The use of benzyloxycarbonyl-[¹²⁵I]iodotyrosylalanyldiazomethane as a probe for active cysteine proteinases in human tissues

Robert W. MASON,* Linda T. BARTHOLOMEW and Bryn S. HARDWICK

Department of Biochemistry, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

The ability of benzyloxycarbonyl-(¹²⁵I)Tyr-Ala-CHN₂ to label cysteine proteinases in a variety of human tissues was investigated. The inhibitor bound only to cathepsin B in tissues homogenized at pH 5.0. When liver was autolysed at pH 4.0 for up to 4 h, the inhibitor also bound to a protein of *M_r* 25000. This was identified immunologically and chromatographically as cathepsin L. Both cathepsins B and L were found primarily in kidney, liver and spleen. In spleen, an additional protein of *M_r* 25000 was also labelled. This protein could not be precipitated by antibodies to any of cathepsins B, H and L. This protein has tentatively been identified as human cathepsin S by its tissue distribution, chromatographic properties and molecular size. This work clearly shows that peptidyl-diazomethanes are specific probes for cysteine proteinases, and that benzyloxycarbonyl-(¹²⁵I)Tyr-Ala-CHN₂ binds to three such enzymes in human tissues.

INTRODUCTION

Peptidyl-diazomethanes have been designed as specific inhibitors of cysteine proteinases. They have been shown to inactivate cysteine proteinases irreversibly, but do not react significantly rapidly with free thiols or other classes of proteinases [1]. The peptide moiety determines the specificity of the inhibitors for individual cysteine proteinases, and a series of inhibitors have been designed that enable the enzymes calpain, cathepsin B and cathepsin L to be distinguished *in vitro* [2]. These types of inhibitors have been used to suggest a role for cysteine proteinases in a number of physiological processes, including elastin degradation and antigen processing [3,4]. Although the specificity of these inhibitors for purified proteinases has been studied extensively [2,5–7], it is not clear whether these compounds have other targets in tissues. The purpose of the present investigation was to use one of these inhibitors containing a radiolabel in order to demonstrate the specificity of such compounds in crude tissue extracts and their use as probes for active cysteine proteinases.

MATERIALS AND METHODS

Z-Tyr-Ala-CHN₂ was a gift from Dr. E. Shaw, Friedrich Meischer Institut, Basel, Switzerland, and was radiolabelled as described previously [8]. Human post-mortem tissues were kindly obtained by Dr. P. Stovin of the Strangeways Research Laboratory.

Human tissues (300 mg) were homogenized in either 50 mM-sodium acetate buffer, pH 5.0, containing 0.1% Brij 35 (1 ml) or 100 mM-acetic acid (1 ml) by use of ultrasound cavitation [a model W-375 sonicator (Heat Systems–Ultrasonics), fitted with a microprobe using an output of 5 for 60 s]. Insoluble material was removed by centrifugation in a Microcentaur microcentrifuge (MSE) at full speed for 10 min. The actual pH values of the supernatants were in the ranges 5.6–6.0 and 4.0–4.2 for

the samples homogenized in the sodium acetate buffer and acetic acid respectively. The pH 5 extracts were immediately labelled with inhibitor as described below, whereas the acetic acid extracts were incubated at 37 °C for 4 h, precipitated protein was removed by centrifugation and the supernatants were then incubated with the inhibitor.

A portion (50 µl) of each supernatant was incubated with 50 µl of 0.4 µM radiolabelled inhibitor and 100 µl of activating buffer (400 mM-sodium acetate buffer, pH 5.0, containing 2 mM-dithiothreitol) at 37 °C for 30 min. After this time 800 µl of 10% (w/v) trichloroacetic acid was added and the complex was stood on ice for 30 min. The samples were then centrifuged at 10000 *g* for 5 min and the pellets were each washed with a further 1 ml of 10% trichloroacetic acid and then 1 ml of acetone. The dried pellets were each then resuspended by boiling in 100 µl of SDS sample buffer for 15 min, and 20 µl of this was subjected to SDS/12.5%-polyacrylamide-gel electrophoresis as described previously [7a]. Gels were stained with Coomassie Brilliant Blue, dried and autoradiographed as described previously [8].

