Inhibition by heparin-modulated antithrombin III of amidolysis catalysed by \(m_F\)-acrosin

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Purified \(m_F\)-acrosin catalysed amidolysis of several \(p\)-nitroanilides with \(C\)-terminal arginine residues. Antithrombin III inhibited amidolysis catalysed by the enzyme. This effect of antithrombin III was potentiated by heparin, and to a modest extent by heparan sulphate, cellulose sulphate, dextran sulphate and xylan sulphate. De-\(N\)-sulphated heparin, de-N-sulphated \(N\)-acetylated heparin, heparin of low relative molecular mass, chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and hyaluronic acid were ineffective.

The plasma glycoprotein antithrombin III is probably the major mammalian physiological inhibitor of thrombin (EC 3.4.21.5), of factor \(Xa\) (EC 3.4.21.6) and perhaps of some other serine proteinases catalysing reactions of the intrinsic coagulation cascade (Barrowcliffe & Thomas, 1981). The inhibitor has been shown to be effective towards a few other proteinases in vitro. These include plasma kallikrein (EC 3.4.21.8; Rosenberg, 1977), plasmin (EC 3.4.21.7; Rosenberg, 1977), urokinase (formerly EC 3.4.99.26; Watson et al., 1981), a proteinase released from mouse macrophages (Seljelid et al., 1980) and a cell-surface neutral proteinase from human leucocytes (Long et al., 1982). In addition, proteinase inhibitors immunologically related to antithrombin III have been detected on guinea-pig T-lymphocyte membranes (Clement et al., 1981) and in numerous normal and malignant tissues (Twining & Brecher, 1977).

Heparins, by a mechanism that is yet to be elucidated, accelerate the inhibitory action of antithrombin III towards at least some of the proteinases mentioned above; this effect forms the basis of the efficacy of heparins as clinical anticoagulants (Barrowcliffe & Thomas, 1981). With the possible exception of heparans, which are structurally related to heparins, other purified glycosaminoglycans do not modulate antithrombin III activity (e.g. Kindness et al., 1980b). Also, with the possible exceptions of \(\mathrm{Cl}\)ts-inhibitor (Caughman et al., 1982), and of partially characterized inhibitors named ‘protease-nexin’ (Baker et al., 1980), ‘antithrombin BM’ (Wunderwald et al., 1982) and ‘heparin cofactor II’ (Tollefsen et al., 1982), proteinase inhibitors other than antithrombin III are not potentiated by heparins.

Vascular-wall heparans may act as immobilized anticoagulants by potentiating the action of plasma or vascular-wall antithrombin III in a heparin-like manner (for discussion, see Long & Williamson, 1983a), and it has been suggested that pericellular heparans in tissues other than blood vessels may also affect pericellular proteinase activities by potentiating the action of proteinase inhibitors similar to, or perhaps identical with, plasma antithrombin III (Long & Williamson, 1983a). In the present paper the activity in vitro of a serine proteinase isolated from a non-plasma source, \(m_F\)-acrosin, is shown to be inhibited by heparin-modulated antithrombin III.

Experimental

Materials

Heparin from porcine intestinal mucosa [Grade 1; lot no. 46C-0035; anticoagulant activity stated by supplier as 170 U.S.P. (United States Pharmacopeia) units/mg] was supplied by Sigma Chemical Co., Poole, Dorset, U.K. Enzymic and electrophoretic analysis of the preparation showed it to contain 3.8% (w/w) of dermatan sulphate (Long et al., 1980). Results reported are from experiments using this heparin. Similar results were obtained when heparin (Third International Standard; code no. 65/69) supplied by the National Institute for Biological Standards and Control, London N.W.3, U.K., was used. A heparin fraction of average \(M\), 6100 (Long et al., 1983), completely de-\(N\)-sulphated heparin (Long & Williamson, 1983b)
and de-N-sulphated N-acetylated heparin (Long & Williamson, 1983b) were prepared and characterized as previously described. Dermatan sulphate from porcine skin was supplied by Seikagaku Fine Biochemicals, Tokyo, Japan (lot no. S 9401). Enzymic and electrophoretic analysis of the preparation showed it to contain less than 0.2% (w/w) heparin (Long et al., 1980). River-sturgeon notochord chondroitins 4-sulphate and 6-sulphate, bovine lung heparan sulphate and human umbilical-cord hyaluronic acid, all produced under contract no. NO1-AM-5-2205 for the U.S.A. National Institutes of Health, were kindly given by Professor M. B. Mathews and Professor J. A. Cifonelli, Department of Pediatrics, University of Chicago, Chicago, IL, U.S.A., from whom analytical information may be obtained in the form of a data sheet. Sulphated cellulose (lot no. 185) was obtained from Kelco, San Diego, CA, U.S.A. The sample had an average degree of substitution of 2.2 sulphate half-ester residues per glucose residue. Sulphated xylan was obtained from Benechemie G.m.b.H., Munich, Federal Republic of Germany. It is a $\beta1\rightarrow 4$-linked $\beta$-xylan, with a degree of polymerization of 6–12 monomers per oligomer and an average degree of substitution of 1.8 sulphate half-ester residues per xylene residue. Sulphated dextran from dextran of $M$, $5 \times 10^6$ was obtained from Pharmacia, Uppsala, Sweden. All polysaccharides were in the form of sodium salts.

