Inhibition by heparin-modulated antithrombin III of amidolysis catalysed by m_{β} -acrosin

William F. LONG and Frank B. WILLIAMSON

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen, AB9 1AS, Scotland, U.K.

(Received 8 August 1983/Accepted 15 November 1983)

Purified m_{β} -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 C1s-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_{β} -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_r 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 $\beta 1 \rightarrow 4$ -linked D-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 xylose residue. Sulphated dextran from dextran of M_r 5×10^5 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) was obtained from Kabi-Vitrum, Ealing, London W.5, U.K. Results reported are from experiments using this antithrombin III. Similar results were obtained when antithrombin III (code no. 75/564; specific activity 3.6 units/mg), supplied by the National Institute for Biological Standards and Control, London N.W.3, U.K., was used. Chromogenic substrates S-2222 (*N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide hydrochloride and its methyl ester), S-2238 (D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride), S-2251 (D-valyl-L-leucyl-L-lysine *p*-nitroanilide dihydrochloride), S-2266 (D-valyl-L-leucyl-L-arginine *p*-nitroanilide dihydrochloride), S-2288 (D-isoleucyl-D-prolyl-L-arginine *p*-nitroanilide dihydrochloride), S-2302 (D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide dihydrochloride) and S-2444 (L-pyroglutamylglycyl-L-arginine *p*-nitroanilide hydrochloride) were from KabiVitrum. Polybrene (*NNN'*-tetramethylhexane-1,6-diamine polymer with 1,3-dibromopropane) was from Sigma Chemical Co. *m* β -Acrosin (specific activity approx. 130 units/mg of protein) purified from ovine spermatozoa

(Harrison, 1982) was kindly given by Dr. R. A. P. Harrison, Agricultural Research Council Institute of Animal Physiology, Cambridge, U.K. This is the major and most stable form of active ovine acrosin (EC 3.4.21.10); for definitions of nomenclature and unit of activity of ovine acrosins, see Harrison (1982). Reagents were dissolved and diluted in buffer (0.11 M-NaCl/0.05 M-Tris/HCl, pH 7.7 at 37°C).

Methods

A 30 μ l portion of polysaccharide was incubated at 37°C for 2.5 min with 20 μ l of antithrombin III (unless stated otherwise 0.035 unit/ml). Then 20 μ l of *m* β -acrosin solution (3 μ g/ml) was added and the mixture incubated for 2.5 min. Next, 20 μ l 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* β -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* β -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 ($\Delta A_{405}^{1.0} \cdot \mu\text{l}^{-1} \cdot \text{min}^{-1}$) (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 $400 \Delta A_{405}^{1.0} \cdot \mu\text{l}^{-1} \cdot \text{min}^{-1}$.

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*_β-acrosin-catalysed amidolysis

[Antithrombin III] (units/ml)	$10^{-3} \times$ Initial reaction rate ($\Delta A_{405}^{1\text{cm}} \cdot \mu\text{l}^{-1} \cdot \text{min}^{-1}$)
0	5.8
0.03	5.7
0.04	5.5
0.06	4.4
0.11	1.5
0.22	0.9
1.11	0.2

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. 1*b*–1*h*). 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*_β-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*_β-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*_β-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*_g-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*_g-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*_g-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.

We thank Dr. R. A. P. Harrison for the gift of the *m*_g-acrosin, Dr. M. W. McLean and Mr. J. Bruce for preparing the chemically modified heparins, and Ms. N. E. Woodhead for preparing the low-*M*_r heparin. We thank the Medical Research Council, the Nuffield Foundation and the Cancer Research Campaign for grants supporting work in the authors' laboratory.

