Heparin-like dextran derivatives as well as glycosaminoglycans inhibit the enzymatic activity of human cathepsin G

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Abstract **Some synthetic dextran derivatives that mimic the action of heparin/heparan sulfate were previously shown to inhibit neutrophil elastase and plasmin. Here we report that these derivatized dextrans also inhibit cathepsin G (CatG). Dextran containing carboxymethyl and benzylamide groups (RG1150) as well as those containing carboxymethyl, sulfate and benzylamide groups (RG1192), were the most efficient inhibitors of CatG activity. RG1192 and RG1150 bind CatG with a K_i of 0.11 and 0.17 nM, respectively, while carboxymethylated sulfated dextran (RG1503) as well as heparin, heparan sulfate and dermatan sulfate bind CatG with a 7- to 30-fold lower affinity. Variation of K_i with ionic strength indicates that ionic interactions account for 26% of the RG1503–CatG binding energy, while binding of RG1192 or RG1150 to CatG is mainly governed by non-electrostatic interactions. This, together with the fact that these compounds both protect fibronectin and laminin against CatG-mediated degradation, suggest that specific dextran derivatives can contribute to the regulation of CatG activity.**

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Key words: Cathepsin G; Regenerating agent; Glycosaminoglycan; Enzyme kinetics

1. Introduction

Cathepsin G (CatG) is one of the major serine proteinases found in an active form within neutrophils and monocytes [1]. CatG has a number of physiological functions which take place during inflammation following tissue injury, and include degradation of phagocytosed proteins, neutrophil migration and extracellular matrix (ECM) remodeling [1]. In vitro, CatG is able to cleave a wide variety of ECM components such as type IV collagen, laminin, fibronectin, elastin and proteoglycans [1]. These findings thus point out an important role for CatG in ECM remodeling during tissue degeneration and repair.

We have previously elaborated on and investigated a new family of polysaccharides, derived from dextran by chemical substitutions and namely regenerating agents (RGTA). The controlled and sequential chemical substitutions of functional groups such as carboxymethyl, sulfate and/or benzylamide along the dextran molecule allowed us to obtain a family of dextran derivatives with different chemical structures and biological activities. We have shown that dextran containing carboxymethyl, sulfate and benzylamide groups (RG1192 compound) can stimulate tissue repair in various in vivo wound-healing models such as cutaneous wound [2], cranial bone defect [3], infarcted myocardium [4], ischemic skeletal muscle [5] and colic ulceration [6]. In addition, we have just reported that carboxymethylated sulfated dextran (RG1503 compound) can also act as a tissue repair agent by both enhancing bone formation and reducing gingival inflammation in a hamster model of periodontitis [7]. In contrast, dextran substituted only with carboxymethyl (RG1100 compound) as well as those containing carboxymethyl and benzylamide groups (RG1150 compound) did not display in vivo tissue repair activity [8], thus pointing out the beneficial contribution of sulfate groups with respect to the in vivo biological activity of these biopolymers. In vitro experiments have, moreover, revealed that RG1503 and RG1192 could act as mimics of heparin/heparan sulfate with regard to their ability to interact with, stabilize and protect various heparin binding growth factors against proteolytic degradation ([9,10] and Rouet, V. et al. Heparan sulfate mimetics enhance VEGF165 activities, manuscript in preparation). In addition, RG1192 and to a lesser extent RG1503, have been shown to directly inhibit the enzymatic activity of neutrophil elastase [11] and plasmin [12], and to protect matrix proteins from proteolytic degradation. Based on their in vitro properties, we propose that RGTA molecules such as RG1192 and RG1503 improve regeneration because they increase the bioavailability of growth factors and can modify certain proteinase activities during the various steps of normal wound healing.

We present here data that support this mechanism of action of RGTA through the in vitro inhibition of CatG enzymatic activity.

2. Materials and methods

2.1. Materials

The substrates succinyl-Ala$_2$-Pro-Phe-*p*-nitroanilide (Suc-Ala$_2$-Pro-Phe-*p*Na) and succinyl-Ala$_2$-Pro-Phe-thiobenzylester (Suc-Ala$_2$-Pro-Phe-SBzl) came from Bachem (Budendorf, Switzerland). Human CatG was purchased from ICN (Irvine, CA, USA) and was active site-titrated with titrated α1-PI [13]. 5,5′-dithiobis(2-nitrobenzoic acid), human fibronectin, Engelbreth–Holm–Swarm mouse sarcoma laminin, polyclonal rabbit anti-fibronectin or laminin Ig, heparan sul-

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Abbreviations: CatG, cathepsin G; RGTA, regenerating agent; GAG, glycosaminoglycan; ECM, extracellular matrix

