The inactivation of the cysteinyl exopeptidases cathepsin H and C by affinity-labelling reagents

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An attempt has been made to extend to the cysteinyl exopeptidases cathepsins H and C affinity-labelling approaches shown to be effective with cysteinyl endopeptidases such as cathepsins B and L and the calcium-activated proteinase. This involved the preparation of amino acid and dipeptide derivatives with unblocked *N*-termini to satisfy the aminopeptidase and dipeptidyl aminopeptidase characteristics of cathepsins H and C respectively. For covalent reactivity, the possibilities examined included diazomethanes ($-\text{CHN}_2$), fluoromethanes ($-\text{CH}_2\text{F}$) and dimethylsulphonium salt [$-\text{CH}_2\text{S}^+(\text{CH}_3)_2$]. A dipeptidylfluoromethane with a free amino group could not be prepared, perhaps due to inherent instability. Cathepsin H was inactivated by $1\ \mu\text{M}$ - H_2N -Phe- CH_2F (the ' H_2N ' indicates a free unblocked amino group) ($k_2 = 1878\ \text{M}^{-1}\cdot\text{s}^{-1}$); this reagent was without effect on cathepsins C and B, even at 100-fold this concentration. Analogous selectivity was shown by H_2N -Ser(OBzl)- CHN_2 and H_2N -Phe- $\text{CH}_2\text{S}^+(\text{CH}_3)_2$, members of other classes of covalently binding reagents. For cathepsin C the dipeptide derivatives H_2N -Gly-Phe- CHN_2 and H_2N -Phe-Ala- $\text{CH}_2\text{S}^+(\text{CH}_3)_2$ caused rapid inactivation near $10^{-7}\ \text{M}$. Higher concentrations inactivated cathepsins H and B, but the rates were slower by two to three orders of magnitude than for cathepsin C.

INTRODUCTION

The development of reagents which inactivate individual cysteinyl proteinases such as cathepsins B, L and the calcium-activated proteinases *in vitro* and within cells seem within reach (Crawford *et al.*, 1988; Kirschke *et al.*, 1988; Mason *et al.*, 1989) and should be helpful in clarifying the biochemical role of these proteinases. A number of exopeptidases are also prominent constituents, at least of the lysosomes, and their contribution to protein turnover remains obscure. Among these are

cathepsins H and C. Cathepsin H has both endo- and exo-peptidase activity (Kirschke *et al.*, 1977a) and in the former capacity acts as an aminopeptidase. Cathepsin C, on the other hand, seems to be limited to removing dipeptides from the *N*-terminus of a protein (McDonald *et al.*, 1969) and is also known as dipeptidyl aminopeptidase I. To derive useful reagents for the inactivation of these proteinases, hopefully with some selectivity, we have attempted to extend previous work with several chemical groupings shown to be useful for affinity-labelling endopeptidases. These are alkylating functions joined to the *C*-terminal residue of amino acids and peptides, resulting in substituted ketones. However, in addition, we have prepared a peptide analogue that contains a sulphonium group instead of a free *N*-terminus (Scheme 1) to explore possible interactions with aminopeptidases.

Scheme 1. Synthesis of a peptide analogue containing a sulphonium group

EXPERIMENTAL

Materials

Cathepsin C was purchased from Boehringer (Mannheim, Germany); cathepsin H (Kirschke *et al.*, 1977a) and L (Kirschke *et al.*, 1977b) were prepared from rat liver, and cathepsin B from pig liver (Evans & Shaw, 1981).

Abbreviations used: the symbols for substituent or protective groups common in peptide chemistry are largely given in Nomenclature and Symbolism for Amino Acids and Peptides (1984); other abbreviations: $-\text{CHN}_2$, diazomethane; $-\text{CH}_2\text{F}$, fluoromethane; $-\text{CH}_2\text{S}^+(\text{CH}_3)_2$, dimethylsulphonium salt; NMec, methylcoumarylamide; TFA, trifluoroacetic acid; $-\text{CH}_2\text{Cl}$, chloromethane; NMM, *N*-methylmorpholine; ' H_2N ' at the extreme left of the peptides indicates that the amino acid residue to its immediate right has a free unblocked amino group and does not in this specific instance indicate an *extra* amino group.

