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INHIBITION OF HUMAN ELASTASE FROM POLYMORPHONUCLEAR LEUCOCYTES BY A GLYCOSAMINOGLYCAN POLYSULFATE (ARTEPARON®)

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Abstract—Human lysosomal elastase from polymorphonuclear leucocytes is inhibited by the glycosaminoglycan polysulfate Arteparon. The inhibition is of a mixed type: hyperbolic uncompetitive. The interaction between inhibitor and enzyme occurs via electrostatic forces, and the binding is very tight (K_i ranges from 10^{-8} to 10^{-7} M). Depending on the chain length of the polysulfated glycosaminoglycan, two, three or five enzyme molecules can be tightly bound by a single inhibitor molecule. These findings suggest a possible therapeutic role of Arteparon as an inhibitor of elastase, a potent mediator of connective tissue breakdown, since the enzyme is inhibited by a drug concentration as small as 1 μg/ml or less.

The destruction of connective tissues by proteolytic attack is a characteristic feature of several inflammatory conditions, including rheumatoid arthritis. Lysosomal elastase, a neutral serine proteinase from the azurophil granules of human polymorphonuclear leucocytes, is an enzyme with a possible function in both physiologic and pathologic degradation of connective tissue. It is an enzyme capable of degrading the four components of the extracellular matrix: collagen, elastin, proteoglycans and structural glycoproteins. *In vitro* studies on cartilage catabolism [1] have shown that elastase degrades proteoglycans in a first phase and collagen in a second phase. Free elastase activity was not detected in synovial fluids of rheumatoid arthritis patients, but it was possible to demonstrate by means of immunohistochemical methods that the enzyme is present in the superficial layer of pannus-free rheumatoid human articular cartilage [2].

The present paper describes the mechanism of inhibition of human lysosomal elastase with the glycosaminoglycan polysulfate Arteparon. It is pointed out that this inhibitor has a possible therapeutic significance, since a very small amount of drug is needed to inhibit the enzyme.

A more detailed investigation regarding the kinetic mechanism of lysosomal elastase, with both an ester and a peptide substrate, will be presented elsewhere (Baici *et al.*, in preparation).

MATERIALS AND METHODS

Enzyme. Human lysosomal elastase (EC 3.4.21.11) was purified from human granulocytes as previously described [3]. The enzyme concentration will be expressed throughout this paper as molar concentration of the enzyme active sites, and was determined with the aza-peptide *N*-acetyl-L-Ala-L-Ala-NHN($CH_2CH_2CH_3$)CO-*p*-nitrophenylester [4] as an active site titrant. The aza-peptide was the generous gift of Dr. J. C. Powers, Atlanta, GA, U.S.A.

Arteparon. Arteparon® fractions with a very limited degree of polydispersity were kindly provided by Luitpold-Werk, Munich, F.R.G., and are listed in Table 1. The molecular weights were determined with the analytical ultracentrifuge except for the fraction with molecular weight around 3000, which was analysed by gel filtration.

Arteparon is a sulfated glycosaminoglycan derivative containing galactosamine, glucosamine, glucuronic acid and sulfate in the following proportions (calculated as μmoles/100 mg): 93.4, 11.1, 94.6 and 425.0, respectively [5]. The sulfate content is about four $-OSO_3^-$ groups per disaccharide unit. From the composition [5] we can calculate a mean molecular weight for a disaccharide unit of about 700. Therefore, a monosaccharide unit, for practical purposes referred to in this paper as the 'mean monomer', has a molecular weight of 350. From this value and the known molecular weights of the Arteparon fractions used, the number of 'mean monomers' present in the chain of each fraction could be calculated. CPK molecular models were constructed for glycosaminoglycan chains and the length of the molecules in the fully extended conformation was measured. The chain lengths expected for the Arteparon fractions used, the molecular weights, and the number of 'mean monomer' units present in the chain are given in Table 1.

