The synthesis, kinetic characterization and application of a novel biotinylated affinity label for cathepsin B

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In this study we report on the synthesis, kinetic characterization and application of a novel biotinylated and active-site-directed inactivator of cathepsin B. Thus the peptidyl diazomethane biotinyl-Phe-Ala-diazomethane has been synthesized by a combination of solid-phase and solution methodologies and has been shown to be a very efficient inactivator of bovine and human cathepsin B. The respective apparent second-order rate constants ($k_{\text{obs.}}/[\text{I}]$) for the inactivation of the human and bovine enzymes by this reagent, namely $\sim 5.4 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $\sim 7.8 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$, compare very favourably with those values determined for the urethane-protected analogue benzyloxycarbonyl-Phe-Ala-chloromethane first described by Green & Shaw [(1981) *J. Biol. Chem.* **256**, 1923–1928], thus demonstrating that the presence of the biotin moiety at the P₃ position is compatible with inhibitor effectiveness. The utilization of this reagent for the detection of cathepsin B in electrophoretic gels, using Western blotting and in combination with a streptavidin/alkaline phosphatase detection system, is also demonstrated. Given that the peptidyl diazomethanes exhibit a pronounced reactivity towards cysteine proteinases, we feel that the present label may well constitute the archetypal example of a wide range of reagents for the selective labelling of this class of proteinase, even in a complex biological milieu containing additional classes of proteinases.

INTRODUCTION

In a previous paper (Cullen *et al.*, 1990), we presented some preliminary results on the active-site labelling of bovine cathepsin B using a biotinylated peptidyl diazomethane, biotinylphenyl-alanylalanyldiazomethane (Bio-Phe-Ala-CHN₂). Here we present the full synthetic details for the preparation of this peptide, report on the kinetic characterization of its inhibitory properties towards bovine and human cathepsin B and describe, in detail, the utilization of this probe for the detection of these proteinases in gels.

EXPERIMENTAL

Materials

Human cathepsin B was purchased from Calbiochem, Nottingham, U.K. Bovine cathepsin B, D-biotin *p*-nitrophenyl ester, Nitro Blue Tetrazolium, 5-bromo-4-chloroindol-3-yl phosphate, *N*-methylmorpholine (NMM) and biotinylated standards were from Sigma Chemical Co., Poole, Dorset, U.K. Pre-stained molecular-mass markers were from Bethesda Research Laboratories, Paisley, Renfrewshire, Scotland, U.K. Nitrocellulose (0.2 μm pore size) was obtained from Schleicher und Schüll, Dassel, Germany. Streptavidin/alkaline phosphatase was purchased from Vector Laboratories, Peterborough, U.K. Benzyloxycarbonylphenylalanyl succinimidyl ester (Cbz-Phe-OSu), tyrosine *t*-butyl ether [*H*-Tyr (OBu^t)-OH], *N*-(*g*-fluorenylmethoxycarbonyl) phenylalanine (Fmoc-Phe-OH) and *N*-(*g*-fluorenylmethoxycarbonyl) alanine (Fmoc-Ala-OH) were purchased from Nova Biochem, Nottingham, U.K. Sasrin resin

(2-methoxy-4-alkoxybenzyl alcohol-derivatized polystyrene) and benzyloxycarbonyl-Arg-Arg-4-methylcoumarin-7-ylamide (Cbz-Arg-Arg-NH-Mec) were purchased from Bachem, Bubendorf, Switzerland.

Synthesis of probe

The probe was synthesized by a combination of solid-phase and classical solution methodologies. In essence, Bio-Phe-Ala-OH (obtained as its *C*-terminal free acid) was synthesized (typically on a 0.25 mmol scale) on a Sasrin polystyrene-based resin containing an acid-sensitive linker (Mergler *et al.*, 1988*a,b*). *L*-phenylalanine and *L*-alanine were incorporated as their *N*-protected Fmoc symmetrical anhydride derivatives in dimethylformamide (DMF) as described by Atherton *et al.* (1978, 1981), the alanine derivative being coupled to the hydroxy function on the solid support in the presence of a catalytic amount of dimethylaminopyridine. The biotin grouping was incorporated into the peptide via its *p*-nitrophenyl ester derivative in the presence of a catalytic amount of 1-hydroxybenzotriazole (König & Geiger, 1970). The completeness of the coupling reactions at each stage was assessed by the Kaiser ninhydrin test (Kaiser *et al.*, 1970). At the end of the coupling cycle for the incorporation of biotin, the resin-bound peptide was washed thoroughly with DMF (20 $\times$ 20 ml). This rigorous washing step is essential in order to remove all traces of the poorly soluble biotin active ester.