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Methods

Cathepsin C was studied in 0.05 M-Mes, pH 6.0, containing 40 μ l of mercaptoethanol/50 ml, using H₂N-Gly-Phe-NMec as substrate at 5×10^{-5} M and a modification of the method of McDonald *et al.* (1969). Before use the enzyme was activated at room temperature in the same buffer without substrate. Cathepsin H was measured in 0.05 M-phosphate buffer, pH 6.8, 1 mM in EDTA and containing cysteine base (0.5 g/100 ml, freshly added), with the use of H₂N-Tyr-NMec as substrate at 6×10^{-5} M (Barrett & Kirschke, 1981). The stored mercury salt was activated in the same buffer at room temperature before use. Cathepsin B was also measured fluorimetrically with Cbz-Phe-Arg-NMec at pH 5.4 (Barrett & Kirschke, 1981).

Incubation mixtures of inhibitor and enzyme in buffer at room temperature were prepared, and samples were removed at timed intervals for assay at 37 °C. From logarithmic plots a typical first-order loss of activity was observed and the half-time for inactivation was determined. Observations were made in duplicate and results obtained were within $\pm 10\%$ of the values reported in the Tables.

Syntheses

Cbz-Phe-Ala-CH₂F (Rasnick, 1985) and Cbz-Phe-Phe-CH₂F (Raubert *et al.*, 1986) were prepared by the literature procedures.

H₂N-Phe-CH₂F TFA. Boc-Phe-CH₂F (8.1 mg, 28.8 μ mol) (Raubert *et al.*, 1986) was deprotected in TFA (0.5 ml). After the mixture had been left for 1 h at room temperature the solvent was evaporated, yielding an orange oil (8.1 mg, 95%) which, after t.l.c. in 20% (v/v) methanol in dichloromethane, gave a single ninhydrin-reactive spot of R_F 0.2.

H₂N-Ser(OBzl)-CHN₂. Cbz-Phe-Ser(OBzl)-CHN₂ (Shaw *et al.*, 1983), 1 mM in 0.1 M-Pipes, pH 7.0, 10% (v/v) in acetonitrile and 0.1% in NaN₃ was cleaved with immobilized chymotrypsin (Green & Shaw, 1981) at 37 °C. The reaction was monitored by h.p.l.c. and was largely complete after 2 h, but was allowed to proceed overnight, after which the supernatant solution was collected and stored at -20 °C.

H₂N-Gly-Phe-CHN₂. Fmoc-Gly-Phe was converted into the diazomethane by way of the intermediate mixed anhydride (Green & Shaw, 1981). The neutral residue eventually crystallized and was thinned with ethyl acetate and hexane and filtered. Recrystallization from the same solvent mixture provided the compound in 50% yield, m.p. 143–144 °C (Found: C, 69.23; H, 5.31; N, 11.82; C₂₇H₂₄N₄O₄ requires C, 69.22; H, 5.16; N, 11.92%). The Fmoc group was removed with piperidine and the product was isolated as described for Leu-Leu-Tyr-CHN₂ (Crawford *et al.*, 1988). The expected M_r , 347, was confirmed by fast-atom-bombardment analysis.

H₃N⁺-Phe-CH₂S⁺(CH₃)₂ 2TFA[−]. Boc-Phe-OH was converted into the diazomethane through the mixed anhydride (Green & Shaw, 1981). The recrystallized (ethyl acetate and hexane) product, m.p. 87–87.5 °C, was obtained in 67% yield (Found: C, 62.17; H, 6.72; N, 14.65; C₁₅H₁₉N₃O₃ requires C, 62.27; H, 6.62; N,

14.53%). The chloromethane was obtained by treating the diazomethane (1.95 g, 6.74 mmol) in ethyl acetate (20 ml) for 30 s at -20 °C with precooled 3 M-HCl in ethyl acetate (5 ml). The reaction mixture was poured over crushed ice and quickly neutralized with aq. NaHCO₃. The neutral fraction was isolated in the usual way and provided 1.85 g (92.2% yield), m.p. 104–105 °C, unchanged on recrystallization from ethyl acetate and hexane (Found: C, 60.59; H, 6.85; N, 4.74; C₁₅H₂₀NO₃Cl requires C, 60.50; H, 6.77; N, 4.70%). Boc-Phe-CH₂Cl so obtained (1.7 g, 5.71 mmol) was converted into Boc-Phe-CH₂SCH₃ as described by Shaw (1988), yielding 1 g (56.6% yield), m.p. 85–86 °C, after crystallization from ethyl acetate and hexane (Found: C, 61.80; H, 7.49; N, 4.59; S, 9.98; C₁₆H₂₃NO₃S requires C, 62.11; H, 7.49; N, 4.53; S, 10.36%).

