins behave similarly. However, the application of the Dnp-pepstatins to the localization of cathepsin D requires the Dnp-labelled inhibitors to

be bifunctional reagents, that is, to bind the proteinase and the antibody to Dnp simultaneously.

The formation of enzyme-inhibitor-antibody complexes by the Dnp-pepstatins at pH 5.0 was demonstrated by gel chromatography. Bimane-labelled univalent fragments (mol.wt. 45 000) of antibody to Dnp (Matthews, 1982) and [³H]-Dnp-pepstatins were used. Human cathepsin D has mol.wt. 43 000 and so that ternary complexes will have mol.wt. 89 000 with associated radioactivity and fluorescence. When *NN'*-(3-pepstatiny laminopropyl-3'-[³H]Dnp-aminopropyl)piperazine was chromatographed on Ultragel Aca 44 at pH 5.0 with an excess of cathepsin D and of bimane-labelled antibody fragments (Fig. 1a) the peak of radioactivity [α (partition coefficient) 0.25] was eluted before the antibody (α 0.39) and the cathepsin D activity (α 0.42). Moreover, the radioactive fractions contained some proteinase activity and a small peak of fluorescence, showing that the high-molecular-weight species was indeed the enzyme-inhibitor-antibody complex.

A mixture of cathepsin D, ³H-labelled inhibitor and antibody fragments was also run on the column at pH 7.4. At this pH value no radioactive species of higher molecular weight was observed, and the radioactivity was eluted in the fractions containing the antibody (α 0.38). This behaviour was that expected if, like other pepstatin derivatives (Knight & Barrett, 1976; Matthews *et al.*, 1981a), *NN'*-(3-pepstatiny laminopropyl-3'-[³H]Dnp-aminopropyl)piperazine did not bind tightly to cathepsin D at the higher pH value.

Similar chromatographic experiments were performed with *N*-pepstatiny-*N'*-[³H]Dnp-3-oxa-1,5-diaminopentane (Fig. 2) and *N*-pepstatiny-*N'*-[³H]-Dnp-3,6-dioxa-1,8-diamino-octane (results not shown). In each case the ternary complex was observed at pH 5.0, but was not seen at pH 7.4. These experiments suggested that Dnp-pepstatins with hydrophilic spacer arms, used in conjunction with fluorescent anti-Dnp antibody, should be useful active-site-directed probes for the subcellular location of cathepsin D.

Subcellular location of cathepsin D in human synovial cells

When fixed human synovial cells were treated at pH 5.0 with *NN'*-(3-pepstatiny laminopropyl-3'-Dnp-aminopropyl)piperazine and stained with fluorescein-labelled fragments of antibody to Dnp, a predominantly granular distribution of fluorescence was observed around the nuclei (Fig. 3a). By marked contrast, no staining was seen when the labelling procedures were made at pH 7.4 (Fig. 3b). Perinuclear granules were also revealed by fluorescent antibody to Dnp in cells treated at pH 5.0 with *N*-pepstatiny-*N'*-Dnp-3-oxa-1,5-diaminopentane

Fig. 1. Chromatography on Ultragel AcA 44 of NN' -(3-pepstatinyl-aminopropyl-3'-[3H]Dnp-aminopropyl)piperazine with both cathepsin D and fragments of antibody to Dnp

A column (1.5 cm $\times$ 78 cm, 138 ml) of Ultragel AcA 44 at 4°C was eluted with (a) 0.05 M-sodium acetate buffer, pH 5.0, or with (b) 0.05 M-sodium phosphate buffer, pH 7.4. Both buffers contained 0.1 M-NaCl and 0.1% Brij 35. The flow rate was 12 ml/h and fractions (0.9 ml) were collected. In (a) NN' -(3-pepstatinylaminopropyl-3'-[3H]Dnp-aminopropyl)piperazine (0.35 nmol, 126 000 d.p.m.) was mixed with cathepsin D (50 units, 1.34 nmol) and bimane-labelled fragments of antibody to Dnp (2.5 nmol) in elution buffer at pH 5.0. The mixture was left at 21°C for 30 min before being applied to the column. In (b) the same mixture of inhibitor, enzyme and antibody was prepared at pH 7.4 and was chromatographed at that pH value. Portions (0.1 ml) of each fraction were taken for the counting of radioactivity (■) and for the assay of cathepsin D activity (□). The remainder (0.7 ml) was taken for the measurement of bimane fluorescence (○). The arrows show the elution volumes of the following markers: 1, Blue Dextran 2000; 2, rabbit IgG (mol.wt. 150 000); 3, avidin (68 000); 4, cytochrome c (11 700).

