

Multiple molecular forms of pyridinoline crosslinks generated by the action of cathepsin B on bone collagen: Influence of high calcium concentrations

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أجريت هذه الدراسة بهدف دراسة قدرة أنزيم، سيستئين بروتيناز اللوزومية، (الكاتبسين ب)، على توليد الصيغ الجزيئية المتنوعة للبيريدينولين من النسيج الكولجيني العظمي من النوع I، وكذلك دراسة تأثير تركيزات متباينة من أيونات ثنائية التكافؤ مثل الكالسيوم (Ca^{2+}) والمغنيزيوم (Mg^{2+}) على هذه العملية. النتائج المتحصل عليها قد أظهرت إن إضافة Ca^{2+} أو Mg^{2+} رفعت كثيرا من تحرير جزيئات البيريدينولين ومن أهم الآثار نذكر تلك التي تحصلنا عليها عند تركيزات ما بين 50-75 ملليمولر. بالدياليز والطرق الكروماتوغرافية على الهلام المرشح قمنا بتتقية وفصل الصيغ الجزيئية للبيريدينولين المرتبطة بقطع ببتيدية مختلفة الأشكال والنتيجة عن هضم النسيج العظمي بواسطة الكاتبسين ب. في محلول وافي خالي من أيونات Ca^{2+} و Mg^{2+} فإن الوزن الجزيئي (و.ج) للبيريدينولين المرتبطة كانت أقل من 3500 Da (80%). إن إضافة $MgCl_2$ (40 ملليمولر) لم يعدل بصورة ملحوظة هذا التوزيع. بينما إضافة $CaCl_2$ غيره بشكل حاد. وبالفعل، ففي وجود تركيزات مختلفة (10-40 ملليمولر) من $CaCl_2$ فإن توازن التحلل البروتيني بواسطة الأنزيم أنتقل نحو جهة توليد البيريدينولين المرتبطة ذات أوزان جزيئية عالية (و.ج > 3500 Da). تشير البيانات إلى أن هذه التركيزات العالية محليا من Ca^{2+} ، كما هو موجود في الميكروبيئي لتحلل النسيج العظمي، استطاعت بفعل أنزيم سيستئين بروتيناز اللوزومية أن تغير مواقع كسرا لروابط الببتيدية لجزيء الكولاجين العظمي.

الكلمات المفتاحية: جزيئات البيريدينولين؛ كالسيوم؛ الكاتبسين

Résumé

La présente étude porte sur la capacité *in vitro* d'une cystéine protéinase lysosomiale, la cathepsine B, de générer les formes moléculaires multiples de pyridinoline, à partir du collagène osseux insoluble de type I, et d'étudier les effets de diverses concentrations des ions bivalents tels que le calcium (Ca^{2+}) et le magnésium (Mg^{2+}) sur ce processus. Les résultats obtenus ont montré que l'addition de Ca^{2+} ou de Mg^{2+} a considérablement augmenté le dégagement des molécules de pyridinoline, les plus importants effets étant obtenus à 50-75 mM. Par dialyse et des méthodes chromatographiques sur gel filtration, nous avons séparé les différentes formes moléculaires de pyridinoline conjuguées résultant de la digestion de l'os par la cathepsine B. Dans un tampon libre de Ca^{2+} et de Mg^{2+} , le poids moléculaire (MW) de pyridinoline conjuguées était au-dessous de 3500 Da (80%). L'addition de $MgCl_2$ (40 mM) n'a pas modifié sensiblement cette répartition, alors que le $CaCl_2$ la changeait nettement. En effet, en présence de différentes concentrations de $CaCl_2$ (10-40 mM), l'équilibre protéolytique de l'enzyme a été décalé vers le dégagement de pyridinoline conjuguées ayant un poids moléculaire plus élevée (MW > 3500 Da). Ces données suggèrent que des concentrations en Ca^{2+} localement élevées, comme celles vues dans le micro-environnement de la résorption osseuse, ont pu modifier les sites protéolytiques du collagène osseux par l'intervention de la cystéine protéinase lysosomiale.

Mots clés : Molécules de pyridinoline; Calcium, Cathepsine; collagène; dégradation de la matrice osseuse.