Dimethylsulphonium salt formation was carried out as for the dipeptide, and the product was purified by adsorption to silica gel when applied as a chloroform solution. After extensive washing with chloroform, the sulphonium salt was eluted with 95% ethanol. It showed a single spot on chromatography on reverse-phase plates in aq. 40% ethanol (R_F = 0.4). It was deblocked in TFA as described for the dipeptide derivative.

H₃N⁺-Phe-Ala-CH₂S⁺(CH₃)₂ 2TFA[−]. Boc-Phe-Ala-CH₂Cl was prepared as described above for Boc-Phe-CH₂Cl and converted into the methylthio ether by reaction with small portions of NaSCH₃ in methanol in the presence of phenolphthalein (Shaw, 1988). On recrystallization of the neutral product from ethyl acetate and hexane there was obtained a 50% yield, m.p. 134–135 °C (Found: C, 60.00; H, 7.41; N, 7.42; C₁₉H₂₈N₂O₄S requires C, 59.34; H, 7.42; N, 7.36%).

The dimethylsulphonium salt was obtained from the sulphide (0.17 g, 0.447 mmol) by the action of methyl iodide and equivalent AgBF₄ (Shaw, 1988) with stirring for 3 h at room temperature. The soluble portion on removal of the solvent eventually crystallized. Thinning with 100% ethanol and di-isopropyl ether provided 0.16 g (74.2% yield), m.p. 136–137 °C, unchanged by additional recrystallization. (Found: C, 49.30; H, 6.28; N, 6.03; C₂₀H₃₁BF₄N₂O₄S requires C, 49.80; H, 6.48; N, 5.81%). The sulphonium salt (80 mg) in TFA (0.5 ml) dissolved with rapid gaseous evolution. After 3 h at room temperature, the reaction mixture was evaporated to dryness in a stream of nitrogen. The residue was desiccated in the presence of KOH pellets and eventually stored at -20 °C.

Z-Leu-DL-Tyr(FAc)-CH₂F. Z-Leu-Tyr (3.0 g, 7 mmol), fluoroacetic anhydride (3.48 g, 25.2 mmol) and triethylamine (2.93 ml, 21 mmol) were cooled to 0 °C. 4-Dimethylaminopyridine (43 mg, 0.35 mmol) and methylene chloride (3 ml) were added. After 15 min the cooling bath was removed and stirring was continued for 2.5 h. The mixture was diluted with ethyl acetate (150 ml), washed with 1 M-HCl (50 ml), satd. NaHCO₃ (50 ml) and satd. NaCl (50 ml), and then dried over anhydrous MgSO₄, filtered and evaporated. The residual oil was chromatographed on silica gel with chloroform as eluent, and the resultant solid was recrystallized twice from chloroform/hexane to yield a colourless solid (204 mg, 6% yield), m.p. 134–138 °C (Found: C, 61.89; H, 5.98; F, 7.66; N, 5.59; C₂₆H₃₀F₂N₂O₆ requires C, 61.90; H, 5.99; F, 7.52; N, 5.55%). N.m.r. δ (p.p.m.)

($^2\text{H}_6$]dimethyl sulphoxide) 0.78, 0.82 (6H, 2 d, J 7 Hz, 2CH_3), 1.00–1.64 [3H, m, $\text{CHCH}_2\text{CH}(\text{CH}_3)_2$], 2.72–2.96, 3.06–3.22 (2H, 2m, AB-part of the ABX system, $\text{OC}_6\text{H}_4\text{CH}_2\text{CH}$), 3.82–4.02 [1H, m, $\text{CHCH}_2\text{CH}(\text{CH}_3)_2$], 4.44–4.66 (1H, m, X-part of the ABX-system, $\text{OC}_6\text{H}_4\text{CH}_2\text{CH}$), 5.02 (2H, s, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$), 4.90–5.36 (2H, m, d of the AB-system, CH_2F), 5.29 (2H, d, J 45 Hz, $\text{C}_6\text{H}_4\text{O-CO-CH}_2\text{F}$), 7.02–7.14, 7.20–7.31 (4H, 2m, C_6H_4), 7.34 (5H, s, C_6H_5), 7.48 (1H, d, J 7 Hz, NH), 8.48, 8.54 (1H, 2d, J 7 Hz, NH , racemate); field-desorption m.s.: M^+ (the molecular ion), 504 ($\text{C}_{26}\text{H}_{30}\text{F}_2\text{N}_2\text{O}_6$ formula M_r 504.530).

