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Paradoxical interactions between modifiers and elastase-2

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The serine endopeptidase elastase-2 from human polymorphonuclear leukocytes is associated with physiological remodeling and pathological degradation of the extracellular matrix. Glycosaminoglycans bound to the matrix or released after proteolytic processing of the core proteins of proteoglycans are potential ligands of elastase-2. *In vitro*, this interaction results in enzyme inhibition at low concentrations of glycosaminoglycans. However, inhibition is reversed and even abolished at high concentrations of the ligands. This behavior, which can be interpreted by a mechanism involving at least two molecules of glycosaminoglycan binding the enzyme at different sites, may cause interference with the natural protein inhibitors of elastase-2, particularly the α-1 peptidase inhibitor. Depending on their concentration, glycosaminoglycans can either stimulate or antagonize the formation of the enzyme-inhibitor complex and thus affect proteolytic activity. This interference with elastase-2 inhibition in the extracellular space may be part of a finely-tuned control mechanism in the microenvironment of the enzyme during remodeling and degradation of the extracellular matrix.

Introduction

The serine endopeptidase elastase-2 (human leukocyte elastase) is a basic protein with an isoelectric point of 10.5. Eighteen of the 19 arginine residues present in the protein are located at the surface of the molecule [1], and can engage in electrostatic interactions with anionic partners [2]. Elastase-2, together with cathepsin G and myeloblastin, released extracellularly from neutrophilic polymorphonuclear leukocytes during inflammation and under a variety of pathological conditions, may be very destructive, degrading several components of the extracellular matrix [3]. Sulfated glycosaminoglycans, constituents of proteoglycans, have been shown to interact with the three leukocytic enzymes and to modulate their enzymatic activity [2,4–9]. In particular, elastase-2 undergoes inhibition by chondroitin sulfate isomers, dermatan sulfate (DS) and related sulfated polysaccharides by a high-affinity, electrostatically driven, hyperbolic mixed-type inhibition mechanism with a predominantly competitive character [2]. Evaluation of these interactions was based on measuring enzymatic activity for increasing concentrations of the modifiers at several fixed concentrations of a suitable substrate until a plateau was reached. We and others [10] observed a puzzling reversal of inhibition, and occasionally complete abolition of the original inhibition, as a result of increasing the concentration of modifiers by orders of magnitude beyond the level that produced inhibition, but this phenomenon was not discussed due to lack of a plausible molecular explanation.

After establishing that the observed effects were not due to experimental artifacts, we describe here the behavior of sulfated polysaccharides as modulators of elastase-2 activity on the basis of a recent theoretical treatment of multiple interactions between enzymes and modifiers [11]. These interactions become important at

Abbreviations
Ch4S, chondroitin 4 sulfate; Ch6S, chondroitin 6-sulfate; DS, dermatan sulfate; MeOSuc, *N*-methoxysuccinyl; pNA, *p*-nitroanilide; PPS, pentosan polysulfate; α_1-PI, α_1 peptidase inhibitor.

the interface between insoluble extracellular matrix components and physiological fluids, where the enzyme is engaged in multiple interactions with glycosaminoglycans bound to the matrix, or released from it, and naturally occurring inhibitors.

Results and Discussion

Inhibition of elastase-2 by sulfated polysaccharides

We previously demonstrated that the interaction between elastase-2 and sulfated polysaccharides resulted in concentration-dependent inhibition of the enzyme activity. We used semi-synthetic glycosaminoglycan derivatives of precisely defined isomeric composition and molecular mass to interpret the effects of specific structural elements of the polysaccharides [2,9]. These effects were based on electrostatic interactions between the positively charged arginine residues at the surface of the enzyme molecule and the negatively charged polysaccharides. The general inhibition mechanism was hyperbolic mixed-type with predominantly competitive character, but could not be precisely analyzed using the specific velocity equation [12] because of cooperative effects and multiple binding of the modifiers at various sites. The affinity between binders and the enzyme was therefore evaluated using the four-parameter logistic Eqn (1). Without assuming a particular mechanism, this empirical model gives a good estimate of the affinity ($K_{0.5}$), an equivalent of the inhibition constant, and of any cooperativity in the binding process, described by the Hill coefficient h. This is a useful approach for comparing the properties of structurally related modifiers.

In nature, sulfated glycosaminoglycans are very polydisperse, and the chondroitin sulfates exist as co-polymers of the 4- and 6-sulfate isomers (Ch4S, Ch6S) with various compositions and mean molecular masses that depend on animal species and tissue. Figure 1 shows the inhibition of elastase-2 by naturally occurring chondroitin and dermatan sulfates, and by a semi-synthetic sulfated polysaccharide of plant origin (PPS) that was used as a reference. Solid curves represent fits to the data using Eqn (1), and the best fit parameters $K_{0.5}$ and h are shown in Fig. 1. Ch4S had the weakest interaction with elastase-2 among the tested polysaccharides and Ch6S the strongest. Two factors contribute to the higher affinity of the 6-isomer: the larger mean molecular mass, with about 130 disaccharide units per chain, compared with only 46 for the 4-isomer (Table 1), and more favorable electrostatic interactions with elastase-2 [2]. DS is sulfated at position 4 of the galactosamine ring, and shows higher affinity with elastase-2 compared with chondroitin 4-sulfate, which has a similar mean molecular mass. The tighter binding is due to higher conformational flexibility that allows the molecule to form strong interactions with several biomolecules [13]. PPS was used in this study as a reference molecule with uniform sulfation and moderate polydispersity. The affinity of this sulfated polysaccharide was high, with a $K_{0.5}$ value of 49 nM and a Hill coefficient of 2.3, indicating cooperative binding to elastase-2, as evidenced by the sigmoid appearance of the saturation curve (Fig. 1D). As discussed previously [2], partial inhibition of elastase-2 by negatively charged polymers can be attributed to