Inhibition studies. Steady-state kinetic measurements were performed with *t*-butyloxycarbonyl-L-Ala-*p*-nitrophenylester as substrate. The release of *p*-nitrophenol was monitored at 400 nm and an extinction coefficient of 12,700/M/cm was used for concentration calculations. The sample cell in the spectrophotometer contained inhibitor plus enzyme, and the reference cell inhibitor alone. The reaction

was started by the addition of a small sample of substrate dissolved in acetonitrile to both the reference and sample cells in order to compensate for the spontaneous hydrolysis of the substrate. The end concentration of acetonitrile in all experiments was 1.6% (v/v). Temperature was 25 ± 1°. The buffer solution was 50 mM phosphate buffer, pH 7.4, ionic strength 0.13. In the experiments with insoluble elastin as substrate the same buffer was used. The enzymatic activity was monitored according to Schwabe [6]. Briefly, 2 mg of elastin from bovine neck ligament (SIGMA) were incubated with enzyme with or without inhibitor in an end volume of 0.4 ml for 120 min at 37°. Enzyme and inhibitor were preincubated for 30 min at 37°. The solution was then made 5% (w/v) with trichloroacetic acid and centrifuged. A 0.1 ml portion of the clear supernatant was added to 3.0 ml of 0.2 M sodium borate buffer, pH 8.5, followed by rapid addition under stirring with 1.0 ml of a fluorescamine solution (15 mg/100 ml in acetone). The fluorescence of the labelled peptides was monitored at 480 nm after excitation at 390 nm. The fluorescence response obtained in the absence of inhibitors was taken as reference (100 per cent activity). Apparatus was a Beckman DB-GT spectrophotometer equipped with a recording system for absorbance measurements and an AMINCO SPF-500 fluorimeter operating in the ratio mode for fluorescence measurements.

RESULTS AND DISCUSSION

Titration of the elastase activity with Arteparon. The five Arteparon fractions listed in Table 1 were employed as inhibitors of the elastase activity in experiments with insoluble elastin as substrate. The enzyme concentration was kept constant in all experiments, while the inhibitor concentration was varied over a wide range. Figure 1 shows an experiment with an Arteparon fraction having a molecular weight of 6500. The first part of the elastase activity decrease is linear and then reaches a plateau value which is not modified even at very high inhibitor concentrations. Due to the tightness of the binding and the enzyme concentration used (0.5 μM) the experiment represents a 'titration' of the enzymatic activity from which the stoichiometry of the binding can be calculated. The titration point corresponds to an inhibitor concentration of 0.25 μM, i.e. one half the enzyme concentration. The same type of experiment was repeated with other Arteparon fractions and results are summarized in Fig. 2. In this

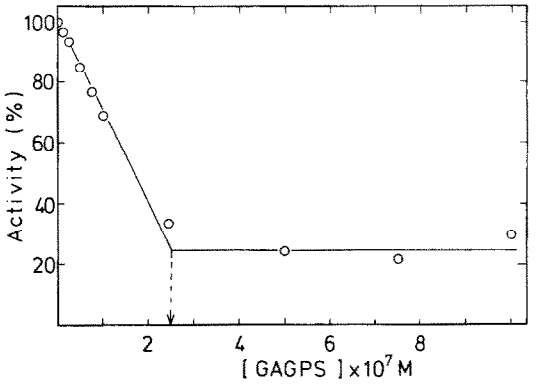


Fig. 1. Titration of the elastase activity with Arteparon. The glycosaminoglycan polysulfate (GAGPS) fraction had molecular weight 6500. The substrate was elastin.

figure the inhibitor concentration is expressed as 'mean monomer', i.e. the molar concentration of the considered fraction multiplied by the number of monomeric units present in the chain, as explained in Table 1. Figure 2 shows that the Arteparon fractions with smaller molecular weight (2100 and 3100) do not produce the sharp enzymatic activity decrease detected with the three fractions of higher molecular weight. With the two low molecular weight fractions an activity decrease of 50–60 per cent is obtained only with high inhibitor concentrations (more than 60 instead of 5 μM, as compared to the other three fractions). The titration point, obtained from the intersection of the initial curve with the plateau value, corresponds to nearly 5 μM of 'mean monomer' units for the fractions having molecular weights of 6500, 9900 and 17,000. This corresponds to the concentration of enzyme multiplied by 10. In other words, about 10 (say 9–10) monomeric units of the inhibitor molecule are needed to bind a single elastase molecule. With this concept in mind we can now understand the stoichiometry of the binding: the fractions with molecular weights of 6500, 9900 and 17,000 bind elastase with inhibitor:enzyme molar ratios of about 1:2, 1:3 and 1:5, respectively. Comparing these results with the values given in Table 1 for the number of 'mean monomer' units present in each Arteparon fraction tested, we find that the fraction with a molecular weight of 6500 has 19 monomeric units in the chain and binds two elastase molecules. The fractions with molecular weights of 9900 and 17,000 have 28 and 48 monomeric units

Table 1. Characteristics of the Arteparon fractions

Arteparon fraction	Molecular weight	Number of 'mean monomer' units	Chain length (Å)
GAGPS A 77038	2100	6	30
GAGPS A 79067	3100	9	45
GAGPS L 980 B	6500	19	95
GAGPS L 980	9900	28	140
GAGPS L 980 A	17,000	48	240