Abstract

We investigated *in vitro* the ability of cathepsin B, a lysosomal cysteine proteinases, to generate multiple molecular forms of pyridinoline crosslinks from insoluble bone type I collagen, and we studied the effects of

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various concentrations of divalent ions such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) on this process. Our results have shown that the addition of either Ca^{2+} or Mg^{2+} greatly enhanced the release of pyridinoline crosslinks, the greatest effects being obtained at 50-75 mM. By dialysis and gel filtration chromatography methods, we attempted to separate the different molecular species of peptides containing pyridinoline crosslinks resulting from the digestion of bone with cathepsin B. In a Ca^{2+} and Mg^{2+} free buffer, the molecular weight of approximately 80% of the peptide fragments containing crosslinks was under 3500 Da. Addition of MgCl_2 (40 mM) did not modified significantly this repartition, while CaCl_2 changed it dramatically. Indeed, in presence of various concentrations of CaCl_2 (10-40 mM), the proteolytic balance of the enzyme was shifted toward the release of peptide-bound crosslinks fragments with higher size (MW > 3500 Da). These data could suggest that locally elevated concentrations of Ca^{2+} , such as those seen in the bone-resorbing microenvironment, could modify the proteolysis of bone collagen mediated by lysosomal cysteines proteinases.

Key words: pyridinoline crosslinks, Calcium, Cathepsin, collagen, bone matrix degradation

1. INTRODUCTION

Throughout life, bone is formed and resorbed in a dynamic process during bone remodeling. In certain pathologic cases, like osteoporosis, there is an imbalance in this process toward increasing bone resorption, which contributes to bone loss. Early detection of an increased level of bone resorption has now become possible thanks to the recent development of several biochemical markers [1] based on the measurement of bone collagen breakdown products, mainly pyridinoline crosslinks : hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), two non reducible crosslinks which stabilize collagen fibers in extra-cellular matrix [2,3]. The crosslinks are released during the osteoclastic bone degradation and ultimately excreted in urine, free of aminoacids or linked to peptides with various molecular weight [4,5]. The measurement of both forms currently constitutes the most reliable index of increased bone resorption [1]. However, the different enzymatic events which generate the various forms of peptide-bound crosslinks from bone collagen have not been well understood and the mechanisms regulating this process are yet unknown. Herein, using a commercially available preparation of cathepsin B - a lysosomal cysteine proteinase which is known to attack insoluble bone collagen [6, 7], we studied its ability to release multiple molecular

forms of pyridinoline crosslinks from bone collagen. Because during bone resorption osteoclasts are exposed to high levels of extracellular Ca^{2+} solubilized from bone mineral (17-40 mM) [8], it is likely that enzymatic degradation of bone collagen take place in presence of high concentration of Ca^{2+} . Therefore, we also investigated the possible influence of various concentrations of Ca^{2+} on bone collagenolysis mediated by cathepsin B.

2. MATERIALS AND METHODS

2.1 Preparation of insoluble bone collagen

We have cut bovine femoral bone into small pieces and pulverized them. The fat was extracted with acetone and the residue dried. We then extracted non mineralized proteins at 4 C for 24H in 4 mol/L guanidine/HCl in 50 mmol/L Tris/HCl buffer, pH 7.4, containing the protease inhibitors aminocaproic acid (0.1 mol/L), benzamidine/HCl (5 mmol/L) and phenyl-methylsulfonyl fluoride (1 mmol/L). After washing the residue, we demineralized it by extracting it five times for 24H with 0.5 mol/L EDTA (pH 7.4). Finally, we repeatedly washed the insoluble residue with distilled water and lyophilized it. As a source of collagen, we also used insoluble type I collagen from bovine achilles tendon, commercially available (Sigma, France).