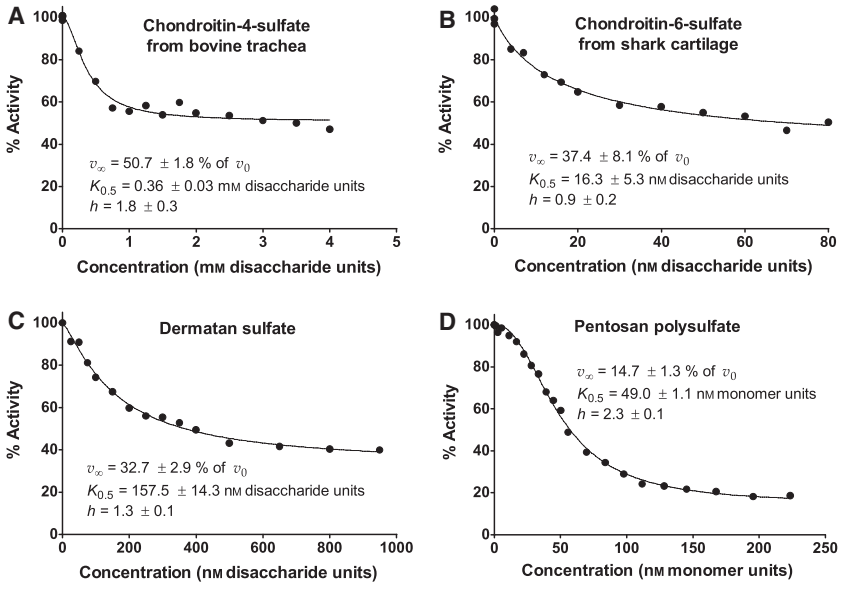


Fig. 1. Inhibition of elastase-2 by sulfated polysaccharides. Equation (1) was fitted to the data, and the solid lines represent the best fit. Parameters from regression analysis are shown together with their standard errors in (A–D). The substrate was MeOSuc-AAPV-pNA at a fixed concentration [S] = K_m = 0.070 mM in 50 mM Tris/HCl buffer, pH 7.40, with NaCl added to an ionic strength of 100 mM and 0.01% v/v Triton X-100; temperature 25 ± 1 °C. The elastase-2 concentration in all assays was 8.6 nM.

Table 1. Molecular mass of the modifiers. Molecular masses are shown as Mn (number average), Mw (weight average) and Mp (molecular mass at the top of the chromatographic peak) measured as described by Bertini et al. [28]. The polydispersity index Mw/Mn is a measure of the molecular mass distribution within a sample. Mp coincides with Mn and Mw for Mn/Mw = 1. DU, disaccharide units; MU, monomeric units. Ch4S was from bovine trachea.

Modifier	Mn	Mw	Mp	Mw/Mn	Average number of units/chain
DS	22 022	26 488	25 297	1.203	Approximately 53 DU
Ch4S	18 843	23 229	20 912	1.233	Approximately 46 DU
Ch6S	58 810	65 668	63 023	1.117	Approximately 130 DU
PPS	3687	5202	3888	1.411	Approximately 15 MU

electrostatic interactions between the 18 positively charged arginine residues at the surface of the enzyme (Fig. 2) and the negatively charged polysaccharides. In particular, when Arg217A interacts electrostatically with polyanions, interference with substrate binding causes partial inhibition. In the crystal structure of elastase-2 irreversibly inhibited by methoxysuccinyl-Ala-Ala-Pro-Ala chloromethyl ketone, Ala in position P4 of the inhibitor interacts at two points with Arg217A, suggesting a strategic role for this residue in the binding of substrates and modifiers [14].

