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Decreased affinity of recombinant antithrombin for heparin due to increased glycosylation

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Recombinant antithrombin produced by baby hamster kidney (BHK) or Chinese hamster ovary (CHO) cells was separated into two fractions, containing comparable amounts of protein, by affinity chromatography on matrix-linked heparin. Fluorescence titrations showed that the more tightly binding fraction had a heparin affinity similar to that of plasma antithrombin ($K_d \sim 20$ nM), whereas the affinity of the more weakly binding fraction was nearly 10-fold lower ($K_d \sim 175$ nM). Analyses of the heparin-catalysed rate of inhibition of thrombin further showed that the fractions differed only in their affinity for heparin and not in the intrinsic rate constant of either the uncatalysed or the heparin-catalysed inactivation of thrombin. The recombinant antithrombin fraction with lower heparin affinity migrated more slowly than both the fraction with higher affinity and plasma antithrombin in SDS/PAGE under reducing conditions, consistent with a slightly higher apparent relative molecular mass. This apparent size difference was abolished by the enzymic removal of the carbohydrate side chains from the proteins. Such removal also increased the heparin affinity of the weakly binding fraction, so that it eluted from matrix-linked heparin at a similar position to the deglycosylated tightly binding fraction or plasma antithrombin. Analyses of N-linked carbohydrate side chains showed that the weakly binding fraction from CHO cells had a higher proportion of tetra-antennary and a lower proportion of biantennary oligosaccharides than the tightly binding fraction. We conclude that the recombinant antithrombin produced by the two cell lines is heterogeneously glycosylated and that the increased carbohydrate content of a large proportion of the molecules results in a substantial decrease in the affinity of these molecules for heparin. These findings are of particular relevance for studies aimed at characterizing the heparin-binding site of recombinant antithrombin by site-directed mutagenesis.

INTRODUCTION

Antithrombin is a plasma glycoprotein that inhibits most proteinases of the coagulation cascade and thus plays a major role in the regulation of blood clotting (for reviews, see Björk & Danielsson, 1986; Björk *et al.*, 1989*b*; Olson & Björk, 1992). The sulphated polysaccharide heparin dramatically accelerates this inhibition, thereby acting as an effective anticoagulant. The accelerating ability of heparin is dependent on the tight binding ($K_d \sim 20$ nM) of a unique pentasaccharide region, occurring in only about one-third of the heparin chains, to antithrombin at a site which has not yet been conclusively identified. This binding induces a conformational change in antithrombin which is sufficient to cause the inhibitor to react more rapidly with some clotting enzymes, e.g. factor Xa. However, the inhibition of other clotting proteinases, such as thrombin, requires the additional binding of the enzyme to the same heparin chain. Heparin thus appears to accelerate the inhibition of these proteinases primarily by bringing together inhibitor and enzyme on the polysaccharide surface.

The amino acid and cDNA sequences of antithrombin, as well as the intron structure of the antithrombin gene, have been determined (Petersen *et al.*, 1979; Bock *et al.*, 1982; Prochownik *et al.*, 1985; Bock *et al.*, 1988), and the protein has been expressed in yeast, insect cells and mammalian cell lines (Zettlmeissl *et al.*, 1987, 1988, 1989; Stephens *et al.*, 1987; Wasley *et al.*, 1987; Bröker *et al.*, 1987; Gillespie *et al.*, 1991). It has thus become feasible to design studies, using site-directed mutagenesis, of the structure and localization of the heparin-binding site of antithrombin. The results of the present work demonstrate a potential complication to such studies, namely that variations in the glycosylation state of antithrombin expressed by mammalian cell lines markedly affect the affinity of the inhibitor for heparin. These variations have to be taken into account in evaluations of the effect of different amino acid substitutions on heparin affinity.