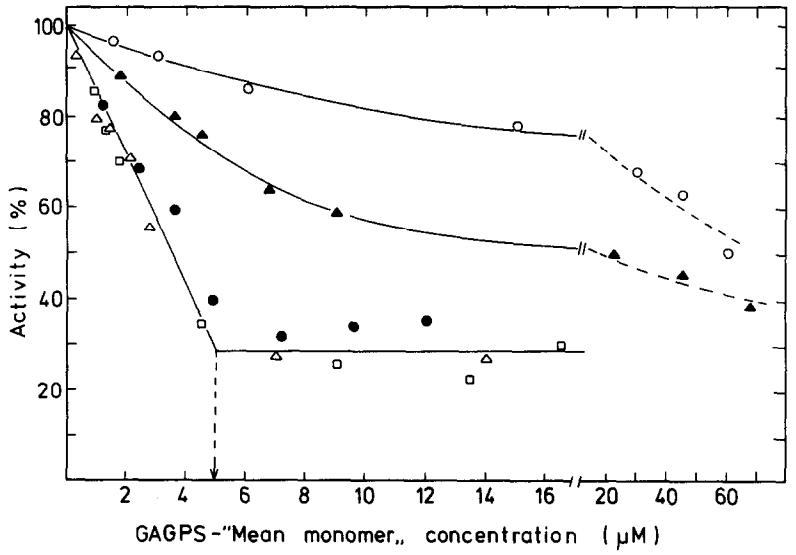


Fig. 2. Titration of the elastase activity with Arteparon. The molecular weights of the glycosaminoglycan polysulfate (GAGPS) fractions were: ○ = 2100; ▲ = 3100; □ = 6500; △ = 9900; ● = 17,000. The concentration is expressed as 'mean monomer' molarity, i.e. the molar concentration multiplied by the number of monomeric units present in the chain (see Table 1). The substrate was elastin.

in the chain, and bind three and five elastase molecules, respectively. This is an accordance with the previously discussed results, which showed 9–10 inhibitor monomeric units to be necessary for binding to a single enzyme molecule.

Phosphate buffer (50 mM), pH 7.4, ionic strength 0.13, was used in all experiments in order to have an ionic strength near to the physiological value. Ionic strengths higher than 0.4 completely abolished the inhibitory power of Arteparon. This suggests that the interaction between the two partners is electrostatic in nature. As a possible interpretation, we could put forward the hypothesis that the interaction occurs via formation of ionic bonds between the negatively charged sulfate groups of Arteparon and the positively charged guanidinium groups of the arginine residues of elastase. The enzyme is in fact a basic protein, with an isoelectric point near 10 [7], because of the high arginine content [8]. However a direct evidence of this hypothesis can only be given by more specific studies as for instance blocking of guanidinium groups. The elastase-Arteparon complexes do not precipitate and are reversibly dissociable with high salt concentrations.

Lysosomal elastase is a globular protein with a molecular weight of about 30,000. Its three dimensional structure is yet unknown, but it can be assumed that the size of the molecule is of the same order of magnitude as that found in other proteins with similar molecular weight [9]. It is reasonable, for the purposes of this paper, to consider lysosomal elastase as a protein having a spherical shape with a diameter of about 40 Å. The expected length for various glycosaminoglycan chains was given in Table 1. Considering the Arteparon fractions with molecular weights of 6500, 9900 and 17,000, the respective chain lengths and the stoichiometry of the binding to elastase, we see that a chain length of 40–50 Å (9–10 monomer units) is needed for binding to a single enzyme molecule. This length corresponds roughly to the enzyme diameter, and the proposed binding model is shown in Fig. 3. The figure describes the complex formed by the Arteparon fraction with a molecular weight of 9900, and three elastase molecules. The inhibitor molecule has 28 monomeric units and has a length of *ca.* 140 Å.

The kinetic mechanism of inhibition. The kinetic mechanism of the inhibition by Arteparon was studied using a soluble ester substrate. The formation of product in the presence of inhibitor was linear from the very beginning of the reaction as demonstrated by stopped-flow measurements (data not shown here). This fact justifies the steady-state approach used. The measurements were very difficult because of the high affinity of the inhibitor for

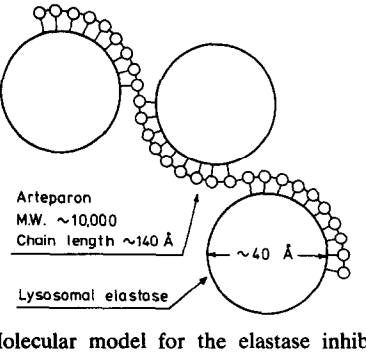


Fig. 3. Molecular model for the elastase inhibition by Arteparon. The figure shows the complex formed by an Arteparon fraction with a molecular weight around 10,000 and three elastase molecules.