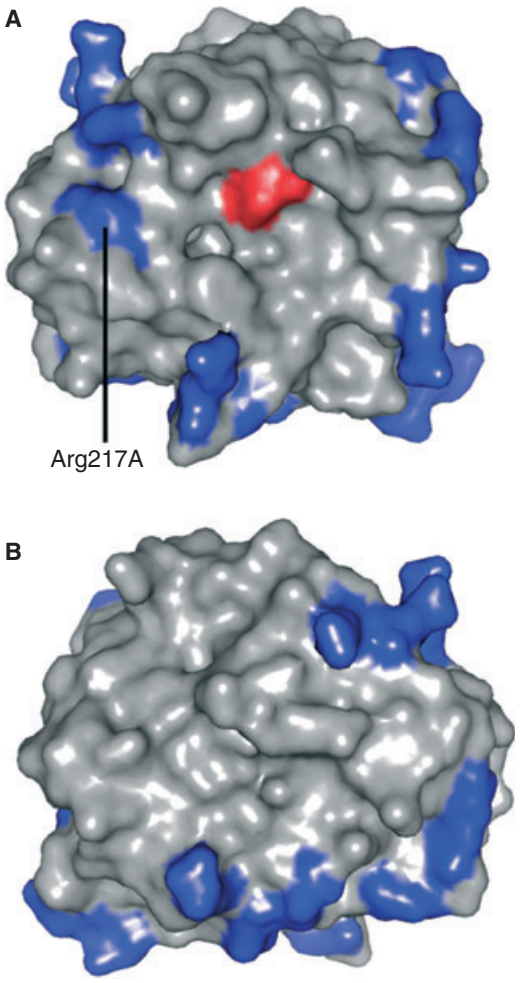


Fig. 2. Three-dimensional structure of elastase-2 (PDB ID 1HNE). Positively charged arginine residues are shown in blue, and the active site is shown in red. The positive charges form three belts around the enzyme molecule, which is shown from the front (A) and the back (B). Arg217A is positioned along the extended hydrophobic substrate binding pocket in such a way as to interfere with substrate binding when masked by interaction with polyanions.

Reactivation of elastase-2 following inhibition

In the intact extracellular matrix, glycosaminoglycans are covalently bound to core proteins, forming a dense network of fixed negative charges available for interaction with elastase-2 released extracellularly. The 'concentration' of glycosaminoglycans is best represented in this situation by measuring the surface available to enzyme binding, as reported in a study of cysteine peptidases binding to insoluble elastin [15]. During matrix remodeling or pathological degradation mediated by several peptidases, small peptides bearing a single glycosaminoglycan chain, as well as small clusters of glycosaminoglycans attached to core protein fragments, are released following hydrolysis of core proteins [16]. Despite the impossibility of direct measurements, it is reasonable to postulate a relatively high local concentration of solubilized glycosaminoglycans at the boundary between the extracellular matrix and the surrounding biological fluid while the degradation process is operating. It is also logical to assume that their concentration progressively decreases after the remodeling or degradative process comes to an end. In order to simulate this plausible natural situation, in which glycosaminoglycans are present at high concentrations in the microenvironment in which elastase-2 is active, we performed measurements as shown in Fig. 3 in which modifier concentrations were increased as much as experimentally possible. In Fig. 3, as in Fig. 1, the concentrations are expressed in terms of repeating units to take into account polydispersity (Table 1). The concentration of the whole molecule is obtained by dividing the numbers on the

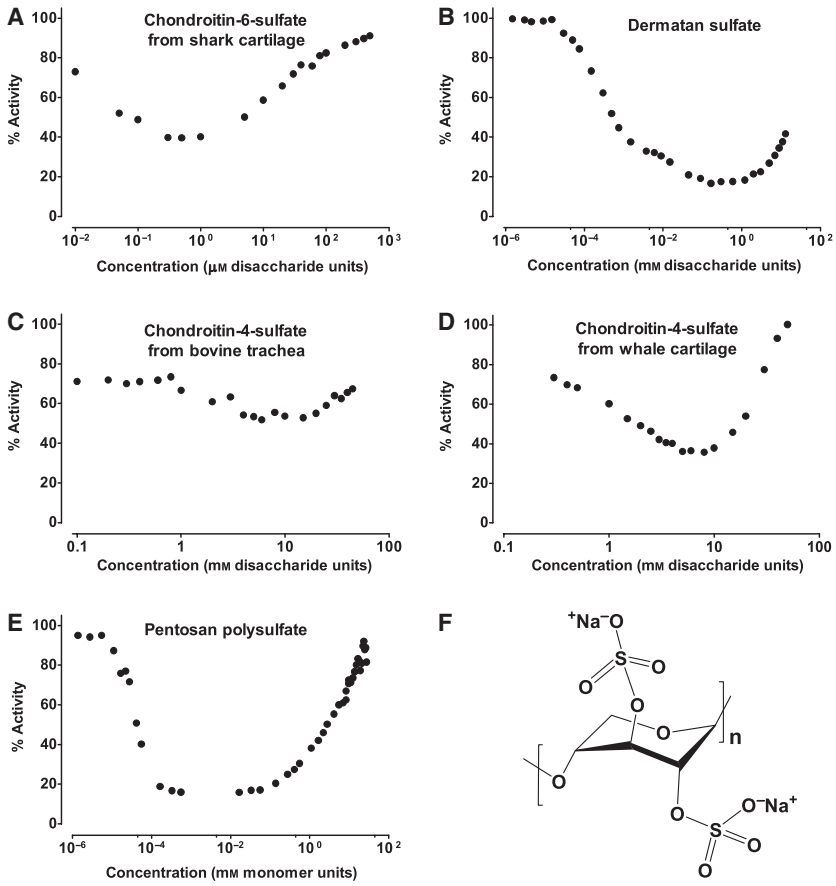


Fig. 3. Inhibition and reactivation of elastase-2 by sulfated polysaccharides. Concentration axes are drawn as a $\log_{10}$ scale of the constitutive units: disaccharide units for chondroitin sulfates and DS (A–D) and monomer units for PPS (E). Experimental conditions are as in Fig. 1. (F) Structure of pentosan polysulfate.