MATERIALS AND METHODS

Proteins

Antithrombin was purified from expired citrated human plasma by affinity chromatography on immobilized heparin (Miller-Andersson *et al.*, 1974; Olson & Shore, 1982; Olson, 1988). The minor antithrombin fraction ('antithrombin β'), with a lower carbohydrate content and a somewhat higher heparin affinity than the major form of the protein (Carlson & Atencio, 1982; Peterson & Blackburn, 1985), was excluded from the pool used. Recombinant human antithrombin was expressed in baby hamster kidney (BHK) cells or Chinese hamster ovary (CHO) cells, as described previously (Zettlmeissl *et al.*, 1987, 1988, 1989). The protein was isolated from the medium by heparin affinity chromatography with stepwise elution, followed by ammonium sulphate precipitation (Zettlmeissl *et al.*, 1989).

The recombinant antithrombin was further fractionated by gradient elution from matrix-linked heparin. About 5 mg of protein was applied to a column (1 cm × 8 cm) of heparin–

Abbreviations used: BHK, baby hamster kidney; CHO, Chinese hamster ovary; HPAE-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection.

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Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden), which was eluted at a flow rate of 0.3 ml/min with a linear gradient (total volume 100 ml) from 0.02 M-sodium phosphate/0.1 M-NaCl, pH 7.4, to the same buffer containing 2 M-NaCl. Fractions of 1 ml were collected. The protein content of the fractions was analysed by tryptophan fluorescence in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL, U.S.A.) with excitation and emission wavelengths of 280 and 340 nm respectively. Appropriate fractions were pooled and concentrated by ultrafiltration.

Concentrations of both plasma and recombinant antithrombin were obtained by absorption measurements at 280 nm with the use of a specific absorption coefficient of 0.65 litres · g^{-1} · cm^{-1} and an M_r of 58000 (Nordenman *et al.*, 1977), unless otherwise indicated.

Human α-thrombin (> 99 % α-form) was generously donated by Dr. John Fenton, New York State Department of Public Health. Stock concentrations were determined by active-site titrations with 4-methylumbelliferyl-*p*-guanidinobenzoate (Jameson *et al.*, 1973). The enzyme was found to be > 90 % active in these analyses (Olson *et al.*, 1991).

Heparin

Heparin with a narrow M_r distribution and with high affinity for antithrombin was isolated by two successive gel chromatographies followed by affinity chromatography on immobilized antithrombin, as described previously (Höök *et al.*, 1976; Olson & Shore, 1982, 1986; Olson, 1988). The high-affinity heparin fraction used had an average M_r of 7900 (Olson, 1988). Its concentration was determined as antithrombin-binding sites by stoichiometric titrations, monitored by fluorescence (see below), of antithrombin at a concentration about 100-fold higher than the dissociation constant (Olson, 1988). This high-affinity heparin fraction was used throughout, except in the studies involving heparin affinity chromatography.

Protein analyses

Amino acid analyses were carried out on an LKB 4151 Alpha Plus amino acid analyser (Pharmacia LKB) after hydrolysis of the samples in 6 M-HCl at 110 °C for 24 and 72 h. *N*-Terminal sequences were analysed as described previously (Björk *et al.*, 1989*a*).

Immunodiffusion was done in a 1 % agarose gel in 0.02 M-sodium phosphate/0.1 M-NaCl, pH 7.4 (Nordenman *et al.*, 1977) with a commercial antiserum against plasma antithrombin (Dakopatts, Glostrup, Denmark).

Heparin binding

Titrations of antithrombin with high-affinity heparin were monitored in the SLM spectrofluorimeter by the increase in tryptophan fluorescence accompanying the interaction (Nordenman *et al.*, 1978; Olson & Shore, 1981; Danielsson *et al.*, 1986). The analyses were carried out at 25 °C by successive additions of heparin to a cuvette containing antithrombin (100–500 nM) in 0.02 M-sodium phosphate/0.1 M-NaCl/100 μM-EDTA/0.1 % (w/v)-poly(ethylene glycol), pH 7.4. The excitation and emission wavelengths were 280 and 340 nm respectively, and the corresponding bandwidths were 4 and 8 nm. Apparent stoichiometries and dissociation constants were evaluated by non-linear least-squares regression analysis of the titration curves (Nordenman & Björk, 1978).