(Fig. 3c) and N -pepstatinyl- N' -Dnp-3,6-dioxa-1,8-diamino-octane (Fig. 3e). These granules were not seen when the experiments were performed at pH 7.4 (Figs. 3d and 3f). Univalent antibodies directed against human cathepsin D revealed a granular distribution of enzyme (Figs. 3g and 3h) that was similar to that seen with the pepstatin probes. We concluded from these results that at pH 5.0 the Dnp-pepstatins were binding selectively to the active conformation of the lysosomal cathepsin D.

Although lysosomal staining by fluorescent anti-Dnp antibody was observed at pH 5.0 with all the Dnp-pepstatins, the intensity of bound fluorescence was greatest with NN' -(3-pepstatinylaminopropyl-3'-Dnp-aminopropyl)piperazine. Moreover, this

derivative stained the lysosomes with minimal non-specific binding (Figs. 3a and 3b). By contrast, both N -pepstatinyl- N' -Dnp-3-oxa-1,5-diaminopentane and N -pepstatinyl- N' -Dnp-3,6-dioxa-1,8-diamino-octane stained the lysosomes less intensely, the former derivative being the least effective. In addition, each of these reagents produced some diffuse cytoplasmic fluorescence. Since this diffuse staining was present at both pH 5.0 (Figs. 3c and 3d) and pH 7.4 (Figs. 3d and 3f), the present observations suggest that these derivatives also bind somewhat non-specifically to human synovial cells.

Although the pepstatin probes clearly revealed the distribution of cathepsin D, the immunohistochemical method gave the most intense fluorescent

Fig. 2. Chromatography on Ultragel Aca 44 of *N*-pepstatiny-*N'*-[^3H]Dnp-3-oxa-1,5-diaminopentane with both cathepsin D and fragments of antibody to Dnp

Chromatography was carried out on a calibrated column of Ultragel Aca 44 as described in the legend to Fig. 1. In (a) a mixture of *N*-pepstatiny-*N'*-[^3H]Dnp-3-oxa-1,5-diaminopentane (0.35 nmol, 126 000 d.p.m.), cathepsin D (50 units, 1.34 nmol) and bimeane-labelled fragments of antibody to Dnp was chromatographed at pH 5.0. In (b) the mixture of inhibitor, enzyme and antibody was made and eluted from the column at pH 7.4. In each experiment the fractions were assayed for radioactivity (■), cathepsin D (□) and fluorescence (○), as described in the legend to Fig. 1.

staining pattern (compare Fig. 3g with Figs. 3a, 3c and 3e). Brighter staining was seen with this method because univalent fragments (Fab) of a sheep immunoglobulin G directed against human cathepsin D were revealed by the multiple binding of fluorescent pig Fab fragments directed against the sheep Fab fragment, yielding a fluorescence amplification not matched by our pepstatin probes.

Discussion

In order to function as active-site-directed localization reagents for cathepsin D, Dnp-labelled pepstatins must bind only to the active enzyme and must also bind to fluorescent anti-Dnp antibody at the same time. We had shown (Knight *et al.*, 1980) that to obtain such a bifunctional inhibitor it was necessary to separate the Dnp and pepstatin moieties by a chain of six carbon atoms. At shorter distances the binding was weakened by protein-protein interactions.

However, in the present study we found that the bifunctional reagent *N*-pepstatiny-*N'*-Dnp-1,6-diaminohexane binds at pH 7.4 to immunoprecipitates containing inactive cathepsin D. Since pepstatin methyl ester did not bind under these conditions, it seemed probable that the hydrophobic nature of the Dnp-aminoethyl moiety (cf. Knight *et al.*, 1980) was responsible for this non-specific interaction. We therefore synthesized some new Dnp-pepstatins with hydrophilic spacer arms and demonstrated that these derivatives had minimal non-specific interactions with immunoprecipitates.

The hydrophilic Dnp-pepstatins were all tight-binding inhibitors of cathepsin D at pH 3.5, with apparent dissociation-constant values of approx. 4×10^{-10} M. These values were close to those obtained with pepstatin (Knight & Barrett, 1976) and the Dnp-aminoalkyl-pepstatins (Knight *et al.*, 1980). Previous work with *N*-pepstatiny-diaminoethane (Knight & Barrett, 1976) had suggested that cationic derivatives of pepstatin were weaker inhibi-