labeling the *x* axes by the mean number of chains (Table 1), for example 130 for Ch6S. Partial or full concentration-dependent reactivation after the original inhibition occurred in all cases, and is best represented on a logarithmic scale. In Fig. 3, Ch4S from whale cartilage is shown in addition to the four polysaccharides shown in Fig. 1 to show that isomer composition and chain length give rise to different effects (compare Fig. 3C and 3D). The paradoxical effects shown in Fig. 3 can be interpreted by considering that at least two molecules of the polyanion concomitantly bind elastase-2, as shown in the double-modifier mechanism shown in Scheme 1 and Eqn (2). According to this mechanism, two hyperbolic inhibitors, or two molecules of the same hyperbolic inhibitor, that bind an enzyme at the same time at two different sites, can induce inhibition at low concentrations of the modifiers and reverse inhibition at higher concentrations [11]. Analysis of such a system for two modifiers that are individually available is straightforward: measurements are first performed with the modifiers separately and then in various combinations of concentrations. In the case of the sulfated polysaccharides, the effector molecules are constituents of the same sample, and their effects on enzyme activity can only be measured by increasing their concentration at a constant ratio. The mole fraction of the individual molecules binding the enzyme at either site is unknown, and any attempt to calculate the individual kinetic constants by regression analysis would be arbitrary. Nevertheless, the simulated inhibition–reactivation profiles shown in Fig. 4, which produce the same effects observed in this study, suggest that a double-modifier mechanism is a plausible model to explain the observed effects. The parameters used to simulate the effects in Fig. 4 were chosen arbitrarily to match experimental results such as those shown in Fig. 3D.

The heterogeneous composition of the glycosaminoglycans does not allow speculation as to which molecular species are responsible for inhibition and its reversal. As there are three arginine residue belts on the surface of the enzyme molecule (Fig. 2), three binding modes can be envisaged. For this reason, PPS, which has a uniform structure (Fig. 3F), was used as a reference. As shown in Fig. 3E, reversal of inhibition was complete, similar to the chondroitin sulfates, suggesting that the same molecule is capable of binding the enzyme at different sites with different effects.

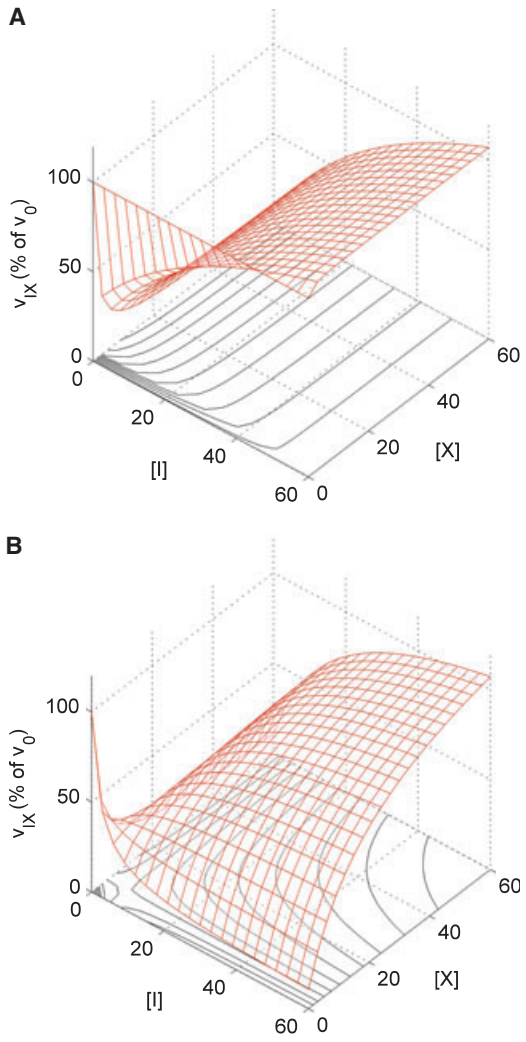


Fig. 4. Simulated enzyme inhibition and reactivation by the concomitant action of two modifiers I and X. Plots of the reaction rate as a function of the concentration (mM) of two modifiers. The kinetic parameters and coefficients are defined in Scheme 1, and simulations were performed with MATLAB® software (The MathWorks, Natick, MA, USA) using Eqn (2) as described previously [11]. In (A), I is a liberator and X is a hyperbolic inhibitor, with the following parameters: $a = 1$, $b = 7.6$, $c = \infty$ (exclusion), $e = 0.77$, $\sigma = 1$, $\beta_I = 1$, $\beta_X = 0.244$, $\beta_{IX} = 1$, $K_I = 63$ mM, $K_X = 0.67$ mM. In (B), I and X are non-exclusive hyperbolic inhibitors, $a = b = 0.32$, $c = \infty$ (exclusion), $e = 1.42$, $\sigma = 1$, $\beta_I = \beta_X = 0.048$, $\beta_{IX} = 1.0$, $K_I = K_X = 4.77$ mM. The curves in the [I]–[X] plane represent isoboles, i.e. equi-effective concentrations of the modifiers obtained by projection of the 3D graphs.