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fate (average M_r 60,000), chondroitin 4-sulfate (average M_r 17 000 Da), chondroitin 6-sulfate (average M_r 32 000 Da), and dermatan sulfate (average M_r 25 000 Da) were obtained from Sigma (St Louis, MO, USA). Porcine intestine heparin (average M_r 12 000 Da) was kindly provided by M. Petitou from Sanofi (France). The average molecular weights (M_r) of these glycosaminoglycans (GAG) were determined by size exclusion chromatography using gel filtration columns (Shodex, Japan). Horseradish peroxidase-conjugated goat anti-rabbit Ig was from Diagnostic-Pasteur (Marne la Coquette, France). Immobilon P and electrochemiluminescence were purchased from Millipore Corporation (Saint-Quentin en Yvelines, France) and Superblock® blocking buffer was from Pierce (Rockford, IL, USA).

RGTA are water-soluble dextran derivatives obtained by controlled and sequential substitutions of CH_2COONa (CM), SO_3Na (Su) and/or $CH_2CONHCH_2C_6H_5$ (CMBn) groups on the OH residues of glucose units. Synthesis, structure, chemical characterization and average M_r determination of these polymers have been previously described in detail [12]. Briefly, compound RG1100 (average M_r 51 000 Da) was synthesized from dextran T40 (average M_r 37 000 Da; Pharmacia, Sweden) by carboxymethylation of OH residues. Compound RG1503 (average M_r 72 000 Da) was synthesized from RG1100 by *O*-sulfonation of the free OH residues. Compound RG1150 (average M_r 72 000 Da) was synthesized from a carboxymethylated dextran by amidation of the carboxylate residues with benzylamine (NH_2-$CH_2C_6H_5$). Compound RG1192 (average M_r 78 000 Da) was obtained by *O*-sulfonation of RG1150. The chemical characterization of all dextran derivatives was based on the degree of substitution (d.s.) of each individual group per glucosidic unit. The d.s. values of each group for the different polymers were as follows: CM = 0.6 for RG1100; CM = 0.6 and Su = 1.1 for RG1503; CM = 0.8 and CMBn = 0.4 for RG1150; CM = 0.8, Su = 0.3 and CMBn = 0.4 for RG1192. Measure of the reductive sugars content indicates that dextran T40 contains an average of 250 glucosidic units per chain. The RGTA polymers present very low anti-coagulant activity, i.e. less than 10 IU/mg as compared to 180 IU/mg for heparin [9].

2.2. Enzymatic methods

Enzymatic kinetics were monitored with a thermostated Labsystem iEMS spectrophotometer on-line with a PC. The progress curves were recorded for 1–10 min, depending upon the reaction velocity and less than 5% of the substrate was hydrolyzed during the rate measurement. The hydrolysis of Suc-Ala_2-Pro-Phe-*p*Na was recorded at 405 nm (ε_{405} = 9650 M^{-1} cm^{-1}) while that of Suc-Ala_2-Pro-Phe-SBzl was monitored at 412 nm in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (0.2 mM), a coupling reagent that transforms benzylthiol, the product of substrate hydrolysis, into a yellow-colored compound (ε_{412} = 13 600 M^{-1} cm^{-1}) [14]. Substrates and 5,5′-dithiobis(2-nitrobenzoic acid) were dissolved in *N*,*N*-dimethyl-formamide whose final concentration in the reaction media was 3% (v/v). The buffer was 50 mM HEPES, 100 mM NaCl, pH 7.4, 25°C, unless otherwise stated. The effect of various polymers on the CatG enzymatic activity was determined by reacting constant concentration of enzyme with increasing concentrations of polymers for 5 min and measuring the residual enzymatic activity with a synthetic substrate. The equilibrium dissociation constant (K_i) values and their standard errors were calculated by non-linear regression using the integral equation editor of the GraphPad Prism software (San Diego, CA, USA).

2.3. Western blot analysis of fibronectin or laminin proteolytic fragments

CatG (500 nM) and polymers were incubated at 37°C for 30 min in 50 mM HEPES pH 7.4, 100 mM NaCl, 0.01% Triton X-100 prior to addition of 50 ng of fibronectin (3.8 nM) or 100 ng of laminin (3.8 nM). Following 1 h of incubation, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and then electrophoretically transferred overnight at 4°C to Immobilon-P membrane in 25 mM Tris pH 8.3, 192 mM glycine. Membranes saturated with Superblock® blocking buffer were incubated with polyclonal rabbit anti-human fibronectin Ig or polyclonal rabbit anti-mouse laminin Ig at 1:1000 dilution in phosphate-buffered saline containing 0.02% (v/v) Tween-20 and 0.3% Superblock® blocking buffer. Antibodies were detected by using horseradish peroxidase-conjugated goat anti-rabbit IgG and electrochemiluminescence according to the manufacturer's recommendations.