Z-Leu-DL-Tyr-CH₂F. Z-Leu-DL-O-(FAC)Tyr-CH₂F (277 mg, 0.55 mmol) was hydrolysed in acidic 80% methanol [1 M-HCl (2.75 ml), water (2.75 ml) and methanol (22 ml)] for 48 h at room temperature. The reaction mixture was concentrated and was then extracted with ethyl acetate (50 ml). The organic phase was washed with satd. NaHCO_3 (10 ml) and satd. NaCl (10 ml), dried over anhydrous MgSO_4 , filtered and evaporated. The resulting viscous oil was chromatographed over silica gel, with chloroform containing 2% methanol as eluent, to yield a colourless solid foam (190 mg, 78% yield) (Found: C, 64.11; H, 6.53; F, 4.29; N, 6.31; $\text{C}_{24}\text{H}_{29}\text{FN}_2\text{O}_5$ requires C, 64.85; H, 6.57; F, 4.27; N, 6.30%). N.m.r. δ (p.p.m.) ($^2\text{H}_6$]dimethyl sulphoxide) 0.72–0.94 (6H, m, 2CH_3), 1.03–1.64 [3H, m, $\text{CHCH}_2\text{CH}(\text{CH}_3)_2$], 2.60–2.82, 2.88–3.04 (2H, m, AB-part of the ABX-system, $\text{HOC}_6\text{H}_4\text{CH}_2\text{CH}$), 3.86–4.04 [1H, m, $\text{CHCH}_2\text{CH}(\text{CH}_3)_2$], 4.34–4.52 (1H, m, X-part of the ABX-system, $\text{HOC}_6\text{H}_4\text{CH}_2\text{CH}$), 4.76–5.40 (2H, m, d of the AB-system, J 47 Hz, CH_2F), 5.02 (2H, s, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$), 6.57–6.70, 6.90–7.06 (4H, 2m, C_6H_4), 7.34 (5H, s, C_6H_5), 7.43 (1H, d, J 7 Hz, NH), 8.34, 8.39 (1H, 2d, J 7 Hz, NH , racemate), 9.20 (1H, d, J 5 Hz, OH , exchangeable with $^2\text{H}_2\text{O}$); field desorption m.s.: M^+ (the molecular ion), 444 ($\text{C}_{24}\text{H}_{29}\text{FN}_2\text{O}_5$ formula M_r 444.503).

D-2-Bromo-2-phenylacetic acid (D- $\text{C}_6\text{H}_5\text{CH}_2\text{CHBrCO}_2\text{H}$, compound 2, Scheme 1). To D-phenylalanine (16.5 g, 100 mmol) in 24% HBr (250 ml) was added NaNO_2 (13.8 g, 200 mmol) in water (20 ml) at 0 °C for 1.25 h. After 1 h the reaction mixture was allowed to warm up to room temperature. 1 M- NaHSO_3 (100 ml) and ethyl acetate (600 ml) were added. The organic phase was washed with satd. NaCl (200 ml), dried over anhydrous MgSO_4 , filtered and evaporated. The resulting oil was chromatographed on silica gel with hexane/ethyl acetate (4:1, v/v) as eluent to yield a slightly yellowish viscous liquid (11.1 g, 48% yield). N.m.r. and mass determination agreed with the assigned structure.

D-2-Bromo-2-phenylacetyl-Ala-Ala methyl ester (D- $\text{C}_6\text{H}_5\text{CH}_2\text{CHBrCO-Ala-Ala-OCH}_3$, compound 3, Scheme 1). D-2-Bromo-2-phenylacetic acid (5 mmol) was activated by the usual mixed-anhydride procedure and allowed to couple to Ala-Ala-OMe for 1 h at –20 °C and then for 5 h at room temperature, followed by the usual work-up. Purification on a silica-gel column with methylene chloride/2% methanol (49:1, v/v) as eluent gave a colourless solid (1.21 g, 63% yield). N.m.r. and mass determination agreed with the assigned structure.