Thus, for only two binding sites, one binding mode is responsible for partial inhibition and the other acts as a liberator (Fig. 4A), or there are two inhibitors that also cause reactivation (Fig. 4B). In the absence of inhibitors or activators, a liberator does not interfere with enzyme activity [11,17].

We were unable to measure the binding of glycosaminoglycans to elastase-2 by a method other than inhibition kinetics, which had allowed confirmation of the existence of two binding sites. Hence our kinetic model is the only experimental support for interpretation of the dual behavior of glycosaminoglycans towards elastase-2. Kinetic analysis was performed by exploiting the spectroscopic properties of a low-molecular-mass synthetic substrate. Considering the physiological relevance of these results, the phenomenon of enzyme inhibition at low modifier concentrations and reactivation at high concentrations should be confirmed in the presence of a macromolecular insoluble substrate of elastase-2. We performed these experiments using insoluble elastin as the substrate in the presence of increasing concentrations of both regular and oversulfated chondroitin sulfates, as previously described (Fig. 2 in [9]). Reactivation after inhibition was qualitatively observed. However, increasing the glycosaminoglycan concentration beyond a certain threshold was impractical because of the exceedingly high viscosity resulting from insoluble elastin particles floating in a jelly-like suspension. This experimental system thus resulted in more artifacts than interpretable results.

Interference of polysaccharides with inhibitors of elastase-2

The interaction between sulfated polysaccharides and elastase-2 may stimulate or dampen the action of naturally occurring protein inhibitors at sites of action of the enzyme. This event is likely to occur at the interface between the extracellular matrix and enzymes engaged in the turnover of proteoglycans. We measured the effects of sulfated polysaccharides on inhibition of elastase-2 by eglin c and α_1 peptidase inhibitor (α_1-PI), whose kinetic mechanisms of inhibition are known [18,19]. We also considered the low-molecular-mass tetrapeptide inhibitor H-TNVV-OMe derived from the active site sequence (amino acids 60–63) of eglin c [20]. The goal of these measurements was to evaluate any disturbance to inhibition by adding polysaccharides at two fixed concentrations representing their inhibitory and reactivation concentration ranges. As eglin c and α_1-PI are slow-acting modifiers of elastase-2, progress curves were obtained at five concentrations of the two inhibitors without added polysaccharides and in the presence of Ch4S from whale cartilage as well as PPS. The reaction profiles are shown in Fig. S1. The purpose of these experiments was to determine the apparent first-order rate constant of the exponential phase (λ) and the steady-state rate (v_s). We therefore fitted an equation for