References

- Abildgaard, U., Gravem, K. & Godal, H. C. (1970) *Thromb. Diath. Haemorrh.* **24**, 224–229
- Baker, J. B., Low, D. A., Simmer, R. L. & Cunningham, D. D. (1980) *Cell* **21**, 37–45
- Barrowcliffe, T. W. & Thomas, D. P. (1981) in *Haemostasis and Thrombosis* (Bloom, A. L. & Thomas, D. P., eds.), pp. 712–724, Churchill Livingstone, Edinburgh, London, Melbourne and New York
- Caughman, G. B., Boackle, R. J. & Vesely, J. (1982) *Mol. Immunol.* **19**, 287–295
- Clement, L. T., Yamashita, U. & Shevach, E. M. (1981) *J. Immunol.* **127**, 1220–1227
- Clinton, M., Long, W. F., Williamson, F. B., Hutchinson, J. S. M. & Seddon, B. (1983) *Biochem. Biophys. Res. Commun.* **111**, 581–587
- Harrison, R. A. P. (1982) *J. Reprod. Immunol.* **4**, 231–249
- Hartree, E. F. (1977) *Biochem. Soc. Trans.* **5**, 375–394
- Kindness, G., Long, W. F. & Williamson, F. B. (1979a) *Thromb. Res.* **15**, 49–40
- Kindness, G., Williamson, F. B. & Long, W. F. (1979b) *Biochem. Biophys. Res. Commun.* **88**, 1062–1068
- Kindness, G., Williamson, F. B. & Long, W. F. (1979c) *IRCS Med. Sci. Libr. Compend.* **7**, 92
- Kindness, G., Long, W. F. & Williamson, F. B. (1980a) *Br. J. Pharmacol.* **68**, 645–649
- Kindness, G., Long, W. F., Williamson, F. B., Edward, M., Winter, J. H. & Bennett, N. B. (1980b) *Thromb. Res.* **17**, 539–544
- Kindness, G., Williamson, F. B. & Long, W. F. (1980c) *Biochem. Soc. Trans.* **8**, 652–653
- Lenz, R. W., Ax, R. L., Grimek, H. J. & First, N. L. (1982) *Biochem. Biophys. Res. Commun.* **106**, 1092–1098
- Long, W. F. & Williamson, F. B. (1983a) *Med. Hypotheses* **11**, 285–308
- Long, W. F. & Williamson, F. B. (1983b) *IRCS Med. Sci. Libr. Compend.* **11**, 163–164
- Long, W. F., Williamson, F. B., Kindness, G. & Edward, M. (1980) *Thromb. Res.* **18**, 493–503
- Long, W. F., Williamson, F. B., Fraser, J. D. & Scott, G. K. (1982) *IRCS Med. Sci. Libr. Compend.* **10**, 208

- Long, W. F., Williamson, F. B. & Woodhead, N. E. (1983) *Biochem. Soc. Trans.* **11**, 97–98
- Morton, D. B. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 445–500, Elsevier/North-Holland Biomedical Press, Amsterdam, New York and Oxford
- Parrish, R. F., Wincek, T. J. & Polakowski, K. L. (1980) *J. Androl.* **1**, 89–95
- Rosenberg, R. D. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 10–18
- Seljelid, R., Bäckström, G. & Lindahl, U. (1980) *Exp. Cell. Res.* **129**, 478–481
- Teien, A. N., Abildgaard, U. & Höök, M. (1976) *Thromb. Res.* **8**, 859–867
- Tollefsen, D. M., Majerus, D. W. & Blank, M. K. (1982) *J. Biol. Chem.* **257**, 2162–2169
- Twining, S. S. & Brecher, A. S. (1977) *Clin. Chim. Acta* **75**, 143–148
- Watson, H. H. K., Long, W. F. & Williamson, F. B. (1981) *IRCS Med. Sci. Libr. Compend.* **9**, 264–265
- Wincek, T. J., Parrish, R. F. & Polakowski, K. L. (1979) *Science* **203**, 553–554
- Wunderwald, P., Schrenk, W. J. & Port, H. (1982) *Thromb. Res.* **25**, 177–191