L-2-Methylthio-2-phenylacetyl-Ala-Ala methyl ester (L- $\text{C}_6\text{H}_5\text{CH}_2\text{CHSCH}_3\text{-CO-Ala-Ala-OCH}_3$, compound 4,

Scheme 1). To D-2-bromo-2-phenylacetyl-Ala-Ala methyl ester (308 mg, 0.8 mmol) in methanol (8 ml) and acetonitrile (2 ml) was added sodium methanethiolate (62 mg, 0.88 mmol) in methanol (1 ml). After stirring for 3 h, the solvents were evaporated. The residue was taken up in ethyl acetate (70 ml), washed with 0.1 M-HCl (20 ml), satd. NaHCO_3 (20 ml) and satd. NaCl (20 ml), dried over anhydrous MgSO_4 , filtered and evaporated. Purification on a silica gel column with methylene chloride/ethyl acetate (3:1, v/v) as eluent yielded, after recrystallization from methylene chloride/hexane, colourless fine needles (186 mg, 66% yield). They had m.p. 134–136 °C (Found: C, 58.10; H, 7.07; N, 8.29; S, 8.99; $\text{C}_{17}\text{H}_{24}\text{N}_2\text{O}_4\text{S}$ requires C, 57.93; H, 6.86; N, 7.95; S, 9.10%) n.m.r. δ (p.p.m.) ($^2\text{H}_6$]dimethyl sulphoxide) 1.20 (3H, d, J 7 Hz, CH_3CH), 1.26 (3H, d, J 7 Hz, CH_3CH), 2.04 (3H, s, SCH_3), 2.94 (2H, AB-part of the ABX system, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 3.60 (1H, t, J 8 Hz, X-part of the ABX system, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 3.62 (3H, s, OCH_3), 4.24 (1H, q, J 7 Hz, CHCH_3), 4.34 (1H, q, J 7 Hz, CHCH_3), 7.14–7.32 (5H, m, C_6H_5), 8.13 (1H, d, J 7 Hz, NH), 8.28 (1H, d, J 7 Hz, NH); field desorption m.s.: M^+ (the molecular ion), 352 ($\text{C}_{17}\text{H}_{24}\text{N}_2\text{O}_4\text{S}$ formula M_r 352.453).

L-2-Dimethylsulphonium-2-phenylacetyl-Ala-Ala methyl ester tetrafluoroborate [L- $\text{C}_6\text{H}_5\text{CH}_2\text{CHS}^+(\text{CH}_3)_2\text{-CO-Ala-Ala-OCH}_3$ BF_4^- , compound 1, Scheme 1]. To D-2-methylthio-2-phenylacetyl-Ala-Ala methyl ester (106 mg, 0.3 mmol) and methyl iodide (93 μl , 1.5 mmol) in methylene chloride (3 ml) was added silver tetrafluoroborate (64 mg, 0.33 mmol). After stirring for 19 h, additional silver tetrafluoroborate (29 mg, 0.15 mmol) was added. After further stirring for 5 h the reaction mixture was diluted with methylene chloride (3 ml). The yellowish precipitate was filtered through Celite. The filtrate was evaporated to yield a colourless oil (59 mg, 43% yield). It had n.m.r. δ (p.p.m.) ($^2\text{H}_6$]dimethyl sulphoxide) 1.27 (3H, d, J 7 Hz, CH_3CH), 1.28 (3H, d, J 7 Hz, CH_3CH), 2.90, 2.92 [6H, 2 s, $\text{S}^+(\text{CH}_3)_2$], 3.34 (2H, AB-part of the ABX-system, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 3.62 (3H, s, OCH_3), 4.26 (1H, q, J 7 Hz, CH_3CH), 4.37 (1H, q, J 7 Hz, CH_3CH), 4.62 (1H, t, J 7 Hz, X-part of the ABX system, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 7.30 (5H, m, C_6H_5), 8.50 (1H, d, J 7 Hz, NH), 8.94 (1H, d, J 7 Hz, NH); positive fast-atom-bombardment m.s.: $(M-\text{BF}_4)^+$, 367; negative fast-atom-bombardment m.s.: $(M+\text{BF}_4)^-$, 541 ($\text{C}_{18}\text{H}_{27}\text{N}_2\text{O}_4\text{S BF}_4$ formula M_r 454.294).