Upon completion of the synthesis, the peptide was cleaved from the support by treatment with a solution (10 ml) of trifluoroacetic acid in dichloromethane (1%, v/v) containing dimethyl sulphide (2.5%, v/v) and anisole (2.5%, v/v), at 25 °C, for 45 min. After removal of the solvents by evaporation under reduced pressure, the product (80% yield based on initial loading

Abbreviations used: NMM, *N*-methylmorpholine; TBS, Tris-buffered saline; Bio, biotinyl; Cbz, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl; Mec, 4-methylcoumarin-7-yl; E-64, *trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane; RFU, relative fluorescence units; Fmoc, *N*-(*g*-fluorenylmethoxycarbonyl)-; OSu, succinimidyl ester; OBu^t, *t*-butyl ester; DMF, dimethylformamide; -CHN₂, diazomethane; HoBt, 1-hydroxybenzotriazole.

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of resin), was dried *in vacuo* over KOH pellets for 12 hours before use in the next stage of the synthesis, when it was used without further purification.

The peptide free acid (1 mmol) was then converted into the diazomethane by the reaction of the mixed anhydride with ethereal diazomethane (Green & Shaw, 1981) at room temperature for 1.5 h and, following the usual work-up, yielded 250 mg (89%) of the desired product, m.p. 125–127 °C (Found: C, 57.5; H, 6.3; N, 18.2. $C_{22}H_{30}N_6O_3S_1$ requires C, 57.6; H, 6.55; N, 18.34%).

The affinity label, Cbz-Phe-Tyr(OBu^t)CHN₂, was prepared by the general procedure of Shaw *et al.* (1983) and had an m.p. of 135–136 °C, in close agreement with the literature value (Kirschke *et al.* (1988).

Kinetic techniques

Inactivation studies (a) Discontinuous assay. A solution of probe in DMF was added to a solution of proteinase (~70 nM) in 100 mM-sodium phosphate buffer, pH 6.4, containing 2 mM-cysteine, 1 mM-EDTA and 0.1% (w/v) Brij 35, at 37 °C, such that the final concentration of inhibitor was 0.13–3.8 μ M. Samples were removed at timed intervals and assayed for residual activity with Cbz-Arg-Arg-NH-Mec. The dilution involved in setting up the assay effectively stopped further reaction with the inhibitor.

(b) Continuous assay. A solution (10 μ l) of the enzyme under study (~5 nM), was added to a solution (1 ml) of Cbz-Arg-Arg-NH-Mec (50 μ M) and probe (2.7–27 μ M) in 100 mM-sodium phosphate buffer, pH 6.4, containing 2 mM-cysteine, 1 mM-EDTA and 0.1% (w/v) Brij 35, at 37 °C. The rate of hydrolysis of substrate was monitored continuously by measuring the rate of increase of fluorescence at 455 nm (excitation wavelength 383 nm) in a Perkin-Elmer MPF 44B spectrofluorimeter.

Determination of the operational molarity of the proteinase solutions. Human and bovine cathepsin B were titrated with accurate amounts of E-64 as described by Barrett & Kirschke (1981). This molarity was then related to the steady-state hydrolysis of a solution (1 ml) of Cbz-Arg-Arg-NH-Mec (50 μ M) in order to have a more convenient measure of enzyme concentration from day to day.

Characterization of labelled proteinases

Labelling of proteinases. To a solution (45 μ l) of proteinase (100–500 ng) in 100 mM-sodium phosphate buffer, pH 6.4, containing 2 mM-cysteine, 1 mM-EDTA and 0.1% (w/v) Brij 35 was added a solution (5 μ l) of Bio-Phe-Ala-CHN₂ (1 mM) in methanol. The solution was then incubated for 30 min at 37 °C. The reaction between proteinase and inhibitor was terminated by addition of an equal volume of Laemmli treatment buffer followed by boiling for 10 min.

Electrophoresis. Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970) with 10%-(w/v)-polyacrylamide slab gels using the Mighty Small Apparatus (Hoefer Scientific). Labelled proteins were denatured by boiling for 10 min in 50 μ l of Laemmli treatment buffer. Pre-stained molecular-mass markers (BRL) and biotinylated standards (Sigma) were included in each gel.