Thrombin inactivation

The heparin-catalysed rate of inactivation of thrombin by antithrombin was measured at 25 °C in 0.02 M-sodium phosphate/0.1 M-NaCl/100 μM-EDTA/0.1 % (w/v) poly(ethylene glycol)/0.1 % (w/v) BSA, pH 7.4. Thrombin (final concentration 0.5–1 nM) was added to a mixture of antithrombin and heparin (final concentrations 5–200 nM and 1 nM respectively) to give a total volume of 50 μl in an acrylic fluorescence cuvette. Identical samples at each antithrombin concentration were allowed to react for various times, after which the reactions were quenched by addition of 1 ml of a 50 μM solution of the fluorogenic thrombin substrate *N*-*p*-tosyl-glycyl-L-prolyl-L-arginyl 7-amido-4-methylcoumarin (Sigma, St. Louis, MO, U.S.A.), containing 50 μg of polybrene/ml. Residual thrombin activity was obtained from the initial rate of substrate hydrolysis (< 1 % of substrate consumption), measured in a Perkin–Elmer 650-10 S (Perkin–Elmer, Norwalk, CN, U.S.A.) or in the SLM spectrofluorimeter with excitation and emission wavelengths of 380 and 440 nm respectively. Before these studies, possibly contaminating heparin in the antithrombin preparations was removed by ion-exchange chromatography on a Mono Q column (Pharmacia LKB). The antithrombin concentrations of the resulting samples were determined by titrations of 50 nM-thrombin with increasing amounts of antithrombin, monitored by measurements of residual thrombin activity with the fluorogenic substrate after incubation at 25 °C for 16 h.

Analyses of deglycosylated protein

Antithrombin was deglycosylated with endoglycosidase F/*N*-glycosidase F (Boehringer, Mannheim, Germany) under denaturing conditions by addition of 0.05 or 2.5 units of enzyme to 70–120 μg of antithrombin (0.7–1.2 mg/ml) in 0.04 M-sodium phosphate/0.1 M-NaCl/2 mM-EDTA/10 mM-β-mercaptoethanol/0.2 % (w/v) Triton X-100/0.2 % (w/v) SDS, pH 6.5. After incubation for 24 or 48 h at 37 °C, the samples were analysed by SDS/PAGE under reducing conditions with the use of a Tris buffer system (Laemmli, 1970).

Oligosaccharides were removed from antithrombin under non-denaturing conditions by addition of 2.5 units of endoglycosidase F/*N*-glycosidase F to 200 μg of antithrombin (0.65–0.85 mg/ml) in 0.04 M-sodium phosphate/0.1 M-NaCl/2 mM-EDTA, pH 6.5. The samples were incubated for 24 h at 37 °C and were then analysed by SDS/PAGE under reducing conditions. Amounts of 160 μg of the digested samples were also applied to an analytical heparin–Sepharose (Pharmacia LKB) column (0.5 cm × 5.5 cm), which was eluted at a flow rate of 0.1 ml/min with a linear gradient (total volume 20 ml) from 0.02 M-sodium phosphate/0.1 M-NaCl, pH 7.4, to the same buffer containing 2 M-NaCl with the use of the Pharmacia LKB h.p.l.c. system. The protein concentration of the effluent was monitored by continuous measurements of absorbance at 280 nm. Controls of undigested antithrombin were analysed in the same manner.

Endoglycosidase H (Boehringer) digestion of antithrombin under non-denaturing conditions was done for 24 h at 37 °C with 0.05 unit of enzyme and 150–170 μg of antithrombin (0.56–0.61 mg/ml) in 0.02 M-sodium phosphate/0.1 M-NaCl/100 μM-EDTA, pH 5.6. The samples were analysed by SDS/PAGE under reducing conditions and by analytical affinity chromatography on immobilized heparin, as described above.

Antithrombin was digested with *Vibrio cholerae* neuraminidase (Behringwerke, Marburg, Germany) for 20 h at 37 °C in 0.05 M-Mes/NaOH/1 mM-$CaCl_2$/0.2 % (w/v) poly(ethylene glycol), pH 6.5. The antithrombin concentration was 0.4–0.8 mg/ml, and the amount of enzyme was 0.5 unit/250 μg of protein. The digested samples were analysed by PAGE under native conditions (Davis, 1964) and by analytical affinity chromatography on immobilized heparin.