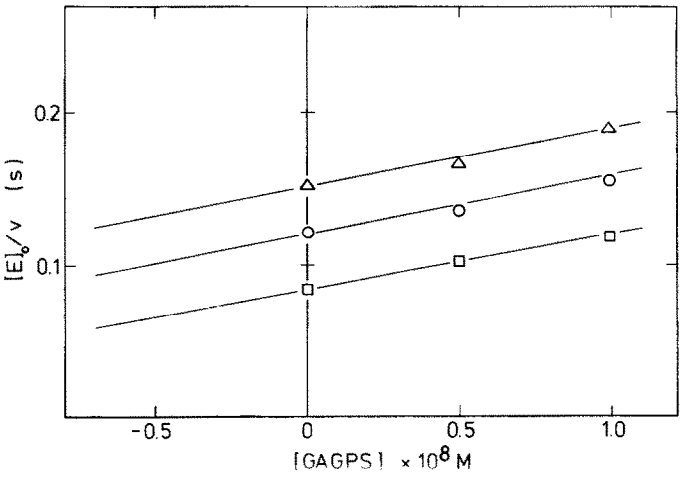


Fig. 4. Inhibition of elastase by Arteparon: Dixon plot. Substrate concentrations (mM): △ = 0.125; ○ = 0.20; □ = 0.40. The substrate was *t*-butyloxycarbonyl-*L*-Ala-*p*-nitrophenyl ester and the Arteparon fraction had a molecular weight of 17,000.

the enzyme. In fact, the correct use of the kinetic equations implies that the total added inhibitor is considered equal to the concentration of free inhibitor. When the affinity is high this is no longer valid, because a part of the added inhibitor is depleted following binding to the enzyme. Three Arteparon fractions were tested (molecular weights of 6500, 9900 and 17,000) using very low enzyme concentrations (of the order of 10^{-8} M), with various substrate and inhibitor concentrations. For the diagnosis of the inhibition type, Dixon [10] and Cornish-Bowden [11] plots were used. The inhibition constants were also determined by the use of the Henderson plot for tightly bound inhibitors [12]. All these methods, although being of very good diagnostic value in determining the inhibition type, cannot give very precise values for the inhibition constants due to the high affinity of the binding. Figure 4 shows the Dixon plot for the Arteparon fraction with a molecular weight of 17,000, and Fig. 5 shows the corresponding Cornish-Bowden plot. The Dixon plot consists of a family of parallel lines whereas the Cornish-Bowden plot gives an intersection above the concentration axis. This feature is characteristic of uncompetitive inhibition. The inhibition is not of a linear type because a saturation effect is observed, i.e. the enzyme cannot be completely inhibited. At high inhibitor concentrations the maximal inhibition capacity is 60–70 per cent and the replot of slopes of the Lineweaver–Burk plot vs inhibitor concentration is hyperbolic. This is in agreement with the titration experiments with elastin as substrate (Fig. 2). The experiments shown in Figs. 4 and 5 were performed with inhibitor concentrations well below the saturation value (the highest inhibition percentage was 30 per cent) in order to use the plots in a part where the inhibition is still more or less linear.

The general kinetic mechanism of inhibition is

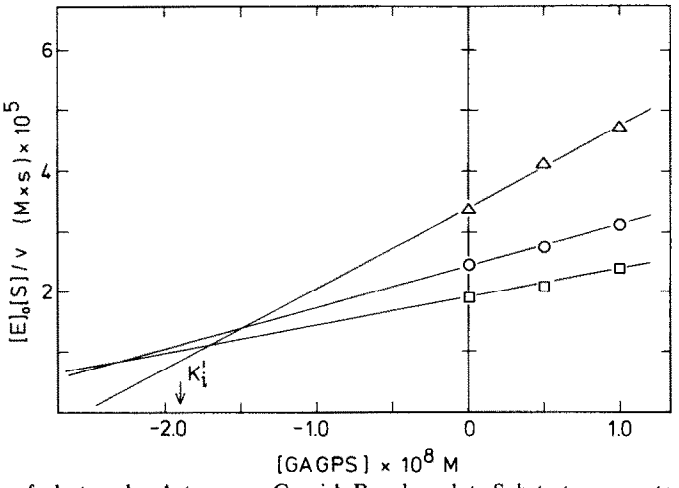
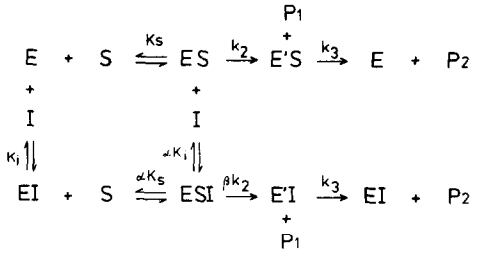


