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EFFECT OF SULPHATED POLYSACCHARIDES ON THE α_1-ANTITRYPSIN INHIBITION OF AMIDOLYSIS CATALYSED BY COAGULATION CASCADE PROTEINASES

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1 The ability of several sulphated polysaccharide anticoagulants to prevent α_1-antitrypsin inhibition of thrombin paralleled their ability to potentiate antithrombin III inhibition of thrombin. None of the compounds examined altered the ability of α_1-antitrypsin to inhibit activated coagulation factor X (X_a).

2 These results are consistent with the possibility that a direct polysaccharide-proteinase interaction may be involved in the sulphated polysaccharide-modulated inhibition of thrombin by antithrombin III.

Introduction

Human plasma contains several proteinase inhibitors that are presumed to regulate the coagulation cascade and other plasma systems involving proteolysis (Ogston & Bennett, 1977). Potentiation of one of these inhibitors, antithrombin III, appears to be the mechanism by which heparins exert anticoagulant effects (Barrowcliffe, Johnson & Thomas, 1978). Several other compounds which are chemically related to heparins also potentiate antithrombin III activity (Kindness, Long & Williamson, 1979a, b, c; 1980a, b; Kindness, Williamson & Long, 1979e, f; 1980d, e; Kindness, Long, Williamson, Edward, Winter & Bennett, 1980c; Long, Williamson, Kindness, Edward & Winter, 1980). In contrast Danishefsky & Pixley (1979) recently reported that heparin prevented inhibition of the clotting proteinase thrombin by another plasma proteinase inhibitor, α_1-antitrypsin. They suggested that the effect involved an interaction between heparin and the proteinase and that the structural requirements in heparins for anticoagulation and for prevention of the α_1-antitrypsin inhibition of thrombin were similar.

In this paper we examine the effects of several sulphated polysaccharide anticoagulants on the inhibitory activity of α_1-antitrypsin towards thrombin and activated coagulation factor X (X_a).

Methods

Colorimetric experiments

Methods used were modifications of those previously described (Kindness *et al.*, 1979b).

To examine the inhibition of thrombin by α_1-antitrypsin, 40 µl of a thrombin solution (3 u/ml) containing polysaccharide (or buffer) was incubated at 20°C for 5 min before addition of α_1-antitrypsin (20 µl) and reincubation of the mixture at 37°C for various times; 30 µl of the mixture was then added to 370 µl of a solution containing substrate S-2238 (0.15 mg/ml), polybrene (0.1 mg/ml) and Tris-HCl (150 mM, pH 7.2 at 37°C). The reaction was stopped after a further 5 min incubation at 37°C by addition of 150 µl of 50% (v/v) acetic acid.

To examine the inhibition of factor X_a by α_1-antitrypsin, 40 µl of a factor X_a solution (1.5 u/ml) containing polysaccharide (or buffer) was incubated at 20°C for 5 min before addition of α_1-antitrypsin (20 µl) and re-incubation of the mixture at 37°C for various times; 40 µl of the mixture was then added to 360 µl of a solution containing substrate S-2222 (0.15 mg/ml), polybrene (0.1 mg/ml) and Tris-HCl (150 mM, pH 7.2 at 37°C). The reaction was stopped after a further 15 min incubation at 37°C by addition of 150 µl of 50% (v/v) acetic acid.

Concentrations of substrates and of polybrene quoted are final concentrations in the mixture after addition of the enzyme. In control experiments (see Results section), polysaccharide, proteinase and α_1-antitrypsin were replaced by appropriate volumes of buffer. The polycation polybrene was included in order to prevent possible effects of the polysaccharides on the chromogenic substrate (Kindness *et al.*, 1979b). Paranitroaniline (PNA) released from substrates was determined at 405 nm with a Cecil spectrophotometer model 272. Inhibition was expressed as the percentage of PNA released from