Protein concentration was determined by the BCA protein assay (Pierce Chemical Co.). Immunoprecipitations of cathepsins B and L were performed as described previously [8,9].

Blocking experiments were performed by preincubation of the tissue extracts with unlabelled inhibitor at 37 °C for 30 min in the presence of 1 mM-dithiothreitol before addition of the radiolabelled inhibitor.

Radiolabelled proteins were partially purified by chromatography on a Mono S column of an f.p.l.c. system (Pharmacia). For these experiments, 1 ml of supernatant was labelled with 0.1 µM inhibitor in the presence of 1 mM-dithiothreitol for 30 min at 37 °C, and free inhibitor was removed and sample was transferred into 20 mM-sodium acetate buffer, pH 4.5, by gel filtration on a PD 10 column (Pharmacia). The sample was filtered through

Abbreviations used: Z, benzyloxycarbonyl; E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane.

* To whom correspondence should be sent, at present address: Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

Fig. 1. Reaction of human tissue extracts with Z-(125 I)Tyr-Ala-CHN $_2$ at pH 5.0

Samples of human tissues were prepared and treated with the radiolabelled inhibitor at pH 5.0 as described in the Materials and methods section. A 20 μ g portion of protein was loaded on to each lane of an SDS/12.5% polyacrylamide gel and after electrophoresis this was autoradiographed as described in the Materials and methods section. Samples were: lane 1, heart muscle; lane 2, brain; lane 3, skeletal muscle; lane 4, liver; lane 5, kidney; lane 6, pancreas; lane 7, spleen. The positions of M_r markers are shown alongside.

a 0.2 μ m-pore-size filter to remove insoluble material and then applied to a Mono S column (HR 5/5). Protein was eluted by a linear gradient of 0–1 M-NaCl in 20 mM-sodium acetate buffer, pH 4.5 (2 $\times$ 25 ml), at a flow rate of 1 ml/min. Radioactivity was monitored with a γ -radiation counter. Fractions were precipitated with 10% trichloroacetic acid and subjected to SDS/polyacrylamide-gel electrophoresis and the gels were autoradiographed as described previously [8].

Fig. 2. Blocking experiments showing specificity of labelling with Z-(125 I)Tyr-Ala-CHN $_2$

Samples of liver (lanes 1 and 4), spleen (lanes 2 and 5) and muscle (lanes 3 and 6) were prepared and treated with radiolabelled inhibitor as described in the Materials and methods section except that they were preincubated without (lanes 1–3) or with (lanes 4–6) 10 μ M-E-64 for 30 min at 37 $^{\circ}$ C. A 20 μ g portion of protein was loaded on to each lane of an SDS/12.5% polyacrylamide gel and after electrophoresis this was autoradiographed as described in the Materials and methods section.

Fig. 3. Effect of autolysis on labelling with Z-(125 I)Tyr-Ala-CHN $_2$

Human liver was homogenized in sodium acetate buffer, pH 5.0 (lane 1), or acetic acid (lanes 2–5) and the supernatant after centrifugation were incubated at 37 $^{\circ}$ C for 0–4 h. The supernatant of each fraction was subsequently incubated with 0.1 μ M radiolabelled inhibitor for 15 min at 37 $^{\circ}$ C before trichloroacetic acid precipitation and loading on to an SDS/12.5% polyacrylamide gel, which after electrophoresis was autoradiographed as described previously [8]. Sample applied to each lane corresponded to 20 μ g of protein before autolysis. Incubation times were 0 h (lanes 1 and 2), 1 h (lane 3), 2 h (lane 4) and 4 h (lane 5).