Human plasma antithrombin III (lot no. 57030) had an approximate specific activity of 5 units/mg of protein; for assay of activity, see Abildgaard et al., 1970; 1 unit is the activity in 1 ml of pooled normal human plasma (W. F. Long and F. B. Williamson, 1984). The sample had an activity of 1.8 units/mg.

A 30 µl portion of polysaccharides was incubated at 37°C for 2.5 min with 20 µl of antithrombin III (unless stated otherwise 0.035 unit/ml). Then 20 µl of $m_{\beta}$-acrosin solution (3 µg/ml) was added and the mixture incubated for 2.5 min. Next, 20 µl of a mixture of chromogenic substrate (0.12 mg/ml) and Polybrene (0.12 mg/ml) was added. Initial reaction rates at 37°C were determined by measuring released $p$-nitroaniline spectrophotometrically. In control experiments, polysaccharide, antithrombin III and $m_{\beta}$-acrosin were in various combinations, replaced by appropriate volumes of buffer. Unless stated otherwise, substrate S-2288 was used. The polycation Polybrene was included in order to prevent possible electrostatic interaction between polysaccharides and chromogenic substrates (Teien et al., 1976). Concentrations quoted are final concentrations in the reaction mixture after addition of substrate and Polybrene.

Results

In the assay system described, $m_{\beta}$-acrosin in the absence of antithrombin III or polysaccharides catalysed the release of $p$-nitroaniline from $p$-nitroanilides containing C-terminal arginine residues at the following initial reaction rates ($\Delta A_{405nm} . \mu l^{-1} . \text{min}^{-1}$) (all $\times 10^{-3}$): substrates S-2288, 5.8; S-2222, 4.0; S-2266, 3.9; S-2238, 3.5; S-2302, 2.7; S-2444, 2.3. Amidolysis of substrate S-2251, a $p$-nitroanilide containing a C-terminal lysine residue, was catalysed at a rate of 400 $\Delta A_{405nm} . \mu l^{-1} . \text{min}^{-1}$.

Incubation of antithrombin III with enzyme before addition of substrate inhibited enzyme activity (Table 1). No amidolysis was observed when antithrombin III was incubated with substrate in the absence of enzyme.

Incubation of heparin with antithrombin III before enzyme addition potentiated the action of antithrombin III; preincubation of Polybrene (0.12 mg/ml) with heparin before antithrombin III addition prevented this action of heparin (Fig. 1a). No amidolysis was observed when, in the absence of enzyme, Polybrene, by itself or together with heparin or antithrombin III, was incubated with substrate. Also, Polybrene did not affect the
Table 1. Effect of antithrombin III on rate of \( m_g \)-acrosin-catalysed amidolysis

<table>
<thead>
<tr>
<th>[Antithrombin III] (units/ml)</th>
<th>Initial reaction rate ( \times 10^{-3} ) (( \Delta A_{580} \cdot \mu l^{-1} \cdot min^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>0.03</td>
<td>5.7</td>
</tr>
<tr>
<td>0.04</td>
<td>5.5</td>
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<tr>
<td>0.06</td>
<td>4.4</td>
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<tr>
<td>0.11</td>
<td>1.5</td>
</tr>
<tr>
<td>0.22</td>
<td>0.9</td>
</tr>
<tr>
<td>1.11</td>
<td>0.2</td>
</tr>
</tbody>
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amidolytic rates observed when, with enzyme, enzyme and heparin, or enzyme and antithrombin III, it was incubated with substrate.