Western blotting. Western blotting was performed by the method of Towbin *et al.* (1979). Transfer of protein from gel to nitrocellulose was performed in 25 mM-Tris/192 mM-glycine/20% (v/v) methanol using a Trans-Blot Cell (Bio-Rad). The running time was 2 h at 200 mA, and a constant temperature of 4 °C was maintained.

Detection of biotinylated proteins. After protein transfer, the blot was stained for biotinylated polypeptides by overlaying the streptavidin/alkaline phosphatase. The nitrocellulose sheet was

first blocked by incubation, for 2 h, with a 3% solution of BSA (Sigma) in Tris-buffered saline (TBS; 0.05 M-Tris/HCl/0.1 M-NaCl, pH 7.4) containing 0.01% (v/v) merthiolate and 0.01% (v/v) anti-foam agent (Sigma). The Streptavidin/alkaline phosphatase stock solution (Vector Laboratories), diluted 1:500 in blocking solution containing 0.25% BSA, was then incubated with the nitrocellulose sheet for 2 h at room temperature with shaking. The blot was washed at least six times with 0.1 M-Tris/HCl, pH 9.5, containing 0.1 M-NaCl and 5 mM-MgCl₂ before the alkaline phosphatase activity was detected by using Nitro Blue Tetrazolium (66 μ l of a 75 mg/ml solution in 70% DMF) and 5-bromo-4-chloroindol-3-yl phosphate (33 μ l of a 50 mg/ml solution in DMF) in 10 ml of the same buffer.

RESULTS AND DISCUSSION

Inactivation studies

Fig. 1 shows the time course for the formation of product (7-amino-4-methylcoumarin), released from the substrate Cbz-Arg-Arg-NH-Mec by bovine cathepsin B, in the presence of increasing concentrations of Bio-Phe-Ala-CHN₂. Similar curves were obtained with human enzyme (results not shown). These progress curves are indicative of the action of an active-site-C directed irreversible inhibitor (see, e.g., Tian & Tsou, 1982; Walker & Elmore, 1984). This kinetic behaviour was predicted, since the probe is modelled on Cbz-Phe-Ala-CHN₂, a known active-site-directed affinity label of cathepsins B and L (Green & Shaw, 1981).

Fig. 1 shows that the initial rates of product formation are independent of inhibitor concentration; this implies that, for the range of inhibitor concentrations employed, there is no non-covalent complex formed before irreversible modification of the enzyme. This view was also reinforced when the Kitz-Wilson (1962) method of analysis was applied to the inhibition process. Fig. 2 shows the semi-logarithmic plots for the loss of activity with time when bovine cathepsin B was incubated with various concentrations of Bio-Phe-Ala-CHN₂ in the absence of Cbz-Arg-Arg-NH-Mec. Over the range of inhibitor concentrations studied,

Fig. 1. Progress curves for the generation of 7-amino-4-methylcoumarin from the bovine cathepsin B-catalysed hydrolysis of Cbz-Arg-Arg-NH-Mec in the presence of Bio-Phe-Ala-CHN₂.

The curves represent the best fit of the data to the equation for irreversible inhibition (Walker & Elmore, 1984) of bovine cathepsin B by Bio-Phe-Ala-CHN₂ in the presence of Cbz-Arg-Arg-NH-Mec. The inactivation studies were carried out with a fixed concentration of substrate (50 μ M) and the following concentrations of inhibitors: ●, 27 μ M; ▲, 15 μ M; ■, 2.7 μ M.

Fig. 2. Semi-logarithmic plots for the inactivation of bovine cathepsin B by Bio-Phe-Ala-CHN₂

Inactivation studies were carried out as outlined in the Experimental section. The range of inhibitor concentrations used was as follows: ■, 0.13 μM; ●, 0.38 μM; □, 3.8 μM; ▲, 1.27 μM. Residual activity was measured against the fluorogenic substrate Cbz-Arg-Arg-NH-Mec.

Table 1. Apparent second-order rate constants ($k_{\text{obs.}}/[I]$) for the inactivation of bovine and human cathepsin B by Bio-Phe-Ala-CHN₂ and Cbz-Phe-Ala-CHN₂

Values are means ± S.E.M. for four determinations.