Analyses of N-linked oligosaccharides

For analyses of the relative proportions of different N-linked

oligosaccharides in recombinant antithrombin fractions I and II, the proteins were reduced and *S*-carboxamidomethylated (Zettlmeissl *et al.*, 1989), and then digested with trypsin (sequencing grade; Boehringer) at an enzyme/protein ratio of 1:20 (w/w) for 6–12 h at 37 °C in 50 mM-Tris/HCl, pH 8.1. Peptides were separated by reverse-phase h.p.l.c. on a C_{18} column (Vydac, Hesperia, CA, U.S.A.) and glycopeptides were detected by carbohydrate compositional analyses essentially as described previously (Zettlmeissl *et al.*, 1989). Total N-linked oligosaccharides were liberated from the pooled glycopeptides by digestion of 5–10 nmol of glycopeptides in 0.5 ml of 50 mM-sodium phosphate/10 mM-EDTA/0.02 % (w/v) NaN_3, pH 7.5, with 5 units of *N*-glycosidase F (Boehringer) for 12 h at 37 °C. The released oligosaccharides were recovered in the first 10 ml of the eluate from a reverse-phase h.p.l.c. on a C_{18} column. Quantitative liberation of the oligosaccharides was indicated by the failure to detect any carbohydrate in the residual peptide material and by the fact that, after neuraminidase treatment of this material, no radiolabel could be introduced by a resialylation attempt with CMP-[^{14}C]NeuAc and Galβ1-4GlcNac-R α2-6 sialyltransferse (Conradt *et al.*, 1987). NeuAc was enzymically released from the oligosaccharide pool by treatment of 1–5 μg of oligosaccharides with 0.01 unit of *Vibrio cholerae* neuraminidase (Calbiochem, San Diego, CA, U.S.A.) in 100 μl of 0.0125 M-sodium acetate/1.25 mM-$CaCl_2$/0.005 % (w/v) NaN_3, pH 5.0, for 1 h at 37 °C. Liberated NeuAc and desialylated oligosaccharides in the treated material were then analysed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Hardy & Townsend, 1988; Nimtz *et al.*, 1990; Conradt *et al.*, 1991; Nimtz & Conradt, 1991). Individual oligosaccharides were identified by comparison with similarly desialylated reference oligosaccharides that had previously been characterized by methylation analyses, fast-atom bombardment mass spectroscopy and ^{1}H-n.m.r. (Conradt *et al.*, 1987; Zettlmeissl *et al.*, 1989; Nimtz *et al.*, 1990; Nimtz & Conradt, 1991). NeuAc was also analysed by HPAE-PAD after being released from the undigested glycoproteins by acid hydrolysis (Hermentin & Seidat, 1991).

RESULTS

Fractionation of recombinant antithrombin

Fractionation of antithrombin produced by BHK cells by affinity chromatography on matrix-linked heparin with the use of gradient elution resulted in two major peaks, designated I and II (Fig. 1). Fraction II, comprising ~ 60 % of the protein, eluted at ~ 0.9 M-NaCl, i.e. similar to a plasma antithrombin control, whereas fraction I, comprising ~ 40 % of the protein, eluted considerably earlier, at ~ 0.6 M-NaCl. The yields from about 5 mg of starting material were 0.9 and 1.2 mg of fractions I and II respectively after concentration of the pools. Antithrombin produced by CHO cells gave a very similar elution pattern and yield.

Fractions I and II from both BHK cells and CHO cells gave one band in SDS/PAGE under reducing conditions (Fig. 2). Moreover, both fractions from the two cell lines had amino acid compositions similar to that of plasma antithrombin and had the *N*-terminal sequence His-Gly-Ser-Pro-Val-, identical with that of the plasma inhibitor. Immunodiffusion analyses showed that the two fractions were immunologically identical with each other and with plasma antithrombin. The protein in both fractions I and II was thus essentially homogeneous antithrombin.