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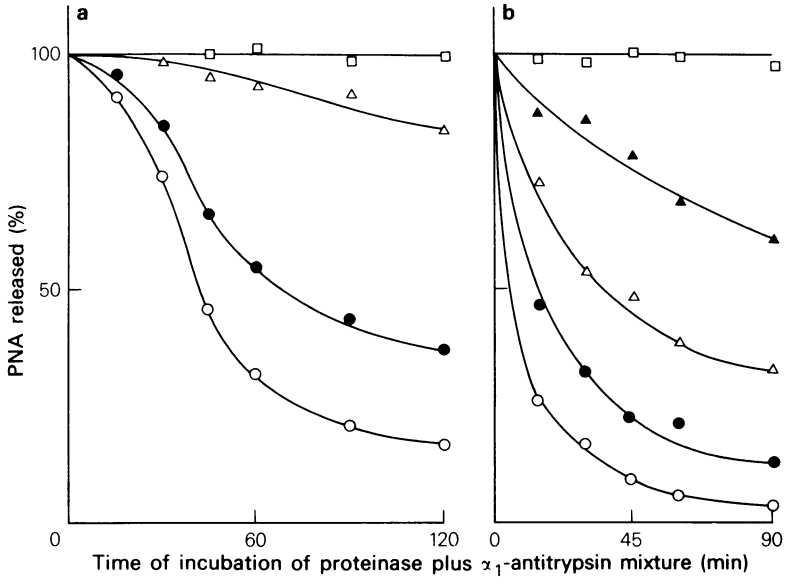

Figure 1 The inhibition of thrombin (a) and factor X_a (b) by α_1-antitrypsin. Concentrations of α_1-antitrypsin: (○) 4 mg/ml; (●) 2 mg/ml; (△) 1 mg/ml; (▲) 0.5 mg/ml; (□) no α_1-antitrypsin.

substrates in the absence of inhibitor or polysaccharides. In the absence of inhibitor or polysaccharides, optical densities of PNA released from S-2238 or S-2222 were 0.65 to 0.72 and 0.30 to 0.34, respectively.

Materials

Sources and properties of heparan, heparin sulphate, dermatan sulphate and chondroitin sulphates A and C (Kindness *et al.*, 1980c), dextran sulphate (Kindness *et al.*, 1979e), cellulose sulphate (Kindness *et al.*, 1980a), xylan sulphate (Kindness *et al.*, 1979f), ι-, κ-, λ-carrageenans (Kindness, Long, Williamson & Boyd, 1979d) and agarose sulphate (Kindness *et al.*, 1979b) have been previously reported. Human α_1-antitrypsin and polybrene were from Sigma Chemical Company Ltd. Chromogenic substrates D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238) and N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride, its methyl ester (S-2222) were from Kabi Vitrum Ltd. Bovine thrombin was from Parke, Davis and Company, and bovine clotting factor X_a from Diagnostic Reagents Ltd. Polysaccharides, α_1-antitrypsin and proteinases were dissolved in buffer (0.11 M NaCl, 0.05 M Tri-HCl, pH 7.4 at 20°C); substrates were dissolved in distilled water immediately before use.

Results

Effect of α_1-antitrypsin on thrombin and X_a activity

α_1-Antitrypsin inhibited amidolytic activity of both thrombin and X_a when the inhibitor was incubated with proteinase before addition of the enzyme to the chromogenic substrate (Figure 1). The degree of inhibition depended upon the concentration of inhibitor present and the length of the preincubation period. Incubation of enzymes in the absence of inhibitor did not affect the subsequent rate of the enzyme-catalysed reactions. No amidolysis was observed in control tubes which contained α_1-antitrypsin but no proteinase.