RESULTS AND DISCUSSION

The extension of earlier affinity-labelling methods to aminopeptidases required the synthesis of reagents with exposed α -amino groups in contrast with the reagents prepared previously. The chemical results depended on whether a single amino acid derivative or a dipeptide was involved, as well as the nature of the covalent-bonding portion of the structure. Thus, in the case of fluoromethanes, Boc-Phe- CH_2F could be deblocked without difficulty and provided the desired $\text{H}_2\text{N-Phe-CH}_2\text{F}$ for enzymic evaluation, as described below. On the other hand, attempts to prepare dipeptide derivatives with a free N -terminal amino group were not successful. Cbz-Tyr-Ala- CH_2F and Cbz-Leu-Tyr- CH_2F were synthesized by application of the Dakin-West reaction (Rasnick, 1985) to the tyrosine-containing peptides. The

choice of tyrosine was based on the possible eventual use of the resultant reagents in an iodinated form. This involved formation of intermediate *O*-fluoroacetyl esters during the Dakin-West reaction, and these could be hydrolysed under acidic conditions to the phenol. However, *N*-deblocking procedures did not lead to the desired products. Trifluoroacetic acid at 50 °C, useful for chloromethanes (Coggins *et al.*, 1974) was attempted with both compounds, and liquid HF with one. The results, independent of the procedure used, indicated that an unexpected loss of fluorine was taking place with the formation of a lower-*M_r* product. We reached the conclusion that the desired dipeptidylfluoromethane with an unblocked amino group was not stable. Dipeptidylchloromethanes have been prepared as synthetic intermediates and elongated to tripeptide derivatives (Coggins *et al.*, 1974), but their stability has not been examined. Difficulties in deblocking blocked dipeptide chloromethanes have been reported (McMurray & Dyckes, 1985) in the form of multiple products.

In the case of the diazomethanes and dimethylsulphonium salts, both a single amino acid and a dipeptide derivative with free α -amino groups were obtainable for evaluation with exopeptidases. In the case of the diazomethanes, acidic deblocking conditions were avoided because of the lability of this functional group. When Fmoc- was used as the temporary protecting group, its removal with piperidine was useful for the synthesis of H_2N -Gly-Phe-CHN₂, but not for H_2N -Phe-CHN₂. It appears that the latter did not survive the reaction conditions. The successful preparation of a single amino acid diazomethane, H_2N -Ser(OBzl)-CHN₂, utilized an enzymic deblocking procedure.

H_2N -Phe-CH₂S⁺(CH₃)₂ and H_2N -Ala-Phe-CH₂S⁺(CH₃)₂ were readily obtainable from the Boc-derivatives. Related to these peptidylsulphonium salts is the synthesis of a peptide in which the sulphonium group occupies the position of the α -amino group, that is, L-C₆H₅CH₂CHS⁺(CH₃)₂CO-Ala-Ala-OCH₃ (compound 1, Scheme 1). D-Phenylalanine was converted with retention of configuration (Fischer & Schoeller, 1907) to

the D-bromo compound 2, Scheme 1) with HBr/NaNO₂, and this was elongated with Ala-Ala-OMe by the standard mixed-anhydride procedure. Treatment with NaSCH₃ yielded, under inversion, the L-methylthio compound (4, Scheme 1), which was then converted with AgBF₄ (Beak & Sullivan, 1982) into the sulphonium salt (1, Scheme 1). It was hoped that this positively charged group might promote binding to exopeptidases as an analogue of the charged amino group. The derivative could be a competitive inhibitor if this was the case, and potentially an affinity-labelling reagent if alkyl transfer ensued. Such a property would be useful not only for exopeptidases, but possibly for the study of ubiquitinylation systems that recognize the *N*-terminus of proteins to be ubiquitinated (Reiss *et al.*, 1988). However, this derivative preincubated at 10⁻⁴ M for 1 h with either cathepsin C or H was without effect on their amidase activity.