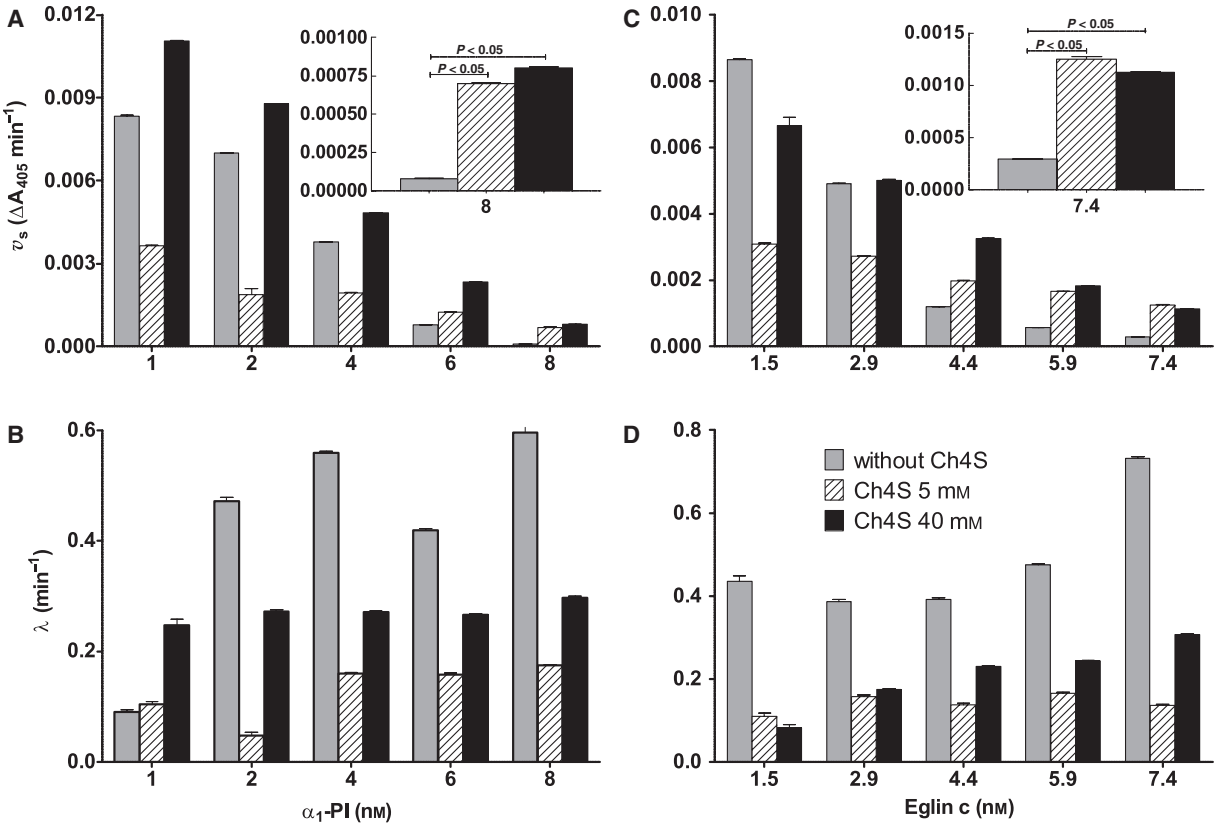


Fig. 5. Effect of Ch4S from whale cartilage on the inhibition of elastase-2 by α_1-PI and eglin c. Bars represent the best fits of parameters ± SE obtained by non-linear regression to the progress curves shown in Figs S1 and S2. The insets in (A) and (B) show enlarged bars for the highest inhibitor concentrations. The steady-state rates in presence of Ch4S were significantly different from those in their absence (one-way analysis of variance and Tukey multiple comparison test). One-way analysis of variance also showed that all values of λ, with the exception of that for α_1-PI at the lowest concentration, were significantly different from one another in all pairwise combinations ($P < 0.05$).

exponential rise followed by steady state without ascribing the results to a particular mechanism (Fig. S1). Problems arising from tight binding did not affect interpretation because the purpose of the experiment was to compare kinetic parameters obtained in the absence or presence of effectors, not to determine absolute values from their dependence on the concentration of eglin c and α_1-PI. The effects of inhibition by α_1-PI and eglin c by Ch4S, calculated by regression analysis of progress curves, are shown in Fig. 5. For increasing α_1-PI and eglin c concentrations, the steady-state rate for substrate hydrolysis leveled off to zero as expected, but, in the presence of glycosaminoglycan, the rate was ten times higher at the highest α_1-PI concentration and four times higher at the highest eglin c concentration (Fig. 5A,C, and insets). The first-order rate constant (λ) for the exponential approach to steady state (Fig. 5B,D) was significantly lower in the presence of Ch4S, and the effect was more appreciable at a low concentration of Ch4S. This retardation effect on the functionality of α_1-PI towards elastase-2 was similar to that caused by heparin, DNA and other polynucleotides on inhibition of the same enzyme by the secretory leukocyte peptidase inhibitor and α_1-PI [21–24]. A reduction in the rate for enzyme–inhibitor complex formation, which can arise for a variety of reasons, is a serious drawback for control of extracellularly acting peptidases [25]. Almost identical behavior with the same trends as shown in Fig. 5 was present when PPS was added to both α_1-PI and eglin c. These data are not shown here, but the trend can easily be deduced from the original progress curves shown in Figs S1 and S2.

The effect of PPS on elastase-2 inhibition by H-TNVV-OMe, a classical, fast-acting linear competitive inhibitor of elastase-2 corresponding to amino acids 60–63 of eglin c, is shown in Fig. 6. The polysaccharide weakened the effectiveness of the inhibitor at low concentrations and potentiated it at higher concentrations. These effects are not predictable by considering the action of the polysaccharide alone at the same

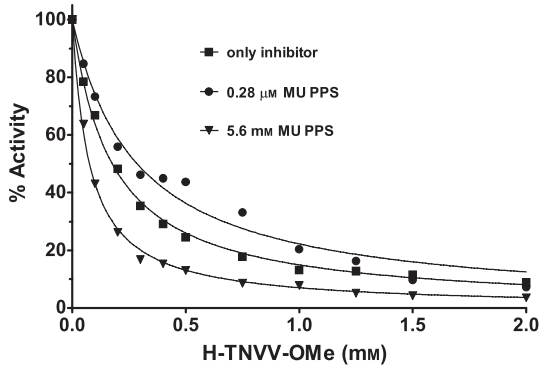


Fig. 6. Inhibition of elastase-2 by H-TNVV-OMe (amino acids 60–63 of eglin c) with and without PPS. The elastase-2 concentration in all assays was 6.9 nM of titrated active sites and other experimental conditions were as described in Fig. 1.

concentration. In fact, 0.28 μM monomer units of PPS reduced enzyme activity by about 80% (Fig. 1D), and 5.6 mM monomer units of this polysaccharide reduced the activity by 40% (Fig. 3E). However, PPS showed an opposite trend in the presence of the tetrapeptide inhibitor. The same experiments were also performed with Ch6S and DS, and the equation for linear competitive inhibition was fitted to the data to calculate the changes in K_i. Curves are not shown for Ch6S and DS, but all numerical results are shown in Table 2. Due to multiple binding interactions resulting from the binding of eglin c and the modifiers, K_i must be interpreted as an apparent K_i. A common trend of the sulfated polysaccharides was to increase the apparent K_i (thus decreasing the affinity of eglin c for elastase-2)

Table 2. Inhibition of elastase-2 by the eglin c-derived tetrapeptide H-TNVV-OMe. Measurement conditions are specified in Fig. 6. The equation for classical competitive inhibition was fitted to the data, and the K_i values, calculated based in an $[S]/K_m$ ratio of 1, are expressed as μM of DU (Ch6S and DS) or μM of MU (PPS). K_i represents the inhibition dissociation constant of the enzyme–inhibitor complex. In the presence of polysaccharides, this must be considered am apparent K_i value. The three groups of experiments (carried out under same conditions as in Fig. 1) were performed on different days with different dilutions of the enzyme solution.