Effect of sulphated polysaccharides on α_1-antitrypsin inhibition of thrombin

Thrombin was separately incubated for 5 min at 20°C with several sulphated polysaccharides (1 to 100 μg/ml) before addition of α_1-antitrypsin (2 mg/ml). Following a further incubation for 90 min at 37°C, the rate of thrombin-catalysed amidolysis was determined (Figure 2). Under these conditions, α_1-antitrypsin in the absence of polysaccharides reduced the rate of the factor X_a-catalysed reaction to 45% of that occurring in the absence of inhibitor or polysaccharide (Figure 1). Heparin, heparan sul-

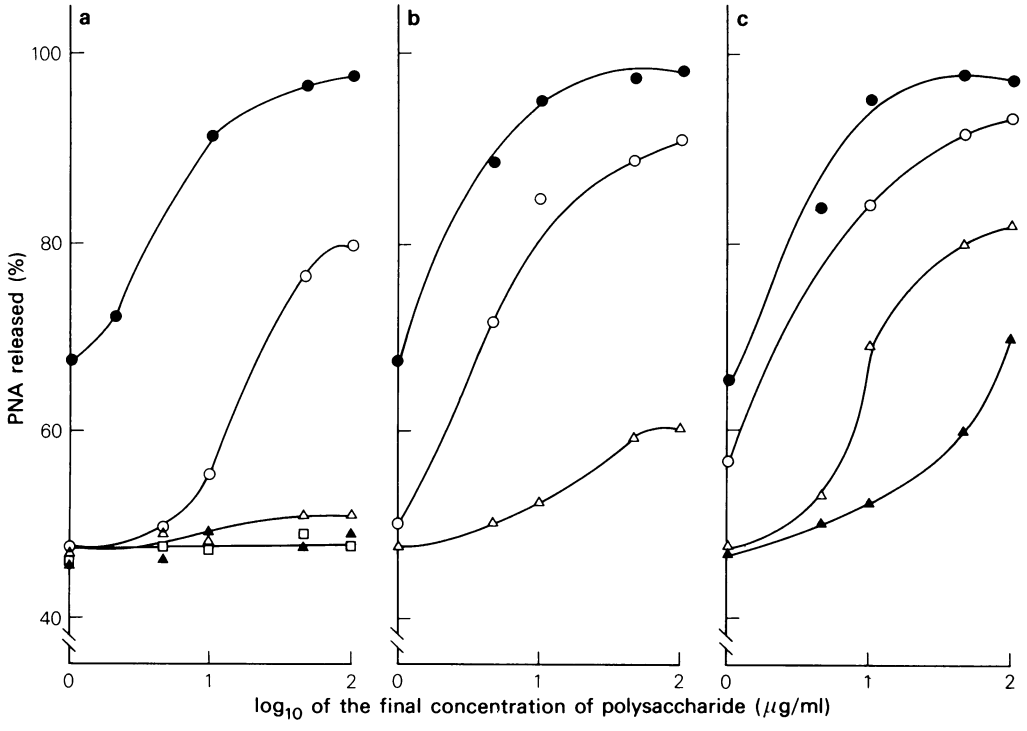

Figure 2 Effect of sulphated polysaccharides on inhibition of thrombin by α_1-antitrypsin: (a) (●) heparin, (○) heparan sulphate, (△) dermatan sulphate, (▲) chondroitin sulphate A, (□) chondroitin sulphate C; (b) (●) dextran sulphate, (○) cellulose sulphate, (△) xylan sulphate; (c) (●) λ-carrageenan, (○) -carrageenan, (△) -carrageenan, (▲) agarose sulphate.

phate, dextran sulphate, cellulose sulphate, xylan sulphate, ι-, κ- and λ-carrageenans and agarose sulphate were capable, to various extents, of preventing α_1-antitrypsin inhibition of the thrombin-catalysed reaction. Dermatan sulphate and chondroitin sulphates exerted little effect. Incubation of sulphated polysaccharides with thrombin in the absence of α_1-antitrypsin did not affect the rate of the catalysed reaction. No amidolysis was observed in control tubes which lacked thrombin but contained polysaccharide or polysaccharide and α_1-antitrypsin.

Effect of sulphated polysaccharides on α_1-antitrypsin inhibition of X_a

Factor X_a was separately incubated for 5 min at 20°C with 1 to 100 μg/ml of the same sulphated polysaccharides, before addition of α_1-antitrypsin (2 mg/ml). Amidolysis was determined after a further incubation for 30 min at 37°C. Under these conditions, α_1-antitrypsin in the absence of polysaccharides reduced the rate of the factor X_a-catalysed reaction to 33% of that occurring in the absence of inhibitor or polysaccharide (Figure 1). None of the polysaccharides examined affected α_1-antitrypsin inhibition of the X_a-catalysed reaction under these conditions. Incubation of polysaccharides with factor X_a in the absence of α_1-antitrypsin did not affect the rate of the catalysed reaction. No amidolysis was observed in control tubes which lacked X_a but contained polysaccharide or polysaccharide and α_1-antitrypsin.