Fig. 4. Reaction of human tissue extracts with Z-(125 I)Tyr-Ala-CHN $_2$ after autolysis at pH 4

Samples of human tissues were autolysed at pH 4.0–4.2 and treated with the radiolabelled inhibitor at pH 5.0 as described in the Materials and methods section. Sample applied to each lane corresponded to 20 μ g of protein before autolysis [except for kidney (lane 5), where a portion of the trichloroacetic acid precipitate was lost]. The SDS/12.5% polyacrylamide gel was subjected to electrophoresis and autoradiographed as described in the Materials and methods section. Samples were: lane 1, heart muscle; lane 2, brain; lane 3, skeletal muscle; lane 4, liver; lane 5, kidney; lane 6, pancreas; lane 7, spleen.

RESULTS

Labelling of different tissues at pH 5.0

Samples of kidney, liver, heart, spleen and brain showed that the major proteins labelled had M_r 31000 and 5000 (Fig. 1). Immunoprecipitation of these bands showed that they were related to cathepsin B, most probably representing the single-chain enzyme and the light chain of the two-chain enzyme. Skeletal muscle contained only very low concentrations of cathepsin B and also showed a labelled band of M_r 13000. The spleen sample also showed an additional similar-sized labelled protein.

Blocking experiments were performed by pretreating samples with 10 μ M-E-64 for 30 min before incubation with radiolabelled inhibitor (Fig. 2). The labelling of the two proteins corresponding to cathepsin B (M_r 31000 and 5000) was blocked almost completely in liver and spleen, and to a lower extent in muscle. The labelling of proteins of M_r approx. 13000 and a number of minor proteins were not blocked. Similar results were seen when labelling was blocked with unlabelled Z-(I)Tyr-Ala-CHN₂. These non-specifically labelled proteins were more evident when inhibitor that had not reacted was not removed before boiling in SDS sample buffer and could be greatly diminished by repeated washing of trichloroacetic acid precipitates or gel filtration of samples to remove excess inhibitor. When free inhibitor was removed by gel filtration and further purification, only cathepsin B was labelled (see Fig. 5a below). The most likely explanation of these results is that the inhibitor reacts non-specifically with free thiols when proteins were boiled in SDS under reducing conditions.

Labelling of tissues at pH 4.0

In order to release active cathepsin L from human tissues, it is necessary to autolyse the homogenized tissues at low pH [10]. We used Z-(¹²⁵I)Tyr-Ala-CHN₂ labelling of a human liver homogenate in order to demonstrate the release of cathepsin L (Fig. 3). Release of a labelled protein of M_r 25000 was seen within 1 h after incubation of the human liver homogenate at pH 4.2 and 37 °C. More of this protein was labelled after prolonged treatment for up to 4 h. This protein was identified as cathepsin L by immunoprecipitation. In addition to release of active cathepsin L, this procedure results in precipitation of 95% of the protein, resulting in a significant purification of the enzymes [10].

When other tissues were treated similarly, an additional labelled protein of M_r 25000 in spleen and kidney, and to a lesser extent muscle, indicating the presence of additional latent or inhibited enzymes in these tissues (Fig. 4).

Separation of labelled proteins by cation-exchange chromatography

Spleen homogenate that had been labelled at pH 5.0 was applied to a Mono S column of an f.p.l.c. system and eluted as described in the Materials and methods section (Fig. 5a). A single broad peak of radioactivity was eluted. SDS/polyacrylamide-gel electrophoresis followed by autoradiography revealed that two labelled proteins, one of M_r 5000 being eluted slightly earlier than the one of M_r 31000. These corresponded to the two molecular

forms of cathepsin B. No other labelled proteins were seen.

Liver that had been autolysed at pH 4.0 at 37 °C for 4 h before the labelling gave a similar peak of radioactivity associated with cathepsin B, and a second peak eluted at a position where cathepsin L would be expected to be eluted (Fig. 5b). This proved to be the case; SDS/polyacrylamide-gel electrophoresis followed by autoradiography revealed that a radiolabelled protein of M_r 25000 was eluted at 400 mM-NaCl that could be precipitated with an antibody to human cathepsin L.