Modifier	K_i (μM)	Fold increase or decrease
None	87.7 ± 2.2	
PPS, 0.28 μM MU	142.5 ± 9.2	1.62
PPS, 5.6 mM MU	37.7 ± 1.2	0.43
None	79.3 ± 4.5	
DS, 0.1 mM DU	229.9 ± 14.5	2.90
DS, 10.0 mM DU	112.0 ± 12.4	1.41
None	104.4 ± 12.8	
Ch6S, 0.2 μM DU	147.8 ± 9.7	1.41
Ch6S, 200 μM DU	94.6 ± 23.2	0.91

when used at a low concentration, i.e. that producing the maximal inhibitory activity when acting on the enzyme alone. At a higher concentration of the polysaccharides, corresponding to the reactivating phase when used alone (Fig. 3), the effects differed, with lowering of the K_i by PPS, a moderately increase in the K_i by DS, and no effect on K_i by Ch6S (Table 2). The various effects of sulfated polysaccharides on inhibition of elastase-2 by eglin c and by the tetrapeptide derived from his sequence suggest a particular binding mode of the polysaccharides to elastase-2. Using the nomenclature described by Schechter and Berger [26], the four amino acids of H-TNVV-OMe bind at positions S_4-S_3-S_2-S_1 in the same order as written, i.e. T binds to S_4 and so on, and eglin c is also expected to occupy the primed positions. The fact that polysaccharides exert concentration-dependent effects on the efficiency of H-TNVV-OMe for the enzyme (Table 2) but always weaken eglin c binding (Fig. 5) suggests an interaction between polysaccharides and arginine residues located next to the primed sites of elastase-2 in such a way that the primed sites are 'covered', thus hindering proper substrate positioning.

Based on the pooled results in this study and our previous contributions to this subject, we conclude with a working hypothesis. Glycosaminoglycans released from connective tissues by the action of hydrolases during inflammation or tissue remodeling may contribute to regulation of elastase-2 by themselves and in association with protein inhibitors. When tissue degradation is required, such as in wound healing, the efficiency of α_1-PI, the major physiological inhibitor of elastase-2, may be finely tuned by the local availability of matrix-bound and solubilized glycosaminoglycans, resulting in slowing down of its activity. After completion of remodeling, it is logical to assume that solubilized glycosaminoglycans will be rapidly removed, allowing efficient inhibition of the no longer required peptidase. If this is true, the same mechanism is likely to be responsible for inefficient inhibition of elastase-2 in pathological situations.

Experimental procedures

Materials

Elastase-2 (EC 3.4.21.37, Merops database identifier S01.131) was obtained from Elastin Product Company (Owensville, MO, USA). The lyophilized enzyme was dissolved at a concentration of 2.5 $mg \cdot mL^{-1}$ in 0.1 M sodium acetate buffer, pH 4.50, and stored in aliquots at −20 °C. The concentration of enzyme active sites was determined by titration with MeOSuc-AAPV-CH_2Cl and measurement of

residual activity using MeOSuc-AAPV-pNA. Inactivator and substrate were purchased from Bachem (Bubendorf, Switzerland).

Chondroitin 4-sulfate (Ch4S) sodium salt from bovine trachea and chondroitin sulfate (mixed isomers) from whale cartilage, as well as chondroitin 6-sulfate (Ch6S) sodium salt from shark cartilage, were obtained from Sigma-Aldrich Chemie (Buchs, Switzerland). DS from porcine intestinal mucosa was purchased from Calbiochem (Nottingham, UK). Although labeled chondroitin 4-sulfate and chondroitin 6-sulfate, these compounds are actually co-polymers of the 4 and 6 isomers within the same chain, and also contain sulfate-free sequences. Ch4S from bovine trachea contained 69% 4-sulfate and 25% 6-sulfate; Ch6S contained 45% 4-sulfate and 54% 6-sulfate; DS contained 98% 4-sulfate. The balance to 100% was non-sulfated material. Analyses were performed by HPLC of the unsaturated disaccharides after digestion with chondroitinase ABC as described previously [27]. Pentosan polysulfate (PPS, structure shown in Fig. 3F) was a generous gift from Bene PharmaChem (Geretsried, Germany). All sulfated polysaccharides were dried for 4 h at 95 °C to remove water, weighed and immediately dissolved in distilled water to produce stock solutions of known concentrations. The molecular masses were kindly determined by Dr Antonella Bisio at the Istituto di Ricerche Chimiche e Biochimiche G. Ronzoni (Milano, Italy). The procedure is based on HPLC combined with a triple detector array comprising right-angle laser light scattering, a refractometer and a viscometer [28]. The isomeric composition and molecular mass of chondroitin sulfate from whale cartilage were not determined (this compound was used only for qualitative comparisons), and the characteristics of the other polysaccharides are summarized in Table 1. Their concentration is expressed as the concentration of the basic unit, which is a monosulfated disaccharide for chondroitin sulfates and DS (M_r = 503.36) and a disulfated monosaccharide for PPS (M_r = 336.27).