2.2 Digestion of bone collagen with Cathepsin B

We suspended 100 mg of the lyophilized residue or insoluble type I collagen from bovine achilles tendon in 3 ml of acetate buffer (pH 4.1), and we then added the cathepsin B solution (purchased from Sigma, France) at the concentration of 8 μ M. The mixture was incubated at 37°C for 24 H and centrifuged. In order to test the effects of divalent ions, CaCl₂ or MgCl₂ were added at various concentrations (10 to 100 mM) to the incubation mixture, and pyridinoline crosslinks were measured, as described below, in the supernatant. The different molecular species of peptide-bound crosslinks released after the action of cathepsin B on insoluble collagen in absence or in presence of MgCl₂ or CaCl₂ (10 to 40 mM) were separated either by dialysis using 3500, and 10000 Da molecular weight (MW) cut-off membranes (Spectra/por, Bioblock Scientific, France) or by gel filtration chromatography on sephadex G25 which had been previously calibrated with known molecular weight peptides

2.3 Pyridinoline crosslinks assay

The pyridinoline crosslinks content (HP+LP) in the supernatant obtained after digestion of bone collagen with cathepsin B and in all dialysis fractions or in fractions collected from the G25 column was determined after hydrolysis by HPLC, according to a method described previously [9]. Briefly, each sample was hydrolyzed with 6M HCL for 3h at 125°C, then extracted with a CF-1 cellulose column (Whatman). The crosslinks were eluted with water, then they were separated on a C18 reversed-phase column. HP and LP were detected by measuring fluorescence with excitation at 297 nm and emission at 380 nm. Free pyridinoline crosslinks were determined by the same method, but by omitting the

hydrolysis step. The inter-assay variation of the HPLC method expressed by the coefficient of variation (CV) was less than 10%. The result of each fraction was expressed as a percent of the total pyridinoline content of whole fractions.

4. RESULTS

As shown in figure 1, the addition of increasing Ca²⁺ and Mg²⁺ concentrations (10 - 100 mM) enhanced the amount of pyridinoline released after bone collagenolysis by cathepsin B, with a maximal effect obtained around 50-75 mM. At these concentrations, the amount was increased by a factor ranged between 2 and 3 as compared to the one obtained in absence of salts. In our conditions, no free pyridinoline were released and only peptide-bound crosslinks have been measured as a result of collagen degradation. By using serial dialysis with several molecular weight cut-off membranes, we attempted to separate the different molecular species of peptides containing pyridinoline generated by the action of cathepsin B on bone collagen.

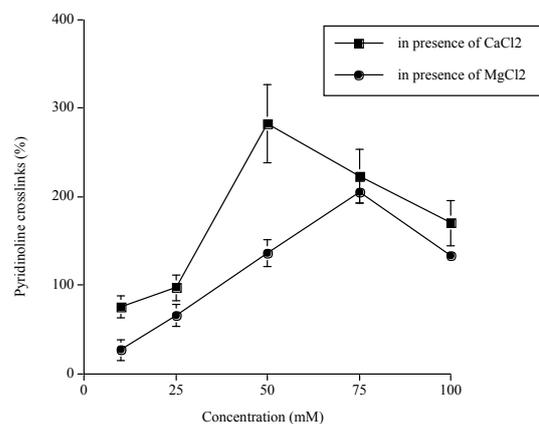


Figure 1. Amount of pyridinoline crosslinks released after bone collagen degradation with cathepsin B in presence of various concentrations of either MgCl₂ or CaCl₂ and expressed as a percentage of control (in absence of divalent ions). Results represent the mean (\pm SEM) of three different experiments.

As shown in figure 2, in the Ca^{2+} and Mg^{2+} -free buffer (control), the MW of more than 80% of bone collagen degradation products containing pyridinolines was under 3500 Da. When Ca^{2+} was added to the buffer (40 mM), the proteolytic balance was shifted toward the release of peptide-bound crosslinks of higher MW (>3500 Da) which then represented the major part of bone collagen degradation products containing crosslinks (approximately 80%). Replacement of Ca^{2+} by Mg^{2+} had no significant effect on the repartition of multiple molecular forms of pyridinoline crosslinks. Similar results have been obtained by using insoluble type I collagen from bovine achilles tendon instead of insoluble bone collagen (*data not shown*).