Cathepsin B	Inhibitor constant ($k_{\text{obs.}}/[I]$) ($\text{M}^{-1} \cdot \text{min}^{-1}$)	
	Cbz-Phe-Ala-CHN ₂	Bio-Phe-Ala-CHN ₂
Bovine	$5.4 \pm 0.6 \times 10^4$	$5.4 \pm 0.5 \times 10^4$
Human	$1.1 \pm 0.1 \times 10^5$	$7.8 \pm 0.9 \times 10^4$

and with both cathepsin B species, we observed a linear relationship between $k_{\text{obs.}}$ and $[I]$, again implying a non-complexing type of irreversible modification. Exactly analogous behaviour was observed for Cbz-Phe-Ala-CHN₂ when it was tested over a comparable range of inhibitor concentrations. Consequently, it was only possible to evaluate the apparent second-order rate constants ($k_{\text{obs.}}/[I]$) for each of these affinity labels and not the individual kinetic constants, k_i and K_i . The $k_{\text{obs.}}/[I]$ values for the inactivation of the bovine and human enzymes with both probes are listed for comparison in Table 1.

A particularly satisfying finding is that the presence of the long alkyl side chain of the biotin moiety does not adversely affect the overall effectiveness of the affinity label. Thus the bovine species is inactivated by both affinity labels with virtually identical effectiveness ($\sim 5 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$), and there are only marginal differences in the overall rate constants for the inactivation of the human species by both reagents, the biotinylated derivative being the slightly less effective of the two ($7.8 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ as against $1.1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$).

Fig. 3. Western-blot analysis of samples of bovine cathepsin B inactivated with the active-site-directed affinity label Bio-Phe-Ala-CHN₂

The samples were inactivated with the biotinylated probe before loading on to a 10%-(w/v)-polyacrylamide gel (run under reducing and denaturing conditions) as outlined in the Experimental section. Lane 1, bovine cathepsin B ($\sim 100 \text{ ng}$) incubated with Bio-Phe-Ala-CHN₂ (70 μM , for 1 h at 37°C); lane 2, bovine cathepsin B pretreated with Cbz-Phe-Tyr(OBu^t)-CHN₂ (0.1 μM , for 17 min at 37°C) before incubation with Bio-Phe-Ala-CHN₂; lane 3, bovine cathepsin B pretreated with Cbz-Phe-Ala-CHN₂ (100 μM , for 1 h at 37°C) before incubation with Bio-Phe-Ala-CHN₂; lane 4, bovine cathepsin B pretreated with iodoacetamide (200 μM , for 1 h at 37°C) before incubation with Bio-Phe-Ala-CHN₂. The position of the prestained molecular-mass markers are indicated by the labelled arrows. After electrophoretic transfer the biotinylated proteins were revealed with streptavidin/alkaline phosphatase as described in the Experimental section.

Labelling studies

Figs. 3, 4 and 5 show the results of the labelling studies employing SDS/PAGE and Western blotting of the biotinylated proteinases.

Figs. 3 and 4 show that incubation of bovine and human cathepsin B with the biotinylated probe results in the labelling of only one main species, of molecular mass 28 kDa, in each instance [Fig. 3, lane 1; Fig. 4, lane 3 (bovine and human enzymes respectively)].

These determined molecular masses are exactly in keeping with the reported values for each proteinase.

That the labelling is active-site directed is shown by the competitive blocking experiments, results of which are also recorded in Figs. 3 and 4. Thus preincubation of either of the bovine (Fig. 3, lane 3) or human species (Fig. 4, lane 4) with Cbz-Phe-Ala-CHN₂ (100 μM , for 1 h at 37°C), completely blocks the incorporation of the biotin label. Similarly, pretreatment of both proteinases with iodoacetamide (200 μM , for 1 h at 37°C) before incubation with Bio-Phe-Ala-CHN₂, also completely blocks the incorporation of this probe into each enzyme (Fig. 3, lane 4; Fig. 4, lane 6: bovine and human enzymes, respectively).

The labelling studies presented in Fig. 5, which utilized different amounts of bovine cathepsin B, give an indication of the sensitivity of the method. This Figure shows the relationship between the amount of biotinylated proteinase applied to the gel and the intensity of the banding pattern obtained. The present

Fig. 4. Western-blot analysis of samples of human cathepsin B inactivated with the active-site-directed affinity label Bio-Phe-Ala-CHN₂.