3. Results and discussion

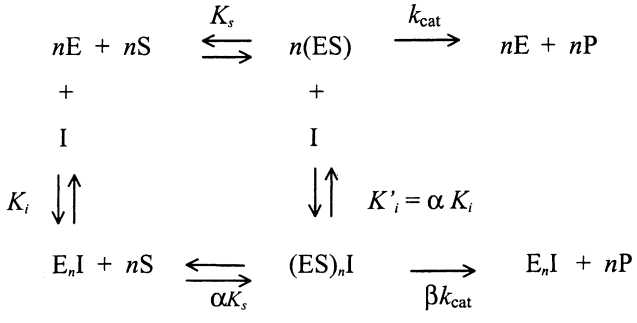
3.1. Effect of GAG and RGTA on the amidolytic activity of CatG

Fig. 1A shows the effect of different GAG on the amidolytic activity of CatG. As previously reported [13,15], heparin inhibits partially the enzymatic activity of CatG, indicating that heparin tightly binds the enzyme to form complexes that are partially active on substrate. We showed, moreover, that heparan sulfate and dermatan sulfate also exhibit an inhibitory action on CatG characterized for both polymers by 40% of residual enzymatic activity. In contrast, chondroitin 4-sulfate and chondroitin 6-sulfate did not affect the activity of CatG even for concentrations greater than 0.5 μM.

We have previously shown that some dextran derivatives, namely RGTA, acted as mimics of heparin with regard to their ability to inhibit neutrophil elastase [11]. Based on this functional homology, we then evaluated their capacity to modulate the enzymatic activity of CatG. Fig. 1B shows that dextran substituted with carboxymethyl groups (RG1100) weakly affected the activity of CatG. Following *O*-sulfonation, the resulting derivatized polymer (RG1503) showed an inhibitory potential toward CatG with 40% of residual enzymatic activity. When hydrophobic benzylamide groups were additionally coupled to a carboxymethylated dextran (RG1150) or to a carboxymethylated sulfated dextran (RG1192), a more potent inhibitory activity was observed, characterized by an inhibition of more than 90% of the CatG enzymatic activity (Fig. 1B).

3.2. Study of the mechanism of CatG inhibition by RGTA and GAG

Heparin, heparan sulfate, dermatan sulfate and all RGTA tested form complexes with CatG that remain partially active on synthetic substrate. This type of inhibition may be described by Scheme 1 where E, S, and I stand for enzyme, substrate, and polymers, respectively, K_i is the equilibrium dissociation constant of the enzyme–inhibitor complex, n is the number of enzyme molecules bound per molecule of polymer, and α and β are dimensionless numbers representing the change in affinity (α) and the change in the catalytic rate constant (β).



Scheme 1.

With tight-binding reversible inhibitors, inhibition depends upon the total enzyme concentration since the concentration of the bound inhibitor [EI] is no longer negligible with respect to that of total inhibitor concentration $[I]_0$ [16]. Such a mechanism has been already described for the inhibition of CatG by heparin [13]. We therefore analyzed whether RGTA molecules as well as other GAG behaved as tight-binding inhib-

itors by measuring the inhibition of CatG using different enzyme concentrations. As shown in Fig. 2, inhibition profile of CatG by RG1192 was modified by an increase of the enzyme concentration $[E]_0$; $IC_{50} = 0.3$ nM and 0.6 nM for $[E]_0 = 1.5$ nM and 3 nM, respectively. Similar results were obtained with RG1503, RG1150, as well as with heparin, heparan sulfate and dermatan sulfate (data not shown), indicating that all these polymers acted as tight-binding inhibitors of CatG. Use was made of this particular behavior to measure the binding stoichiometry of each polymer–CatG complex using high reactant concentration of enzyme ($[E]_0/K_i \gg 100$) to ensure pseudo-irreversible binding of CatG to inhibitor [16]. In the case of the RG1192–CatG complex (see Fig. 2, inset), the inhibitor/enzyme titration curve intercepted the abscissa at $[I]_0/[E]_0 = 0.25$, indicating that about four molecules of CatG bound to one molecule of RG1192 ($n = 4$). The n values for