On the other hand, the enzymic properties of the newly synthesized derivatives having a reactive group at the C-terminus clearly expand the scope of affinity-labelling reagents to discriminate among cysteine proteinases. For example, H_2N -Phe-CH₂F at 1 μ M inactivates cathepsin H with a *t*_{1/2} of 6 min (Table 1), whereas a 100-fold higher concentration has no measurable inhibitory activity towards cathepsin C (Table 2) or cathepsin B (Table 3). This is consistent with the aminopeptidase properties of cathepsin H (Kirschke *et al.*, 1977a) not shown by the other two cysteinyl proteinases. It is reminiscent of previous observations with H_2N -Leu-CH₂Cl (Kirschke *et al.*, 1976), which also inactivates cathepsin H at 1 μ M concentration, although the conditions of the present study are slightly different. However, it appears that H_2N -Phe-CH₂F is more selective, since we found no inactivation of cathepsin B with 10⁻⁴ M inhibitor even after a 42 min preincubation (Table 3), whereas H_2N -Leu-CH₂Cl at 10⁻⁵ M causes a measurable inactivation of cathepsin B (Kirschke *et al.*, 1976). Blocked forms of H_2N -Phe-CH₂F, such as Cbz-Phe-CH₂F and Cbz-Phe-Phe-CH₂F also inactivate cathepsin H (Table 1), but a considerably higher concentration is

Table 1. Inhibition of rat cathepsin H by various peptidyl derivatives at pH 6.8

Inhibitor	[I] (μ M)	<i>t</i> _{1/2} (s)	10 ³ × <i>k</i> _{app.} (s ⁻¹)	<i>k</i> ₂ (M ⁻¹ · s ⁻¹)
H_2N -Phe-CH ₂ F	1	369	1.88	1878
Cbz-Phe-CH ₂ F	50	471	1.47	29
Cbz-Phe-Phe-CH ₂ F	50	940	0.74	15
Cbz-Leu-Tyr-CH ₂ F	10	560	1.24	124
H_2N -Ala-CHN ₂	10	402	1.72	172
H_2N -Ser(OBzl)-CHN ₂	0.5	540	1.28	2567
H_2N -Gly-Phe-CHN ₂	50	540	1.28	26
Cbz-Phe-Ala-CHN ₂	100	> 6000	—	—*
Cbz-Phe-Ser(OBzl)-CHN ₂	50	1965	0.35	7
Cbz-Leu-Tyr-CHN ₂	3	1785	0.39	129
H_2N -Phe-CH ₂ S ⁺ (CH ₃) ₂	15	1326	0.52	35
H_2N -Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂	200	423	1.64	8
Cbz-Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂	1	639	1.08	1085
E-64 [L- <i>trans</i> -epoxysuccinyl]-leucylamido-(4-guanidino)butane]				4000†

* 37 °C; Kirschke *et al.* (1980).

† 40 °C; Barrett *et al.* (1982).

Table 2. Inactivation of cathepsin C by peptidyl affinity-labelling reagents at pH 6.0

Inhibitor	[I] (μM)	$t_{1/2}$ (s)	$10^3 \times k_{\text{app.}}$ (s^{-1})	k_2 ($\text{M}^{-1} \cdot \text{s}^{-1}$)
H ₂ N-Phe-CH ₂ F	100	—	—	—*
Cbz-Leu-Tyr-CH ₂ F	50	—	—	—*
H ₂ N-Ser(OBzl)-CHN ₂	100	—	—	—*
H ₂ N-Gly-Phe-CHN ₂	0.25	414	1.67	6697
Fmoc-Gly-Phe-CHN ₂	200	3000	0.23	1.2
Cbz-Phe-Ala-CHN ₂	—	—	—	18†
H ₂ N-Phe-CH ₂ S ⁺ (CH ₃) ₂	50	—	—	—*
H ₂ N-Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂	0.05	1080	0.64	12836
Cbz-Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂	4	1635	0.42	106

* No change in 40 min.

† Green & Shaw (1981).