Discussion

α_1-Antitrypsin is the most abundant proteinase inhibitor present in human plasma, and inhibits a range of proteinases *in vitro* but its physiological roles are uncertain (Ogston & Bennett, 1977).

Danishefsky & Pixley (1979) demonstrated that the addition of heparin to thrombin protected the enzyme from subsequent inhibition by α_1-antitrypsin and that structural modification of heparin resulted in parallel changes in the anticoagulant activity of the polysaccharide and in its ability to prevent α_1-antitrypsin inhibition of thrombin. Heparins apparently act as clinical anticoagulants by potentiating the antithrombin III inhibition of coagulation cascade proteinases. The sequence of reaction between polysaccharide, proteinase and proteinase inhibitor

is in some dispute (Barrowcliffe *et al.*, 1978). Parallel changes in anticoagulant activity of heparin and its ability to affect interactions between α_1-antitrypsin and thrombin are consistent with the possibility that direct thrombin-heparin interaction may play a role in the anticoagulant activity of the polysaccharide.

Our results demonstrate that several other sulphated polysaccharides interfere, like heparins, with the ability of α_1-antitrypsin to inhibit thrombin activity. Of these compounds, heparan sulphate, dextran sulphate, cellulose sulphate and xylan sulphate are capable, like heparins, of potentiating inhibition by antithrombin III of the amidolytic activity of purified proteinases (Kindness *et al.*, 1979b, c, e, f; 1980a, d). In addition, the anticoagulant activity of these polysaccharides in plasma appears, like that of heparins, to involve potentiation of antithrombin III (Kindness *et al.*, 1979a, c, e, f; 1980a, b, c, d; Long *et al.*, 1980). The potency with which these compounds prevent α_1-antitrypsin inhibition of thrombin parallels their reported ability to potentiate the inhibition by antithrombin III of the amidolytic activity of thrombin.

Of the other sulphated polysaccharides, only the algal sulphated galactans, carrageenans and agarose sulphate, interfered with α_1-antitrypsin inhibition of thrombin. These compounds are capable of potentiating inhibition by antithrombin III of thrombin-catalysed amidolysis *in vitro* (Kindness *et al.*, 1979b), although antithrombin III does not seem to be involved in their anticoagulant activity (Kindness *et al.*, 1979e; 1980b; Long *et al.*, 1980). The action of α_1-antitrypsin was not affected by dermatan sulphates, which do not potentiate antithrombin III inhibition of proteinases *in vitro* (Kindness *et al.*, 1979b), and whose anticoagulant activity is probably independent of antithrombin III (Teien, Abildgaard & Höök, 1976; Kindness *et al.*, 1979a; 1980c; 1981; Long *et al.*, 1980). Similarly, chondroitin sulphates, which are neither anticoagulant nor affect antithrombin III activity *in vitro* (Kindness *et al.*, 1980d; Teien *et al.*, 1976), did not alter α_1-antitrypsin activity.

We have not directly demonstrated binding between thrombin and these polysaccharide anticoagulants; however, the direct relationship between the relative ability of a polysaccharide to potentiate inhibition of thrombin by antithrombin III and to interfere with inhibition of thrombin by α_1-antitrypsin suggests that such an interaction may be important in the polysaccharide-thrombin-antithrombin III interactions which lead to inhibition of the proteinase. In contrast, it has been suggested that heparin potentiation of antithrombin III inhibition of X_a may not require a polysaccharide-proteinase interaction (Holmer, Lindahl, Bäckström, Thunberg, Sandberg, Söderström & Andersson, 1980). This view is supported by our demonstration that the polysaccharides examined do not alter α_1-antitrypsin inhibition of X_a.

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