Fig. 5. Inhibition of elastase by Arteparon: Cornish-Bowden plot. Substrate concentrations (mM): □ = 0.125; ○ = 0.20; △ = 0.40. The substrate was *t*-butyloxycarbonyl-*L*-Ala-*p*-nitrophenyl ester and the Arteparon fraction had a molecular weight of 17,000.



Scheme 1. The kinetic mechanism of the elastase inhibition by Arteparon. E = enzyme, S = soluble substrate, I = inhibitor, P_1 and P_2 = products.

given in Scheme 1. The mechanism can be classified as hyperbolic uncompetitive and belongs to a mixed type [13]. The system is characterized by the fact that the α and β coefficients are nearly equal. β represents the fraction of the maximum velocity of the reaction when both substrate and inhibitor are saturating. α is a measure of the affinity change of the substrate for the enzyme in the presence of inhibitor [13]. If $\alpha>1$ the inhibitor decreases this affinity, and if $\alpha<1$ the affinity is increased. Since for the elastase inhibition by Arteparon α is less than 1, the presence of inhibitor paradoxically favours the binding of substrate. The drug is, however, a good inhibitor because the catalytic efficiency of the ternary enzyme–substrate–inhibitor (ESI) complex is decreased by a factor β. The β coefficient for the Arteparon fractions with molecular weights 6500, 9900 and 17,000 is 0.35 ± 0.05, i.e. the maximal inhibition capacity is 60–70 per cent and α is very close to this value. α and β can be easily calculated from intercept replots vs inhibitor concentration [13]. The value of the inhibition constant, K_i', can be calculated from the Cornish-Bowden plot. K_i' is the dissociation constant of the ESI complex, whereas K_i is the dissociation constant of the enzyme–inhibitor (EI) complex. Plots as those shown in Figs. 4 and 5 were made also for the Arteparon fractions with molecular weights of 6500 and 9900, and results were as those shown in Figs. 4 and 5. From Fig. 5 we can calculate a K_i' value of $1.9 \pm 0.5 \times 10^{-8}$ M. The same parameter was also calculated from experiments with inhibitor concentrations higher than those shown in Fig. 5 with the method of Henderson for tightly bound inhibitors [12]. The value was *ca* 2×10^{-8} M, in good agreement with the estimate obtained from Fig. 5. However, we do not feel that values for K_i or K_i' can be given with such a precision for the system presented here for the reasons outlined above, and prefer to say that the inhibition constants for the Arteparon fractions with molecular weights between 6500 and 17,000 fall between 10^{-8} and 10^{-7} M. The binding is so tight, that a very precise evaluation of the equilibrium constant by means of kinetic measurements is not possible for technical reasons. It remains clear that even an inhibition constant of the order of 10^{-7} M is representative of a powerful inhibitor. We are further investigating the binding properties of Arteparon to elastase and other basic enzymes with the use of equilibrium techniques.

Recently, it was demonstrated that Arteparon, after intramuscular injection in patients with osteoarthrosis, is transported into the joints and is found in cartilage in variable concentrations depending on the observation time and on the cartilaginous layer. For instance, 24 and 72 hr after injection, the superficial layer of cartilage contained 1.43 and 0.94 μg Arteparon/g cartilage, respectively [14]. These concentrations should be compared with the results presented in the present paper. With an inhibition constant of the order of 10^{-8} to 10^{-7} M, an Arteparon fraction with molecular weight 10,000 can inhibit human lysosomal elastase with concentrations ranging from 0.1 to 1 μg/ml.

Although the role of lysosomal enzymes in osteoarthrosis (the illness for which Arteparon is basically used) is still unclear, these results represent a possible model for drugs, transported into the joint by virtue of their structural features. Although the drug–lysosomal enzyme interaction is probably nonspecific for a given enzyme, the high inhibition degree achieved with low drug doses suggests a possible application of Arteparon in those processes which are characterized by cartilage damage through the action of basic enzymes of essentially different cellular origin.

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