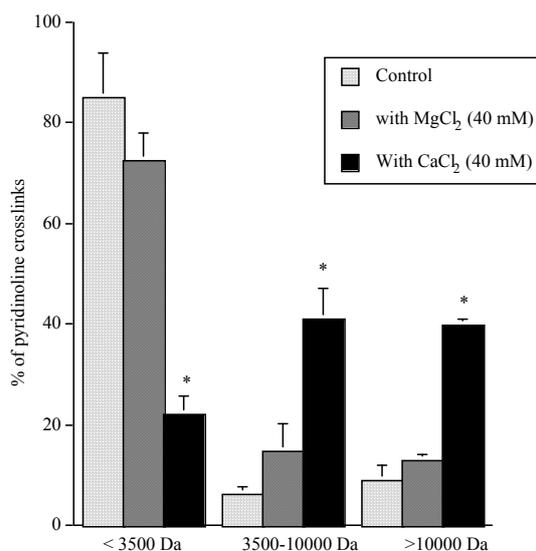


Figure 2. Multiple molecular forms of peptide-bound pyridinoline crosslinks resulting from the action of cathepsin B on bone collagen in absence or in presence of 40 mM either of CaCl_2 or MgCl_2 , and separated by dialysis using 3500, and 10000 Da molecular weight cut-off membranes. (Each bar represents the mean \pm SEM of four different experiments, the pyridinoline content of each fraction was expressed as a percent of pyridinoline content of whole fractions, * : $p < 0.001$ versus control by unpaired two-tailed student's *t*-test.).

Because the nominal molecular weight cut-offs for dialysis membrane are not really reliable, we also used a more specific method such as gel filtration chromatography (fig. 3). This method allowed us to obtain results which were comparable to those obtained with dialysis membranes. In order to see whether the generation of high molecular weight fragments was merely due to interactions between Ca^{2+} and peptides, we also analyzed bone collagen breakdown products by gel filtration chromatography in presence of EDTA. As it is shown in figure 3, the addition of EDTA did not modify the elution profile of the reaction products obtained in presence of Ca^{2+} .

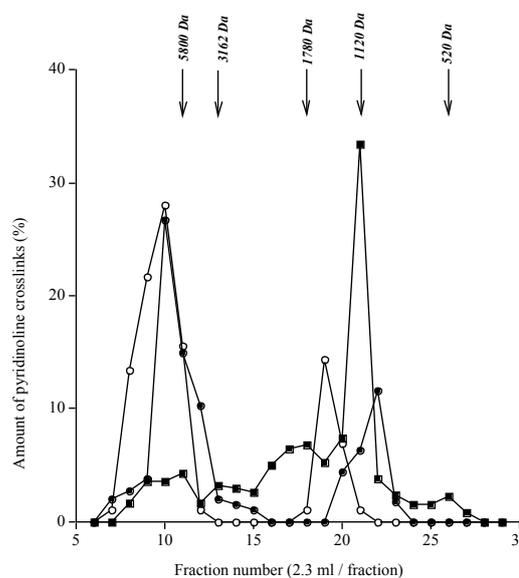


Figure 3. Separation by Gel filtration chromatography on sephadex G25 of bone collagen breakdown products containing pyridinoline crosslinks and resulting from the action of cathepsin B in absence \blacksquare , and in presence of Calcium (40 mM) eluted without \bullet or with EDTA \triangle . (The results of each fraction are expressed as percent of whole fractions; the arrows indicate the elution position of peptide standards with known MW).

By using various Ca^{2+} concentrations (0 - 40 mM), we have shown (fig. 4) that the size of the different peptide fragments

containing pyridinoline crosslinks released from bone collagen varied in a dose dependent manner according to Ca^{2+} concentration.

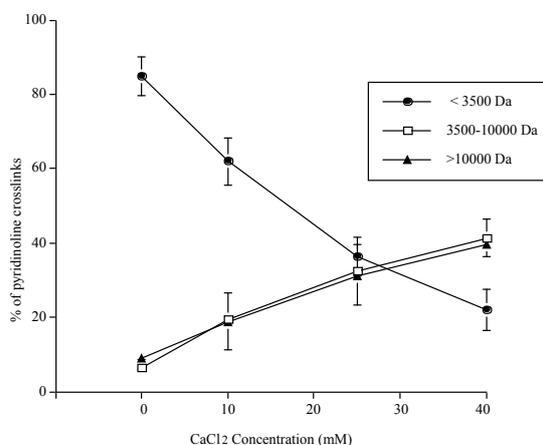


Figure 4. Multiple molecular forms of peptide-bound pyridinoline crosslinks resulting from the action of cathepsin B on bone collagen in presence of different concentrations of Calcium (each data point represents the mean \pm SEM of four different experiments).