Autolysed spleen samples were subjected to similar treatment (Fig. 5c). Again two peaks of radioactivity were eluted. The major peak fractions contained the two labelled cathepsin B proteins and also a major additional protein of M_r 25000. This could not be identified as any of cathepsins B, H and L by immunoprecipitation. The later-eluted peak contained a labelled protein of M_r 25000 that was identified as cathepsin L.

DISCUSSION

We have studied the specificity of a radiolabelled inhibitor as a probe for cysteine proteinases. In homogenates of human liver Z-(¹²⁵I)Tyr-Ala-CHN₂ reacts with two molecular forms of cathepsin B. Reaction with an additional protein of M_r 25000 was observed on treatment of the homogenate at pH 4 at 37 °C for up to 4 h. This protein was identified immunologically as the heavy chain of cathepsin L. The two labelled proteinases behaved similarly to the uninhibited enzymes when chromatographed on a Mono S column at pH 4.5, cathepsin B being eluted at 250 mM-NaCl and cathepsin L at 400 mM-NaCl [10]. This indicates that the inhibitor does not significantly affect the charge of labelled enzyme and could be used as a tracer in the purification of enzymes.

The tissue distribution of cathepsin B in human samples determined by this method is in close agreement to results obtained previously by using immunological assays of extracts of rat tissues [11], with the major sources of enzyme being kidney, liver and spleen. Upon autolysis, these tissues also revealed a protein of M_r 25000 that proved to be cathepsin L by immunological and chromatographic criteria in liver and kidney. In spleen, however, only a small proportion of this 25000- M_r protein proved to be cathepsin L. Most of this protein was co-eluted with cathepsin B from a Mono S column, and could not be precipitated by antibodies to any of cathepsins B, H and L. Furthermore, Z-(I)Tyr-Ala-CHN₂ does not react with purified human cathepsin H (R. W. Mason, unpublished work). Labelling of this protein was blocked by E-64, another inhibitor of cysteine proteinases. This suggests that it is a cysteine proteinase that has not previously been identified in human tissues. It is possible that this enzyme is the human counterpart of cathepsin S, which to date has only been isolated from bovine spleen [12]. In addition to the tissue-specificity of the enzymes, they also have similar molecular sizes and chromatographic properties. Further purification and characterization of the human enzyme are required to establish its similarity to the bovine enzyme.

The usefulness of this inhibitor as a probe to show tissue distribution and suitable extraction methods for the known enzymes cathepsin B and L have been demonstrated. The ability to use the inhibitor as a probe

Fig. 5. Separation of radiolabelled proteins by cation-exchange chromatography

Tissues (300 mg) were homogenized and treated with radiolabelled inhibitor as described in the Materials and methods section. After gel filtration to remove excess inhibitor and equilibrate the samples into running buffer, the protein fractions were loaded on to a Mono S column of an f.p.l.c. system and eluted as described in the Materials and methods section. Peak fractions of radioactive proteins were precipitated with trichloroacetic acid, and 20 % of each fraction was loaded on to SDS/polyacrylamide gels, which were subjected to electrophoresis and autoradiographed. The light chain of cathepsin B was run off of the gel autoradiographed in (c) in order to enhance the separation of bands. Samples are: (a) human spleen; (b) autolysed human liver; (c) autolysed human spleen.

for other as-yet-undetermined enzymes has also been established. These results clearly show that cysteine proteinases are the only targets of this inhibitor. This inhibitor has also proved to be non-toxic to live cells [8], and hence it can be used as a probe for the functions of cathepsins B, L and S.

This work was supported by grants from the Council for Tobacco Research-U.S.A. Inc. and the Medical Research Council. We also thank Donna Wilcox and Elliott Shaw for valuable discussions.

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Received 8 May 1989; accepted 14 June 1989