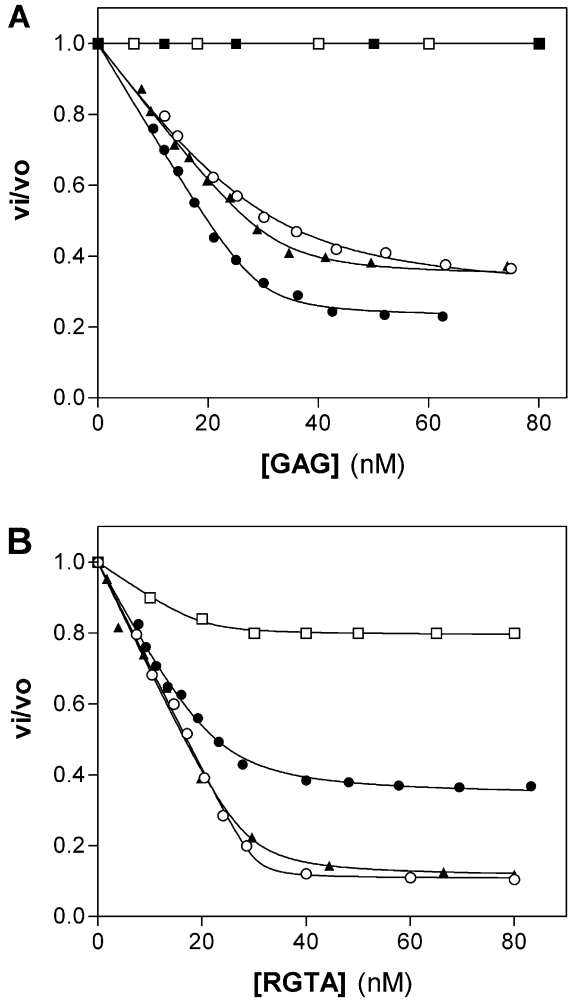


Fig. 1. Effects of different GAG (A) and RGTA (B) on the enzymatic activity of CatG. Increasing concentrations of chondroitin 4-sulfate (□, A), chondroitin 6-sulfate (■, A), heparan sulfate (○, A), heparin (●, A) or dermatan sulfate (▲, A) as well as RG1503 (●, B), RG1100 (□, B), RG1150 (▲, B), RG1192 (○, B) were added to CatG (30 nM) at pH 7.4, 25°C, and the residual enzymatic activity was measured with Suc-Ala_2-Pro-Phe-*p*Na (2 mM). Abscissa, [GAG] or [RGTA], nanomolar concentrations of GAG or RGTA in the assay multiplied by the number of binding sites, respectively. Ordinates, v_i/v_0, initial rate in the presence of inhibitor/initial rate in its absence.

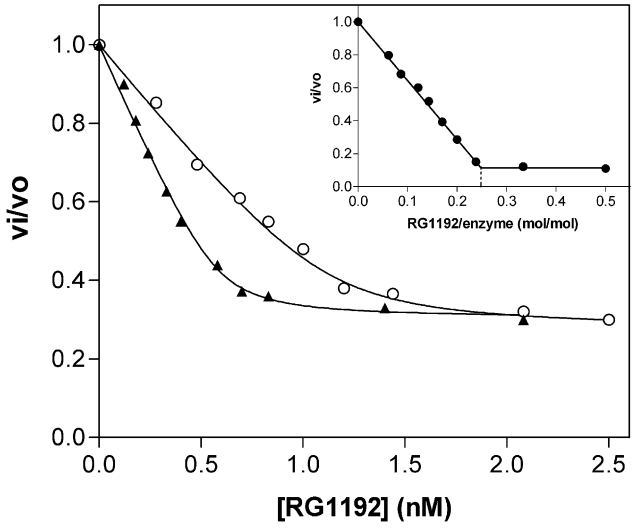


Fig. 2. Determination of the equilibrium dissociation constant and the stoichiometry of the RG1192/CatG complex at pH 7.4 and 25°C. The CatG concentrations ($[E]_0$) were 1.5 nM (▲), 3 nM (○) and 30 nM (●, inset). The substrate was Suc-Ala_2-Pro-Phe-SBzl (80 μM) for $[E]_0 = 1.5$ and 3 nM and Suc-Ala_2-Pro-Phe-*p*Na (2 mM) for $[E]_0 = 30$ nM. The inset shows a titration experiment, which allows determining the RG1192/CatG binding stoichiometry. [RG1192], nanomolar concentration of RG1192 in the assay. v_i/v_0, see legend to Fig. 1.

other polymer–CatG complexes could also be determined in this way (Table 1).