Table 3. Observations with cathepsin B at pH 5.4

Inhibitor	[I] (μM)	$t_{1/2}$ (s)	$10^3 \times k_{\text{app.}}$ (s^{-1})	k_2 ($\text{M}^{-1} \cdot \text{s}^{-1}$)
H ₂ N-Phe-CH ₂ F	200	—	—	—*
Ac-Phe-CH ₂ F	—	—	—	0.5†
Cbz-Phe-Ala-CH ₂ F	—	—	—	53333‡
Cbz-Leu-Tyr-CH ₂ F	2	1068	0.65	325
H ₂ N-Ser(OBzl)-CHN ₂	100	744	0.93	9.3
H ₂ N-Gly-Phe-CHN ₂	100	18000	0.04	0.4
Cbz-Phe-Ser(OBzl)-CHN ₂	—	—	—	8819§
Cbz-Phe-Ala-CHN ₂	—	—	—	1249
H ₂ N-Phe-CH ₂ S ⁺ (CH ₃) ₂	100	—	—	—*
H ₂ N-Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂	50	516	1.34	27
Cbz-Phe-Ala-CH ₂ S ⁺ (CH ₃) ₂	0.2	720	0.96	4814¶

* No change in 42 min.

† Shaw *et al.* (1986).‡ Rauber *et al.* (1986).§ Shaw *et al.* (1983).

|| Green & Shaw (1981).

¶ Shaw (1988).

necessary than with the unblocked amino acid derivative. This is consistent with observations made with other classes of affinity-labelling reagents such as the diazo-methanes and sulphonium salts, as discussed below.

Ser(OBzl)-CHN₂ is also an effective inactivator of cathepsin H, causing a rapid loss of activity at less than micromolar concentrations (Table 1). The benzyl ether side chain apparently contributes to binding in S₁ with this cysteinyl proteinase as it does with its homologues, cathepsins B and L (Shaw *et al.*, 1983; Kirschke *et al.*, 1988), providing a more effective inhibitor than H₂N-Ala-CHN₂. Here again, blocked derivatives are less active as observed previously by Schwartz & Barrett (1980). However, the tripeptide derivative, Cbz-Leu-Leu-Tyr-CHN₂, from a study of calpain inactivators (Crawford *et al.*, 1988), which take advantage of the affinity of that proteinase for Leu-Leu binding in S₂ and S₃ (Sasaki *et al.*, 1984), was unexpectedly effective as an inactivator of cathepsin H. When examined at 3 μM it produced an inactivation with $t_{1/2}$ of 13 min (Table 1). This inhibitor has been shown to be 1000-fold more effective in inactivating cathepsin L than B and to be an effective

calpain inactivator (Crawford *et al.*, 1988). This result suggests that cathepsin H appears to have some similarity to cathepsin L in this region of the active centre. However, if, as suggested by Takahashi *et al.* (1988), the function of cathepsin H is largely as an aminopeptidase, one would expect this part of the active centre, i.e., S₂ and S₃, to exclude the binding of peptides.

Cathepsin C, a dipeptidyl aminopeptidase, was resistant to single amino acid derivatives such as H₂N-Phe-CH₂F, H₂N-Ser(OBzl)-CHN₂, and H₂N-Phe-CH₂S⁺(CH₃)₂, which, when examined at 10⁻⁴ M or 5 × 10⁻⁵ M for 40 min, were without effect (Table 2). This demonstrates the specificity of these reagents as cysteinyl-aminopeptidase inactivators. We could readily have detected a 10% loss in activity in 40 min, which corresponds to a $t_{1/2}$ of 260 min; for an inhibitor at 5 × 10⁻⁵ M, this corresponds to a rate of about 1 M⁻¹·s⁻¹. The 'better' reagents for cathepsin H, i.e. H₂N-Phe-CH₂F and H₂N-Ser(OBzl)-CHN₂, at 1878 M⁻¹·s⁻¹ and 2567 M⁻¹·s⁻¹ are therefore about three orders of magnitude more effective for cathepsin H than for cathepsin C. On the other hand, H₂N-Phe-Ala-CH₂S⁺(CH₃)₂ gives

a rate for the inactivation of cathepsin C of $12836 \text{ M}^{-1} \cdot \text{s}^{-1}$ compared with $8 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cathepsin H (Table 1) and $27 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cathepsin B (Table 3). This is, this reagent is three orders of magnitude more specific for cathepsin C than for H or B.

These findings extend to the exopeptidases cathepsin H and C reagent types found useful for cysteinyl endopeptidases such as cathepsins B and L, by exploiting their ability to bind a single amino acid derivative or a dipeptide. The new reagents may be helpful in clarifying the cellular function of these exopeptidases.

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