Eglin c from the leech *Hirudo medicinalis* (Merops database identifier I13.001) was purified and characterized as described previously [18,29], and its protein concentration was confirmed by amino acid analysis. A tetrapeptide inhibitor based on the amino acid sequence 60–63 of eglin c, H-TNVV-OMe [20], was obtained from Bachem. Human α_1 peptidase inhibitor (α_1-PI, Merops database identifier I04.001) was obtained from CLS Behring (King of Prussia, PA, USA).

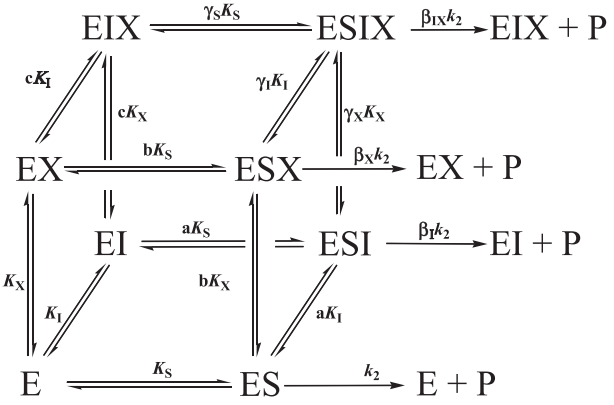
Kinetic methods

Kinetic measurements were performed using disposable acrylic cuvettes at 25 ± 1 °C in 50 mM Tris/HCl buffer with NaCl added to an ionic strength of 100 mM; the pH was 7.40 and 0.01% Triton X-100 was added to prevent adsorption of the enzyme to the cuvette. The buffer was prepared and used at 25 °C. The substrate MeOSuc-AAPV-pNA was dissolved in dimethyl sulfoxide before dilution into the assay buffer, and the final assay concentration of dimethyl sulfoxide was < 0.1% v/v. K_m was determined by fitting the Michaelis–Menten equation by non-linear regression to data with substrate concentrations ranging from 0.2–5 K_m. The reaction progress was monitored at 405 nm using a Cary 50 spectrophotometer, (Varian, Palo Alto, CA, USA), ranging from 0.2 K_m to 5 K_m and the concentration of released *p*-nitroaniline was calculated using an absorption coefficient of 9920 $M^{-1} \cdot cm^{-1}$. Regression analysis was performed using GRAPHPAD PRISM version 5.02 for Windows (GraphPad Software, San Diego, CA, USA http://www.graphpad.com). Inhibition of elastase-2 by sulfated polysaccharides was analyzed using the four-parameter logistic equation adapted to kinetic measurements [2]:

$$v_i = v_0 - \frac{(v_0 - v_\infty)[I]^h}{K_{0.5}^h + [I]^h} \quad (1)$$

where v_i is the inhibited velocity, v_0 is the velocity in the absence of modifiers, v_∞ is the velocity after reaching the plateau (saturating concentration of inhibitor I), $K_{0.5}$ is the inhibitor concentration for which the velocity equals $(v_0 - v_\infty)/2$, and h is the Hill coefficient (usually not an integer). All measurements were performed at a known fixed substrate concentration.

Double enzyme–modifier interactions were treated as described by Schenker and Baici [11] according to the mechanism shown in Scheme 1 and Eqn (2):



Scheme 1. Simultaneous interaction of two modifiers I and X on the enzyme E [11]. S, substrate; P, product. The coefficients *a* and *b* describe the proportions of competitive and uncompetitive inhibition in mixed inhibition. The coefficient c defines four types of interaction between the modifiers I and X on the free enzyme: facilitation ($0 < c < 1$), independence ($c = 1$), hindrance ($1 < c < \infty$) and exclusion ($c = \infty$). The coefficients γ_S, γ_I and γ_X characterize the interactions between reactants in formation of the quaternary complex ESIX.

$$v_{IX} = v_0(1+\sigma)\frac{1+\beta_I\frac{[I]}{aK_I}+\beta_X\frac{[X]}{bK_X}+\beta_{IX}\frac{[I][X]}{eK_IK_X}}{1+\frac{[I]}{K_I}+\frac{[X]}{K_X}+\frac{[I][X]}{cK_IK_X}+\sigma\left(1+\frac{[I]}{aK_I}+\frac{[X]}{bK_X}+\frac{[I][X]}{eK_IK_X}\right)} \quad (2)$$

where v_{IX} represents the rate in the presence of the two modifiers I and X, v_0 represents the rate in the absence of modifiers, and $\sigma = [S]/K_m$. The coefficients a, b and c are those in Scheme 1, and $e = a\gamma_X = b\gamma_I = c\gamma_S$.

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Supporting information

The following supplementary material is available:
Fig. S1. Progress curves for the inhibition of elastase-2 by α_1-PI and interference by sulfated polysaccharides.
Fig. S2. Progress curves for the inhibition of elastase-2 by eglin c and interference by sulfated polysaccharides.

This supplementary material can be found in the online version of this article.

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