With lower CatG concentrations, the inhibition curves were concave so that the data could be used to calculate K_i by non-linear regression analysis (see Fig. 1A for GAG, Fig. 1B for RG1503 and Fig. 2 for RG1192). Since these polymers behaved as tight-binding inhibitors, K_i was calculated from the complex steady-state rate equation derived by Szedlacsek et al. [17] which takes into account both the tight-binding character and the incompleteness of inhibition (equation not shown). Table 1 summarizes the parameters characterizing the inhibition of CatG by heparin, heparan sulfate and dermatan sulfate. As previously described [13,15], heparin inhibited CatG according to a reversible, tight-binding, hyperbolic

Table 1
Parameters characterizing the inhibition of CatG by RGTA and GAG

Polymer	α	β	K_i (M)	n
RG1503[a]	1.4	0.43	$(1.2 \pm 0.3) \times 10^{-9}$	22
RG1150[b]	7	0.7	$(0.17 \pm 0.02) \times 10^{-9}$	4
RG1192[b]	7	0.9	$(0.11 \pm 0.04) \times 10^{-9}$	4
Heparin[a]	1.4	0.27	$(0.8 \pm 0.2) \times 10^{-9}$	3
Heparan sulfate[a]	1.4	0.36	$(3.0 \pm 0.4) \times 10^{-9}$	12
Dermatan sulfate[a]	1.5	0.42	$(0.8 \pm 0.2) \times 10^{-9}$	5

K_i and its standard error were calculated by non-linear regression analysis and the given value corresponds to the best estimate obtained from one fit curve among three independent experiments for each polymer. Standard deviation between the three experiments is less than 15%. The buffer was 50 mM HEPES, 100 mM NaCl, pH 7.4, 25°C.

[a] α, β and K_i were measured with Suc-Ala_2-Pro-Phe-*p*Na (2 mM) ($K_m = 2.3$ mM, $k_{cat} = 3.2$ s^{-1}) and [CatG] = 25 nM whereas n was determined with [CatG] = 500 nM and the same substrate.

[b] α, β and K_i were measured with Suc-Ala_2-Pro-Phe-SBzl (80 μM) ($K_m = 50$ μM, $k_{cat} = 30$ s^{-1}) and [CatG] = 3 nM whereas n was determined with [CatG] = 30 nM and Suc-Ala_2-Pro-Phe-*p*Na (2 mM) as substrate.

mixed-type mechanism ($0<\beta<1$ and $\alpha>1$). Here we show that heparan sulfate and dermatan sulfate also inhibit CatG according to the same mechanism. Analysis of the inhibition parameters reported in Table 1 reveals that heparin and dermatan sulfate bind CatG with identical affinities (0.8 nM) while heparan sulfate displays a 4-fold lower affinity. This result is surprising since it does not well correlate with the negative charge content of the polysaccharide studied. Indeed, dermatan sulfate and heparan sulfate both contain two negative charges per disaccharide unit (one carboxylate and ~one sulfate group) while one disaccharide unit of heparin bears an average of 3.5 negative charges (one carboxylate and ~2.5 sulfate groups) [18]. These results suggest that the polysaccharide–CatG interaction is not strictly dependent on charge density and that glycosidic linkage, type of saccharide present, and molecular weight may also be important in displaying the interacting groups of the polysaccharide in a productive manner. However, the fact that the poorly sulfated chondroitin 4- and 6-sulfates (~0.15 sulfate group/disaccharide unit) do not bind to CatG suggests an essential role of negatively charged groups in the enzyme/polysaccharide complex formation, most probably by establishing ionic interactions between the two partners (see the paragraph below). The 12-kDa heparin chain used in this study was shown to bind three molecules of CatG and, in a more general way, results indicated that stoichiometry increased with the increasing average M_r of the polysaccharides studied; $n=3$, 5, 12 for heparin (M_r 12 000 Da), dermatan sulfate (M_r 25 000 Da) and heparan sulfate (M_r 60 000 Da), respectively. Considering that one disaccharide unit of heparin has a molecular mass of about 0.6 kDa [18], the 12-kDa heparin chain used here is composed of about 20 disaccharide units. Hence, we may conclude that 13–14 glucosidic units of heparin were required for a 1:1 heparin:CatG binding stoichiometry. It is noteworthy that this size of binding site is similar to the one involved in the binding of a 5.1-kDa heparin fragment to CatG [15] as well as in the binding of heparin with leukocyte elastase [19], mucus proteinase inhibitor [20] or secretory leukocyte protease inhibitor [21].

