Volume 308, number 3, 305–308 FEBS 11448 August 1992

Quantitative study of the binding of cysteine proteinases to basement membranes

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Received 15 June 1992; revised version received 7 July 1992

Binding of cysteine proteinases of the papain superfamily (papain and cathepsins B, B-like and L) to basement membranes was studied by using the enzymatic activity of these proteinases against their specific fluorogenic substrates. Papain inactivated by E64 was used for K_d determination by competition experiments. The binding was characterized using the following parameters, the equilibrium constant, K_d, and the number of substrate sites, n, values of which were in the range of 10^{-7} M and 10^{-8}, respectively. Such results would be of significant interest for the understanding of the biological role of cysteine proteinases in tumour invasion and other types of tissue remodeling.

Cysteine proteinase; Quantitative binding; Basement membrane

1. INTRODUCTION

Cysteine proteinases of the papain superfamily were able to degrade intact basement membranes in vitro through a slow, time-dependent process, i.e. over 24–72 h [1]. Binding of cathepsin B to basement membranes was observed previously in this laboratory [1]. Consequences of such interaction could be the unusual in vitro stability of bound cysteine proteinases, associated with the degradation of the substrate. Binding of pepsin to collagen II was also reported [2]. Different pathological states, such as tumor invasion and metastasis, occur through the action of proteinases against basement membrane components [3]. Thus, the study of the binding mechanism by cysteine proteinases to basement membranes would be of interest. As a consequence of changes in their intracellular processing [3,4] these lysosomal proteinases are secreted by a lot of transformed cells [3,5]. In order to characterize these binding sites, we have undertaken a quantitative study. Using saturation and competition experiments, several parameters of the interaction of papain and cathepsins B, B-like and L with the bovine lens capsule were determined, including K_d values and n, i.e. the number of binding sites. These experiments were performed by using the enzymatic activity of the proteinases against the fluorogenic substrates Z-Arg-Arg-NHMEC, Z-Phe-Arg-NHMEC and papain inactivated by E64 for competition experiments instead of radiolabelled enzymes. A covalent linkage between papain and E64 was demonstrated by the crystal structure of this complex [6], permitting us to develop an experimental approach.

Abbreviations: Cathepsin B, EC 3.4.22.1; Papain, EC 3.4.22.2; Cathepsin L, EC3.4.22.15; E64, L-*trans*-epoxysuccinyl-L-leucylamino-4-guanidinobutane; Z, benzyloxycarbonyl; NH Mec, 4-methyl-7-coumarylamide; EDTA, ethylene diamine tetraacetate disodium salt; DTE, dithioerythritol.

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2. MATERIALS AND METHODS

2.1. *Materials*

Papain (twice crystallised) was purchased from Sigma (St. Louis, MO, USA). The fluorogenic substrates Z-Phe-Arg-NHMEC, Z-Arg-Arg-NHMEC and the epoxide inhibitor E-64 were provided by Nova-Biochem (Laufelfingen, Switzerland) and the Protein Research Foundation (Osaka, Japan). Fluorescence measurements were carried out on a Kontron SFM 25 spectrofluorometer at λ_{ex} 347 nm and λ_{em} 440 nm using NH2-MEC for calibration.

2.2. *Isolation of bovine lens capsules*

Eyes were removed from cows killed less than 4 h previously. Posterior and anterior lens capsules were taken and the adherent material gently removed with a blunt instrument under a magnifying glass. Before digestion, lens capsules were stored at −20°C.

2.3. *Purification of human cysteine-proteinases*

Human liver cathepsins B and L were purified using a new HPLC method from this laboratory [7]. The cathepsin B-like proteinase from human malignant ascitic fluid was isolated as previously reported [8]. Purified proteinases were characterized using SDS-PAGE, gel-electrofocusing, and catalytic properties against synthetic substrates [7,8]. Cathepsin B and B-like were also studied by immunoblotting [7,9]. The active site of these proteinases was titrated with E64, thus the concentrations given here refer to active concentrations.

2.4. *Inactivation of papain by E64*

2 μl of $1.46 \cdot 10^{-3}$ M E64 was added to 2 ml of $2.4 \cdot 10^{-6}$ M papain in the activation buffer. Binding was performed over 10 min at room temperature. Excess of inhibitor was removed by dialysis overnight by

shaking against the starting buffer as a control experiment, the enzymatic activity of blocked papain was checked against Z-Phe-Arg-NHMEC.