Incubation of antithrombin III with de-N-sulphated heparin, de-N-sulphated N-acetylated heparin, a low-\( M_r \) fraction of heparin, chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate or hyaluronic acid before addition of enzyme did not alter the amidolytic rate (Figs. 1b–1h). In contrast, modest potentiation of antithrombin III action was observed at high concentrations of heparan sulphate, cellulose sulphate, dextran sul-

Fig. 1. Effect of sulphated polysaccharides on antithrombin III inhibition of amidolysis catalysed by \( m_g \)-acrosin
(a) ●, Heparin; ○, heparin preincubated with Polybrene; (b) de-N-sulphated heparin; (c) de-N-sulphated N-acetylated heparin; (d) low-\( M_r \) heparin; (e) chondroitin 4-sulphate; (f) chondroitin 6-sulphate; (g) dermatan sulphate; (h) hyaluronic acid; (i) heparan sulphate; (j) cellulose sulphate; (k) dextran sulphate; (l) xylan sulphate. Reaction rates are shown as percentages of control rates, i.e. the rates observed when \( m_g \)-acrosin was, under the conditions specified, incubated with substrate and Polybrene only. Representative absolute reaction rates are quoted at the start of the Results section. Incubation of \( m_g \)-acrosin, under the conditions specified, with antithrombin III, substrate and Polybrene and in the absence of polysaccharides, decreased the initial reaction rate to 98% of the control rate.
phate and xylan sulphate (Figs. 1i–1l). No amidolysis was observed when any of the polysaccharides, with or without antithrombin III, were incubated with substrate in the absence of enzyme. None of the polysaccharides, when incubated with enzyme in the absence of antithrombin III, altered the amidolytic rate.

Discussion

The action of heparin and antithrombin III on acrosin-catalysed amidolysis of a well-defined arginine-terminated p-nitroanilide was studied because the physiological substrates of acrosin are not defined. For thrombin, plasmin and factor Xa, heparin-modulated antithrombin III inhibition of both amidolysis and proteolysis of the natural substrate has been demonstrated (e.g. Rosenberg, 1977; Kindness et al., 1979a, 1980c).

We have not demonstrated the mechanism by which heparin modulation of the antithrombin III inhibition of $m_f$-acrosin occurs. However, its prevention by Polybrene, and the ineffectiveness of the chemically modified and of the low-M$_r$ heparin preparations resemble similar effects seen when heparin-modulated antithrombin III inhibition of other serine proteinases is examined (Teien et al., 1976; Long & Williamson, 1983b; Long et al., 1983). The ineffectiveness of other glycosaminoglycans in potentiating antithrombin III action towards $m_f$-acrosin resembles their ineffectiveness in potentiating antithrombin III action towards other serine proteinases (Kindness et al., 1979a, 1980c; Watson et al., 1981). The modest effect of the heparan sulphate, seen here and elsewhere in other antithrombin III–serine-proteinase systems (Teien et al., 1976; Kindness et al., 1979a, 1980c; Watson et al., 1981) needs to be assessed with caution because of the high heparin content of the source of this glycosaminoglycan. Modest effects of sulphated cellulose, dextran and xylan on the antithrombin III inhibition of $m_f$-acrosin resemble similar effects on antithrombin III inhibition of other serine proteinases (Kindness et al., 1979b,c, 1980a,c; Watson et al., 1981).

Acrosin, largely in zymogen form, is present within the acrosome, an intracellular spermatozoon organelle. Prevention of fertilization in vitro and in vivo by acrosin inhibitors suggests the importance of acrosin during fertilization, possibly in enabling spermatozoon penetration of the zona pellucida of the ovum and/or in participation in the acrosome reaction, a series of events leading to release or exposure of acrosomal contents. These considerations have stimulated interest in the effectiveness of proteinase inhibitors on acrosin activity, and in their possible function as physiological modulators of acrosin activity and possible use as pharmacological contraceptives (Hartree, 1977; Morton, 1977). Sulphated glycosaminoglycans are synthesized in ovarian tissue (Clinton et al., 1983); in vitro, some may affect the acrosome reaction (Lenz et al., 1982) and the production of acrosin from its zymogen (Wincek et al., 1979; Parrish et al., 1980). The possibility of endogenous or exogenous sulphated polysaccharide modulation of antithrombin III or antithrombin III-like effects on spermatozoal proteinases in vivo should be considered.

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References

Antithrombin III-inhibited amidolysis