Table 1 also described the parameters characterizing the inhibition of CatG by RG1503, a carboxymethylated sulfated dextran, and by RG1150 and RG1192, two RGTA compounds containing hydrophobic benzylamide groups. First, it can be seen that RG1503 bound CatG with a K_i value in the nanomolar range and inhibited its enzymatic activity according to a hyperbolic mixed-type mechanism. Moreover, one molecule of RG1503 interacted with 22 molecules of CatG ($n=22$), indicating that about 12 glucosidic units of RG1503 were involved in the binding of one CatG molecule. These results, compared to those described above, suggest that this carboxymethylated sulfated dextran inhibits the CatG enzymatic activity according to a heparin-like mechanism. Interestingly, it appears also from Table 1 that RG1150 and RG1192 thoroughly differ from RG1503 in their ability to inhibit the CatG activity. Indeed, (i) they have a 10-fold higher affinity for CatG than RG1503 and, (ii) they form with CatG a 1:4 binding stoichiometry ($n=4$), a 5-fold lower value than the one determined for the RG1503–CatG complex. If we assume that all RGTA contain the same content of glucosidic units per chain, the difference in stoichiometry observed between benzylamided and non-benzylamided RGTA could be interpreted as a difference in displaying their interacting groups in a productive manner toward the protein, that is to say a difference in their three-dimensional conformation. This idea is supported by preliminary studies (D. Papy, personal communication) using computer analysis, which showed that benzylamided dextran (RG1150 and RG1192) can form globular-like structures in aqueous solution, while non-benzylamided dextran (RG1503) preferentially adopts a linear and helicoidal-like conformation. In any event, these results clearly indicate that chemical substitution of polysaccharides by both anionic and hydrophobic residues leads to a very efficient class of CatG inhibitor, represented here by RG1150 and RG1192 compounds. Moreover, this class of molecules constitutes to our knowledge, one of the most powerful reversible inhibitors of CatG, characterized by K_i values lower or far lower than those previously reported including natural GAG (our results and [15,22]), DNA [23], proteins [24,25] as well as synthetic molecules [26–28].

3.3. *Influence of ionic strength on the* K_i *of the RGTA–CatG complexes*

CatG is a very basic protein (pI~12) characterized by an extremely large number of positively charged residues (34 arginines, four lysines), 36 of which are not compensated by neighboring counter charges [29]. Most of the arginine residues are arranged on the surface site opposite to the active site of the protein, although three of them are located in the immediate vicinity of the active site [29]. It was previously shown that binding of heparin to CatG was governed by electrostatic interactions [15,19]. In order to determine whether ionic interactions are also involved in its binding to RG1503, RG1150 or RG1192, the ionic strength dependence of the equilibrium dissociation constants K_i was analyzed according to the theory of macromolecule–polyelectrolyte interactions [30,31]. The theory predicts that binding of a protein (CatG) to a polyelectrolyte (polyanionic polymer) in the presence of a counterion (Na^+) will result in the stoichiometry

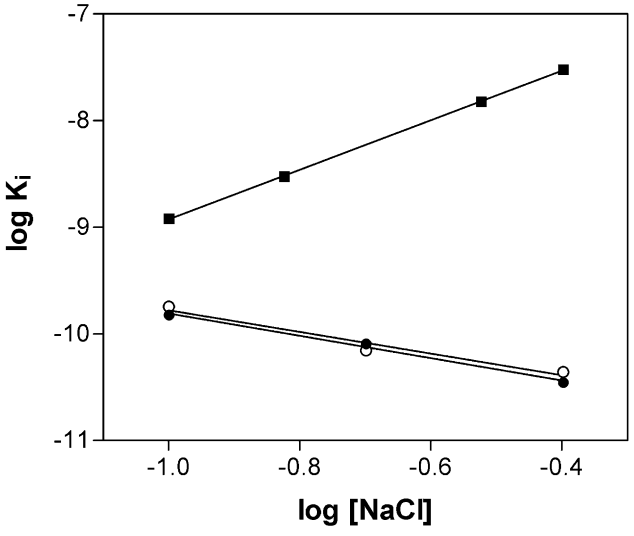


Fig. 3. Influence of ionic strength on the equilibrium dissociation constant K_i of RG1503–CatG (■), RG1150–CatG (○) and RG1192–CatG (●) complexes measured at pH 7.4 and 25°C (50 mM HEPES buffer). K_i values were plotted as a function of [NaCl] (0.1–0.4 M) in accordance to Eq. 1 and the data were analyzed by linear regression. K_i were measured with Suc-Ala_2-Pro-Phe-SBzl (80 μM) and [CatG] = 3 nM for RG1150 and RG1192 and with Suc-Ala_2-Pro-Phe-*p*Na (2 mM) and [CatG] = 25 nM for RG1503.