2.5. *Binding assay*

Binding studies were performed in 0.1 M phosphate buffer containing 1 mM DTE and 2 mM EDTA at pH 6.8 for both papain and cathepsin B-like, and at pH 6.0 for cathepsins B and L, respectively. For washing, lens capsules were thawed and incubated at 37°C for 24 h with continuous shaking in 10 ml of the chosen buffer. Binding kinetics were determined in the following way: 2 ml of buffer with or without a washed lens capsule was incubated under shaking with various concentrations of papain, or one of the three cathepsins. The assay without enzyme was used as control. At different times, aliquots of 20 μl were removed and assayed for the enzymatic activity of the cysteine proteinase against Z-Phe-Arg-NHMEC or Z-Arg-Arg-NHMEC for papain, cathepsin L and the two other cathepsins, respectively. The assay was stopped as in [7], and the fluorescence of the NH_2MEC was estimated [7]. For the time dependency of the binding of papain and cathepsin B, an assay was performed every 15 min for 2 h. For the cathepsin B-like proteinase, the time interval of the binding study was shortened to 1 h. In this latter case, the time interval between the assays was shortened to 5 min. Results are expressed as 1/E vs. T for both binding and control experiments. Using these plots, the total enzyme concentration (E_t) and the free enzyme concentration (E_f) were calculated from the control and the binding experiments, respectively. The bound proteinase (E_b) was deduced from $E_t - E_f$. From these results, the binding curve E_b vs. E_f could be drawn.

Displacement experiments of bound proteinases were performed under saturating conditions. After binding, the lens capsules were washed quickly in 10 ml of activation buffer, and incubated in 2 ml of the same buffer containing a variable amount of E64–papain complex. 20 μl aliquots of both binding and displacement media were taken for enzymatic activity measurements, as described above. Concentration of bound proteinases was calculated as above. Quantity of displaced proteinases were estimated after subtraction of non-specific displacement, i.e. by a control experiment carried out under the same conditions without addition of papain. The displacement was calculated from the ratio:

$$\frac{\text{E displaced}}{\text{E bound}}$$

Scatchard plot, i.e. E_b/E_f vs. E_b was used to calculate K_d values where E_b = maximal concentration bound × fraction displaced. E_t = concentration of E64-papain-complex used for the displacement, and $E_f = E_t - E_b$.

3. RESULTS AND DISCUSSION

From binding kinetics, the maximal binding was observed within 30 min with complete saturation. A saturable dose-dependent binding curve was found at 37°C with the four cysteine proteinases used in this study (Fig. 1A–D). Displacement of these proteinases by E64–papain complex is shown in Fig. 2A–D. These latter results allow us to determine the dissociation constant for each proteinase basement membrane equilibrium (Fig. 3A–D). Data are summarized in Table I. Dissociation constants were found in the 10^{-7} M range and the number of binding sites was in the 10^{12} range. The weight of a lens capsule was estimated at 50.64 ± 15.4 mg (n = 100) [1]. On this basis, about 1 binding site was present for 50 pg of basement membrane. Nevertheless, a high degree of variation was observed in the competition experiments when the proteinase was changed (Fig. 2). It could be linked to the use of the same competitor, i.e. E64 papain, for the displacement of the four different cysteine proteinases. The source of such variations could be some structural differences in the binding site of each cysteine proteinase for the basement membrane. However, these differences were not significant on the Scatchard plot. This method was unable to analyze these small molecular differences.