The proteolysis profile raises the possibility that Ca^{2+} dependent proteases such as calpain, potentially present in the cathepsin B preparation, could be involved in the degradation of collagen molecules. But by using purified calpain, we checked that these enzymes were unable to degrade the substrate in our conditions (*ie* pH 4.1).

5. DISCUSSION

So far, the mechanisms by which osteoclastic enzymes degrade collagenous matrix during bone resorption are not completely elucidated. Both lysosomal cysteine proteinases [10-12] and matrix metalloproteinases (MMP) [13-15] have been involved in bone resorption process. Although both classes of enzymes may cooperate in bone collagen degradation, the acidic pH generated in the bone resorbing compartment by proton pump [8] is not compatible with MMP's

activities and it has been proposed that the degradation of organic matrix is due firstly to the collagenolytic action of lysosomal cysteine proteinases [16]. These enzymes solubilize collagen by initially cleaving the non helical telopeptide extensions that are involved in the crosslinking of the molecules. They then attacked the helical region, which allowed a rapid degradation to low molecular weight peptides [6]. Among peptides resulting from bone collagen degradation, many interests have been focused during recent years on the N- and C- crosslinked telopeptide fragments of type I collagen [17-19], because these latter have proved their usefulness as biochemical markers of bone resorption [1]. The aim of our investigation was to study the ability of cathepsin B to generate multiple molecular forms of pyridinoline crosslinks, and since there is likely to be a high concentration of calcium at the site of bone resorption, to test the effects of calcium salts on this process. The amount of collagen crosslinks released after digestion of bone with cathepsin B is greatly enhanced in presence of CaCl_2 , the greatest effects being obtained around 50 mM, but this effect was not specific for calcium, since the addition of MgCl_2 was also effective. Similar results have been previously reported by Etherington et al. [20] and by Eeckhout [21] who have shown by measurement of hydroxyproline that the amount of collagen solubilized in presence of high concentrations of calcium and magnesium salts by lysosomal cysteine proteinases was increased more than four-fold. In our conditions, no free pyridinoline were released suggesting that cathepsin B was unable to degrade completely insoluble bone collagen, and only peptide-bound crosslinks have been measured after digestion of bone. Cathepsin B led to the release of a mixture of different molecular species of pyridinoline, principally those with low molecular weight (< 3500 Da).

The addition of CaCl_2 but not MgCl_2 , changed dramatically and in a dose-dependent manner the repartition of multiple molecular forms of pyridinoline crosslinks. Indeed, in presence of 40 mM of Ca^{2+} (which corresponds to the greatest concentration found in sub-osteoclastic resorption zone) the size of peptide fragments containing pyridinoline were much higher than those obtained in absence of Ca^{2+} or in presence of Mg^{2+} . The mechanism whereby Ca^{2+} could exert its effects remains unclear, but recently Eeckhout [21] suggested that these effects could be on substrate rather than on enzyme activity. Interaction between Ca^{2+} and collagen could induce changes in the substrate conformation as it has been shown for other bone proteins such osteocalcin [22] and osteonectin [23] or non bone proteins such chromatin [24], and troponin [25]. These conformational changes may facilitate protease-mediated cleavage by increasing the accessibility of susceptible bonds in the substrate leading to the release of higher MW peptides. Recent studies [26,27], using synthetic peptides mimicking the cleavage site in the native collagen by collagenase, have shown with structural data derived from Circular Dichroism spectroscopy and Fourier Transformation Infrared Spectroscopy, that Ca^{2+} bound to peptides and caused changes in the substrate conformation, which are responsible for the specific cleavage site of collagen. These findings are consistent with our hypothesis that, in the Ca^{2+} rich extracellular fluid, modifications in the conformational state of bone collagen could play an important role in the cathepsin-mediated cleavage of bone collagen. Our present results do not in themselves give any direct indication of the likely physiological role of cathepsin B on bone collagen degradation and it has been shown recently that cathepsin B is much less expressed than cathepsin K in human osteoclasts, which seems to be the predominant cysteine proteinase present

in bone [28,29]. However, if Ca^{2+} acts on collagen substrate rather than on enzyme activity, one may speculate that the results obtained herein using cathepsin B, could be extended to cathepsin K. Further experiments are in progress in our laboratory to verify these suggestions.

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