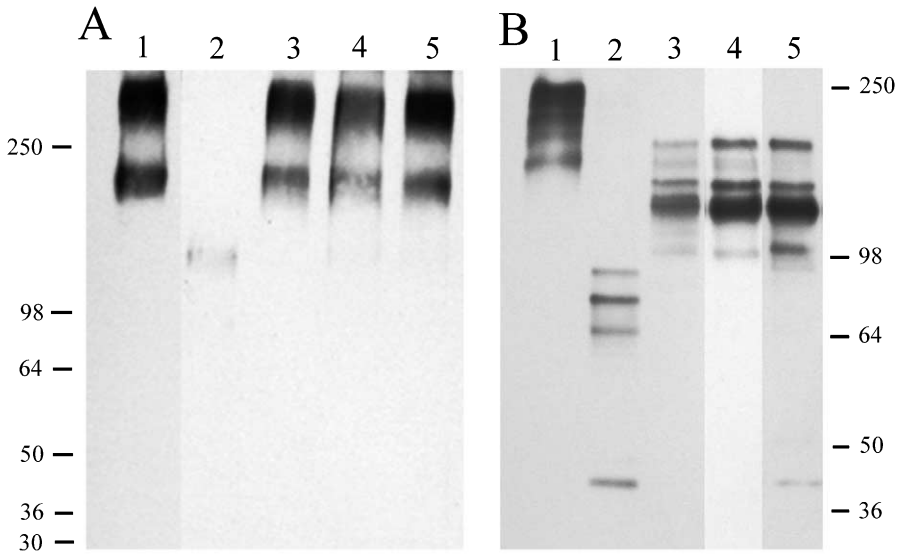


Fig. 4. Effect of RG1503, RG1150 and RG1192 on the CatG-mediated fibronectin and laminin proteolysis. CatG and the different polymers were preincubated at 37°C prior to addition of laminin (A) or fibronectin (B). Samples were then analyzed under reducing conditions by SDS–PAGE followed by Western blotting assays with antibodies to human fibronectin or mouse laminin. A: Laminin (3.8 nM) and CatG (500 nM) were incubated without (lane 2) or with 100 nM of RG1503 (lane 3), RG1150 (lane 4) or RG1192 (lane 5). B: Fibronectin (3.8 nM) and CatG (500 nM) were incubated without (lane 2) or with 20 μM of RG1503 (lane 3), RG1150 (lane 4) or RG1192 (lane 5). Laminin (3.8 nM) (A, lane 1) and fibronectin (3.8 nM) (B, lane 1) were loaded as control of electrophoretic migration. The molecular mass of the marker proteins (M) are shown in kDa.

release of Na^+ from the polyanionic polymer. At equilibrium, the process may be described as follows:

$$\mathrm{CatG}^{Z+} + \text{polymer-Na}^+ \underset{}{\overset{K_i}{\rightleftharpoons}} \text{CatG-polymer} + Z\psi\mathrm{Na}^+$$

Scheme 2.

where Z is the number of purely ionic interactions formed between CatG and the polymer, which result in the displacement of $Z\psi$-bound Na^+ from the polymer and ψ represents the fraction of Na^+ bound to the polymer per unit charge. A value of $\psi = 0.9$ has been experimentally determined[1] for all RGTA compounds, thus indicating that about 90% of their negatively charged groups (sulfates and/or carboxylates) were ionized. The observed equilibrium dissociation constant, K_i, characterizing the equilibrium detailed in Scheme 2 is related to the non-ionic equilibrium dissociation constant, $K_{i(\text{non-ionic})}$, through the relationship:

$$\log K_i = \log K_{i(\text{non-ionic})} + Z\psi \log[Na^+] \quad (1)$$

where $K_{i(\text{non-ionic})}$ may be considered as the dissociation constant for non-ionic protein–polymer interactions so that the contribution of ionic interactions to the binding at 0.1 M NaCl may be calculated [30]. Fig. 3 shows that the plots of $\log K_i$ vs. $\log [Na^+]$ are linear for the three CatG–RGTA complexes but only the one corresponding to the CatG–RG1503 complex has a positive slope, as predicted by Eq. 1. This indicates that the contribution of ionic interactions in the different RGTA–enzyme complexes depends upon the nature of the RGTA compound, i.e. the nature of the chemical groups substituted to the dextran.

In the case of RG1503, the value of Z calculated from the positive slope and using $\psi = 0.9$ was found to be 2.30 ± 0.02. This indicates that about two ionic interactions are involved in the RG1503–enzyme binding. For [NaCl] = 1 M, $\log K_i = \log K_{i(\text{non-ionic})}$ and $K_{i(\text{non-ionic})} = 0.22$ μM. At 0.1 M NaCl, the ionic strength used throughout this study K_i was 1.2 nM (see Table 1) while $K_{i(\text{non-ionic})} = 0.22$ μM. Comparison of the logarithms of these numbers indicates that ~26% of the RG1503–CatG binding free energy is derived from purely ionic interactions at pH 7.4 and 25°C. These results are very similar to those reported by Ermolieff et al. [32] which indicate that about 22% of the heparin–CatG binding energy is due to ionic interactions and that an average of two ionic interactions were required for a 1:1 heparin:CatG binding stoichiometry. On the other hand, chemical characterization of RG1503 indicates that one glucosidic unit of this polymer bears an average of 1.7 negatively charged group (sulfate+carboxylate; see Section 2). Assuming that 90% of them are ionized ($\psi = 0.9$), we may conclude that one molecule of RG1503 carries about 380 negatively charged groups that are able to interact with the positively charged groups of CatG. Hence, considering the n and Z binding parameters calculated above, it appears that about 11% of the ionized groups of RG1503 are involved in the binding of CatG. In comparison, the binding of heparin to CatG involves about 9% of the polysaccharide's charged groups [15,32]. All together, these results as well as those derived from Table 1 confirm that this RGTA compound behaves as a real functional mimetic of heparin in term of inhibitor of the amidolytic activity of CatG.