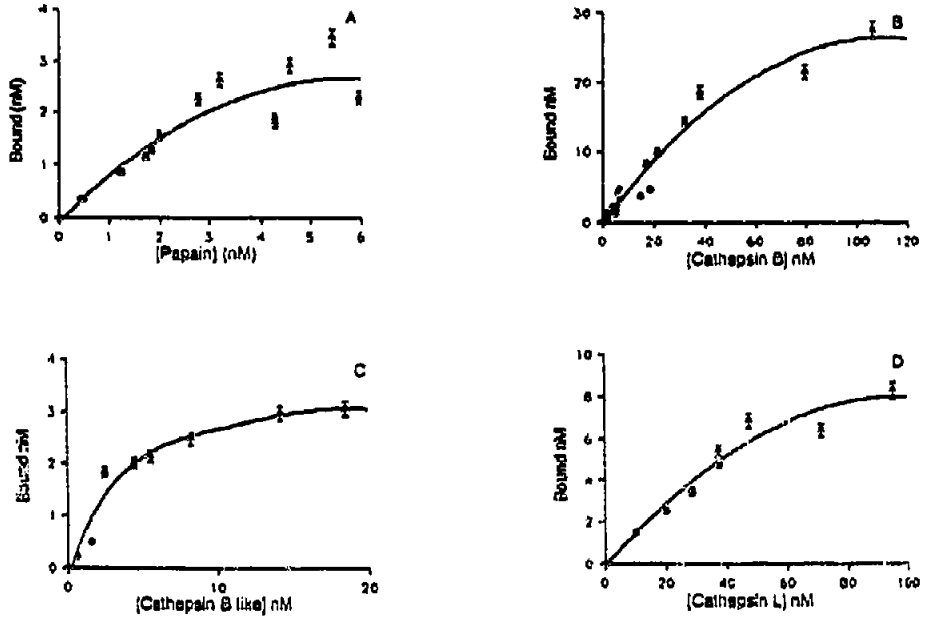

Fig. 1. Specific binding curve of four cysteine proteinases to bovine lens capsules at 37°C. Each point is the mean ± S.D. of triplicate determinations. (A) Papain, pH 6.8. (B) Cathepsin B, pH 6.0. (C) Cathepsin B-like, pH 6.8. (D) Cathepsin L, pH 6.0.

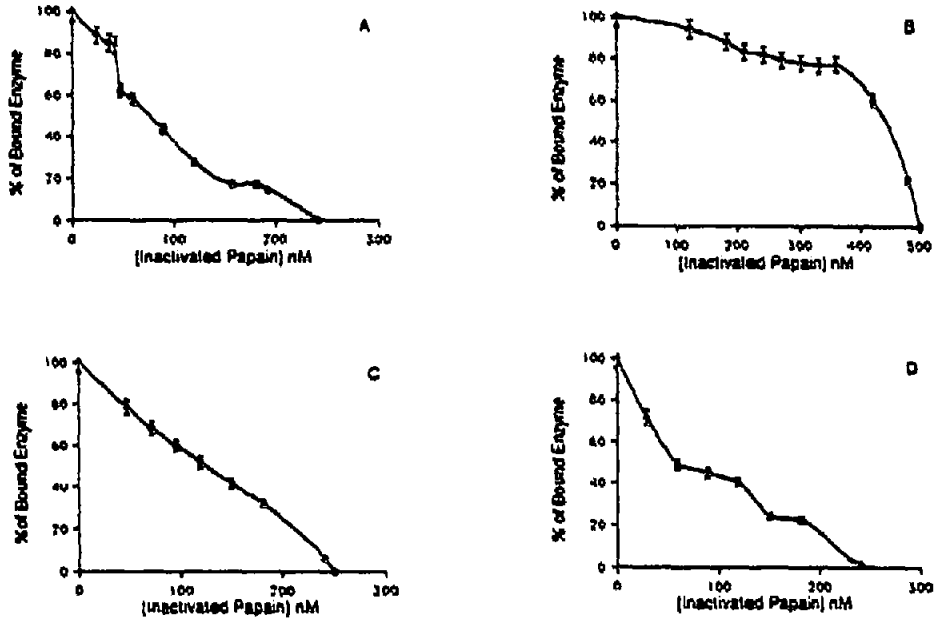

Fig. 2. Displacement of cysteine proteinases by the papain–E64 complex at 37°C. Each point is the mean ± S.D. of triplicate determinations. (A) Papain, pH 6.8. (B) Cathepsin B, pH 6.0. (C) Cathepsin B-like, pH 6.8. (D) Cathepsin L, pH 6.0.

The present results characterize the binding phenomenon of cysteine proteinases to basement membranes. As shown from both K_d and binding sites values, the active site of the proteinases was not involved in the binding on the basis of the following assumption: (i) displacement of active cysteine proteinases was found using E64 inactivated papain, i.e. papain with a blocked active site [6] and (ii) similar K_d values were found for the four cysteine proteinases: in contrast, when peptide substrates were used, different K_m values were observed. For example, with Z-Phe-Arg-NHMEC as a substrate, K_m values were 390 and 6 μM for cathepsin B and cathepsin L, respectively [7]. The mechanism of basement membrane digestion could be the following: active cysteine proteinases are bound, many peptide bond cleavages occur simultaneously, and numerous peptides are solubilized. A 72 h time dependency was observed for the digestion [1]. This could be a consequence of the binding since bound cysteine proteinases did not autolyze, in constrast to diluted purified cysteine proteinases [1].