Fig. 3. Localization of cathepsin D in human synovial cells in culture

Details of the cell culture and staining methods are given in the Materials and methods section. (a) Cells treated at pH 5.0 with *NN'*-(3-pepstatinylaminopropyl-3'-Dnp-aminopropyl)piperazine and fluorescein-labelled anti-Dnp Fab fragments showed a bright perinuclear array of granules, which were not seen at pH 7.4 (b). (c) Although perinuclear granules were stained by *N*-pepstatinyl-*N'*-Dnp-3-oxa-1,5-diaminopentane only at pH 5.0, the staining was much less intense than in (a) and some diffuse non-specific staining was seen at pH 7.4 (d). (e) The perinuclear granules were clearly revealed by *N*-pepstatinyl-*N'*-Dnp-3,6-dioxa-1,8-diamino-octane at pH 5.0, but some diffuse non-specific staining was seen at pH 7.4 (f). (g) Cells treated at pH 7.4 with monospecific sheep Fab fragment directed against human cathepsin D, followed by a fluorescein-labelled pig Fab fragment directed against sheep Fab fragment. This indirect immunohistochemical method revealed a similar pattern of numerous intensely fluorescent granules around the nuclei (cf. a, e). (h) When normal sheep Fab fragment was used in the immunohistochemical procedure, the cells were unstained.

tors of cathepsin D, but this was not the case with *NN'*-(3-pepstatinylaminopropyl-3'-Dnp-aminopropyl)piperazine.

The interactions of the hydrophilic Dnp-pepstatis with cathepsin D and anti-Dnp Fab were characterized by gel-chromatographic studies with ³H-labelled inhibitors. In each case at pH 5.0 the radioactivity was eluted from the column before the excess of proteinase and antibody, whereas at pH 7.4 the radioactivity was eluted in the fractions that contained the antibody (Figs. 1 and 2). These results clearly demonstrated that the bifunctional nature of the Dnp-pepstatis was expressed only when the cathepsin D was in the native active conformation.

The elution characteristics of the ternary complexes at pH 5.0 were dependent on the length of the hydrophilic spacer arm. Thus the cathepsin D and anti-Dnp Fab associated with *NN'*-(3-pepstatinylaminopropyl-3'-[³H]Dnp-aminopropyl)piperazine were eluted from the column clearly resolved from the excess of enzyme and antibody (Fig. 1a). With *N*-pepstatinyl-*N'*-[³H]Dnp-3-oxa-1,5-diaminopentane (Fig. 2a), however, the radioactivity was eluted over a larger volume, and the associated enzyme and antibody were seen as shoulders on the leading edges of the main peaks. It is probable that this elution pattern reflects steric hindrance in the enzyme-inhibitor-antibody complex. We demonstrated previously (Knight *et al.*, 1980), by using double immunodiffusion in agarose gels, that decreasing the chain length of the Dnp-aminoalkyl-pepstatis from six to four carbon atoms increased the dissociation of the ternary complex. The nitrogen atoms of the 3-oxa-1,5-diaminopentane spacer arm are separated by approx. 0.72 nm, compared with 0.87 nm for 1,6-diaminohexane and 0.62 nm for 1,4-diaminobutane.

A decrease in the stability of the ternary complex due to steric interactions between cathepsin D and anti-Dnp Fab fragment probably accounts for the much weaker staining of human synovial-cell lysosomes by *N*-pepstatinyl-*N'*-Dnp-3-oxa-1,5-diaminopentane (Fig. 3c). For example, the binding of *N*-pepstatinyl-*N'*-biotinyl-1,2-diaminoethane to cathepsin D (Matthews *et al.*, 1981b) showed a marked dissociation in the presence of avidin, and the value of the apparent dissociation constant increased from 0.36 nM to 2.3 nM. This compound, when used in conjunction with tetramethylrhodamine-labelled avidin, failed to stain human synovial-cell lysosomes (Matthews *et al.*, 1981b).

NN'-(3-Pepstatinylaminopropyl-3'-Dnp-aminopropyl)piperazine was the most satisfactory localization reagent for the lysosomal cathepsin D. Bright staining of the enzyme was seen at pH 5.0 (Fig. 3a), and little staining was seen at pH 7.4 (Fig. 3b). *N*-Pepstatinyl-*N'*-Dnp-3,6-dioxo-1,8-diamino-octane gave less intense staining at pH 5.0 (Fig. 3e), and

non-specific staining was seen at pH 7.4 (Fig. 3f). An additional disadvantage of the latter compound was the very poor synthetic yield.

In conclusion, *NN'*-(3-pepstatinylaminopropyl-3'-Dnp-aminopropyl)piperazine represents a significant improvement over our previous labelled pepstatin probes (Matthews *et al.*, 1981a,b), for it gives a more intense fluorescent staining of the lysosomal cathepsin D with minimal non-specific staining.

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