When the same experiments were performed with RG1150

[1] ψ was determined by acidimetric titration for CH_2COOH and SO_3H content before and after complete acidification of the polymer using an amberlite column. As control, we determined ψ for heparin according to the same protocol and, as previously described [30], a value of $\psi = 0.8$ was found.

and RG1192, the ionic strength dependence of the equilibrium dissociation constants K_i was thoroughly different to that observed with RG1503. Indeed, the presence of benzylamide groups fully abolished the ionic interactions between the polymer and the enzyme, as evidenced by the negative slope of the curves shown in Fig. 3. Moreover, a 10-fold increase in ionic strength leads to a 13-fold decrease in the K_i of RG1150–CatG and RG1192–CatG complexes ($K_{i(non\text{-}ionic)} = 120$ pM for both compounds). It is interesting to note that *O*-butyrylation of heparin affects the heparin–CatG interaction in a way similar to that observed with our benzylamided RGTA compounds [32]. Taken together, these results suggest than CatG no longer binds electrostatically with RG1150 and RG1192 and that their binding with the enzyme is mainly governed by non-ionic interactions. Interestingly, CatG contains a shallow hydrophobic pocket which borders the active site of the enzyme [29]. This hydrophobic surface depression may then make non-ionic interactions with benzylamide groups of the polymers and thus could act as additional hydrophobic binding site. From these results, we then speculate that in the benzylamided RGTA–CatG complexes, ionic interactions brought by sulfate and/or carboxylate groups serve as guides for the formation of an initial complex which then isomerizes into a stable complex in which the partners are held together by non-ionic interactions. Such a combination of ionic and non-ionic interactions which could take place during the complex formation may thus account for the very high affinity of these inhibitors toward CatG and for their ability to form tight complexes with the enzyme.

3.4. *Effect of RGTA on the CatG-mediated fibronectin and laminin proteolysis*

Dextran derivatized such as RG1192 and RG1503 were shown to promote tissue remodeling in various in vivo models [2–7]. Based on their in vitro properties, we proposed that these biopolymers could protect heparin binding growth factors as well as ECM components from proteolysis mediated by proteinases activated during the inflammatory process [9,11,12]. To further support this hypothesis, we studied the in vitro effect of RG1503, RG1150 and RG1192 on the CatG-mediated proteolysis of fibronectin and laminin, the major non-collagenous components of ECM and basement membranes. SDS–PAGE analyses followed by immunoblotting assays showed that laminin was efficiency cleaved by CatG, as evidenced by the disappearance of the 400-kDa and 200-kDa laminin chains (Fig. 4A, lane 2). In the presence of each RGTA, however, laminin is protected completely from cleavage by CatG (Fig. 4A, lanes 3–5). When the same experiment was done with fibronectin as the natural substrate (Fig. 4B), all RGTA tested were also able to prevent its CatG-mediated proteolysis (Fig. 4B, lanes 3–5). However, no complete inhibition of this natural substrate against proteolysis was attained even for concentrations up to 20 μM (data not shown).

4. Concluding remarks

In this study, we provide in vitro evidence that some dextran derivatives could contribute to the regulation of CatG activity as evidenced by their ability to inhibit its enzymatic activity toward synthetic as well as natural substrates. We show that the extent of inhibition varied with the nature of the substituted groups and revealed that a combination of both anionic and hydrophobic groups yielded to a very efficient inhibitor. Kinetic studies indicate that at physiologic ionic strength, the best CatG inhibitor (RG1192) has a K_i value of 0.11 nM, a value lower or far lower than those previously reported for CatG reversible inhibitors [15,22–28]. Interestingly, while RG1150 and RG1192 inhibited CatG activity and protected matrix proteins from proteolysis in a similar way, only RG1192 presented in vivo tissue repair activity. This indicates that CatG inhibition is not involved as a key or unique mechanism of action of these compounds and points out the very high degree of complexity and the numerous factors involved in tissue healing.

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