From this mechanism, the digestion was dependent on the location of the proteinase binding sites inside the basement membranes. These sites probably represent the number of substrate sites accessible to proteinases. Nevertheless, the number of binding sites did not correspond to the number of peptide bonds hydrolyzed: as reported before [1] only a partial digestion of basement membranes by cysteine proteinases was observed in vitro. The number of binding sites represent only the number of potent digestion sites. These sites could be located on the collagen IV network: this major protein constituent of basement membranes binds other membrane constituents i.e. laminin, proteoglycan and fibronectin [10]. Consequently, it could be the source of the proteinase binding. Another type of collagen (type II collagen) was also found to be able to bind pepsin [2]. Nevertheless, some binding sites could be associated with the supramolecular structure of collagen IV and linked to the spatial organisation of the basement membrane. Further studies using isolated basement membrane constituents and combinations of such constituents could be useful for the molecular study of the binding sites.

In vivo, a large number of proteinase binding sites could be associated with the dissolution of basement membrane observed during tumour invasion [11]. A proteolytic cascade [12] linked to the secretion of proteinases of different classes by malignant cells, could increase the efficiency of both binding and digestion. Some years ago, cysteine proteinases, such as cathepsin B, were located outside malignant cells and around tumour islets by immunohistological methods [5]. This

Table I

Cysteine proteinase binding parameters to bovine lens capsules

	K_d (M)	Number of binding sites/ capsule
Papain	$3.0 \cdot 10^{-7}$	$4.6 \cdot 10^{12}$
CB	$6.6 \cdot 10^{-7}$	$22.5 \cdot 10^{12}$
CB-like	$5.6 \cdot 10^{-7}$	$13.2 \cdot 10^{12}$
CL	$1.1 \cdot 10^{-7}$	$12.8 \cdot 10^{12}$

These results were calculated from the Scatchard plots drawn in Fig. 3.

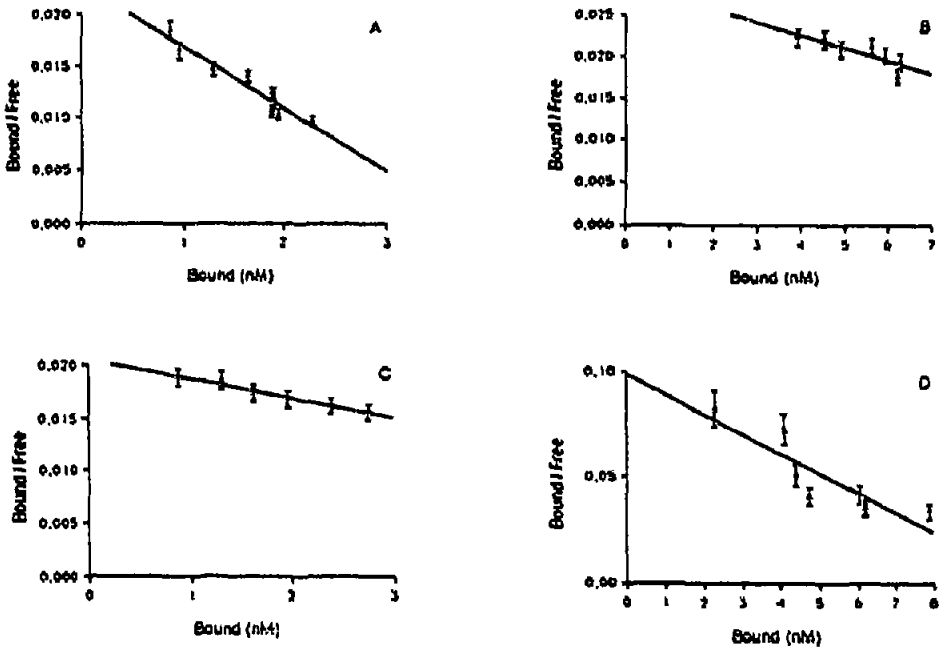

Fig. 3. Scatchard plots for cysteine proteinases binding to bovine lens capsules at 37°C. Each point is the mean ± S.D. of triplicate determinations. (A) Papain, pH 6.8. (B) Cathepsin B, pH 6.0. (C) Cathepsin B-like, pH 6.8. (D) Cathepsin L, pH 6.0.

extracellular location argues for a basement membrane binding. In conclusion, in this report the molecular basis of basement membrane digestion is pointed out. In future, a better understanding of tumour invasion and other cellular migration processes could be dependent on the development of such relationships.

Acknowledgements: This work was supported by Grant 6862 to M.P. from the 'Association pour la Recherche sur le Cancer', Villejuif, and by the Université Pierre et Marie Curie. N.G. is a recipient of a Fellowship from the 'Ministère de la Recherche et de la Technologie.'

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