The samples were inactivated with the biotinylated probe before loading on to a 10% PAGE (run under reducing and denaturing conditions) as outlined in the Experimental section. Lane 1, biotinylated molecular-mass markers; lane 2, blank; lane 3, human cathepsin B (~100 ng) incubated with Bio-Phe-Ala-CHN₂ (70 μ M, for 1 h at 37 °C); lane 4, human cathepsin B pretreated with Cbz-Phe-Ala-CHN₂ (100 μ M, for 1 h at 37 °C) before incubation with Bio-Phe-Ala-CHN₂; lane 5, human cathepsin B pretreated with Cbz-Phe-Tyr(OBu^t)-CHN₂ (0.1 μ M, for 17 min at 37 °C) before incubation with Bio-Phe-Ala-CHN₂; lane 6, human cathepsin B pretreated with iodoacetamide (200 μ M, for 1 h at 37 °C) before incubation with Bio-Phe-Ala-CHN₂; lane 7, prestained molecular-mass markers. After electrophoretic transfer the biotinylated proteins were revealed with streptavidin/alkaline phosphatase as described in the Experimental section. The position of the biotinylated cathepsin B is indicated by the labelled arrow.

Fig. 5. Western-blot analysis of active-site biotinylated bovine cathepsin B

Samples of Bio-Phe-Ala-CHN₂-modified bovine cathepsin B were prepared as described in the Experimental section. The samples were then subjected to electrophoresis on a 10% (w/v) polyacrylamide gel run under reducing and denaturing conditions. Amounts of active-site-biotinylated cathepsin B applied to the gel were as follows. Lane A, 500 ng; lane B, 340 ng; lane C, 170 ng; lane D, 86 ng; lane E, 43 ng; lane F, 21 ng. The biotinylated standard markers are arrowed. After electrophoretic transfer the biotinylated proteins were revealed with streptavidin/alkaline phosphatase as described in the Experimental section.

system is clearly capable of detecting between 500 ng (lane A) and 20 ng (lane F) of active enzyme.

We are by no means convinced that this represents the absolute limit of detection for this system, and we are keen to explore potential methods for improving the sensitivity. For example, it may be necessary to incorporate a spacing unit between the

biotin moiety and the peptide recognition sequence bearing the diazomethyl grouping in order to permit the maximum interaction between the streptavidin label and the inactivated proteinase. Additionally, by choosing a luminescent detection system based, for example, on the luciferase/luciferin system, the limit of detection could probably be extended by a further order of magnitude at least, given the inherent increase in sensitivity of luminescence versus spectrophotometric detection.

In the present study we have been concerned solely with demonstrating how cathepsin B can be labelled with this biotinylated affinity probe. However, given that cathepsin L is readily inactivated by Cbz-Phe-Ala-CHN₂ (Kirschke & Shaw, 1981), it is likely that Bio-Phe-Ala-CHN₂ may function as an effective label for this proteinase also. This should not necessarily be considered as being an insuperable source of ambiguity in, for example, situations where one is attempting to positively identify a proteolytic species as being cathepsin B-like while dealing with a sample which may contain both B and L activities. In instances like these, one should be able to discriminate easily between the two proteinases on the basis of blocking experiments using the affinity label Cbz-Phe-Tyr(O-But)-CHN₂. This affinity reagent inactivates cathepsin L with an apparent second-order rate constant ($k_{\text{obs}}/[I]$) of $1.2 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, some 25000-fold more readily than it does cathepsin B (Kirschke *et al.*, 1988). Thus, for example, incubating cathepsin L with this reagent at a concentration of 0.1 μ M for 15 min (exposure time equivalent to eight half-lives), should result in the complete inactivation of the enzyme, while having no demonstrable effect on cathepsin B. Preincubation of a sample containing both proteinases with the unbiotinylated inhibitor at the above stated concentration and exposure time, before labelling with the biotinylated probe, should therefore permit the selective labelling of cathepsin B. In the present study we have already gone some way towards proving this thesis, since preincubation of either bovine or human cathepsin B with Cbz-Phe-Tyr(OBu^t)-CHN₂ (0.1 μ M, for 15 min) before incubation with Bio-Phe-Ala-CHN₂ (final concn. 50 μ M) has no effect on the incorporation of the label. This is shown clearly in Figs. 3 and 4, which show that there is no diminution in the extent of labelling of bovine or human cathepsin B when they are pretreated with the blocking reagent (Fig. 3, lane 2, and Fig. 4, lane 5, respectively) and with this pretreatment step omitted (Fig. 3, lane 1, and Fig. 4, lane 3, respectively).

This probe may be used to detect cathepsin B-like proteolytic activity in various tissues and fluids derived from normal and pathological samples. For example, in the accompanying paper (Cullen *et al.*, 1992), we describe how the probe can be used to characterize the molecular forms of cathepsin B-like proteolytic activity produced by breast-tumour cells in culture.

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