Heparin- and thrombin-binding properties of recombinant antithrombin fractions

The difference in heparin affinity between fractions I and II indicated by affinity chromatography was established by fluorescence titrations (Table 1). High-affinity heparin bound to fraction II from both BHK cells and CHO cells with a dissociation constant of ~ 20 nM, i.e. with an affinity similar to that measured for plasma antithrombin (Nordenman *et al.*, 1978; Olson *et al.*, 1981). In contrast, the dissociation constant for the binding of high-affinity heparin to fraction I from both BHK cells and CHO cells was nearly 10-fold higher, i.e. ~ 175 nM. The fluorescence increase at saturation of the protein with high-affinity heparin was similar to that with plasma antithrombin (Nordenman *et al.*, 1978; Olson *et al.*, 1981) for fraction II, but somewhat lower for fraction I. The slightly low apparent heparin–antithrombin binding stoichiometry of fraction I, and possibly also of fraction II, from BHK cells may reflect the presence in the preparations of some protein with weak or no heparin affinity. However, such

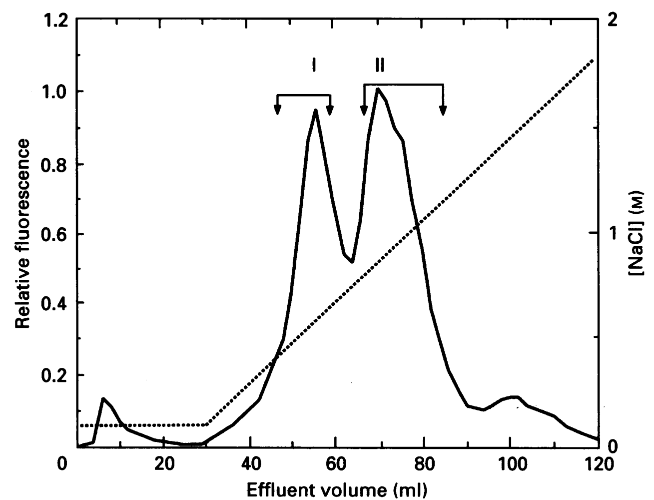

Fig. 1. Affinity chromatography on matrix-linked heparin of recombinant antithrombin from BHK cells

——, Relative fluorescence; ········, NaCl concentration. The arrows denote the fractions pooled for further analyses.

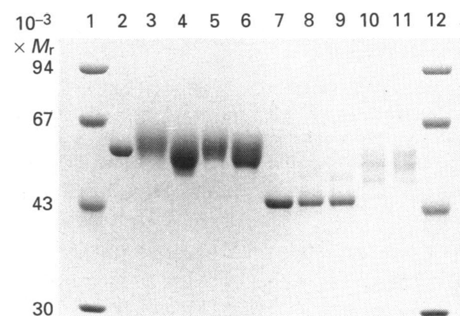

Fig. 2. SDS/PAGE under reducing conditions of plasma antithrombin and recombinant antithrombin fractions I and II from BHK and CHO cells, before and after digestion with endoglycosidase F/*N*-glycosidase F under denaturing conditions

Lanes 1 and 12, standard proteins with M_r values indicated; lanes 2–6, samples before digestion; lanes 7–11, samples after digestion. Lanes 2 and 7, plasma antithrombin; lanes 3 and 8, fraction I from BHK cells; lanes 4 and 9, fraction II from BHK cells; lanes 5 and 10, fraction I from CHO cells; lanes 6 and 11, fraction II from CHO cells. The samples were digested for 24 h with 0.05 unit of enzyme/ ~ 100 μg of protein. Amounts of 18 μg of undigested recombinant proteins and 6–12 μg of other samples were applied to each well.

Table 1. Apparent stoichiometries, dissociation constants and maximal fluorescence enhancements, derived from titrations monitored by tryptophan fluorescence, for the binding of high-affinity heparin to fractions I and II of recombinant antithrombin from BHK and CHO cells

The analyses were done at 25 °C, pH 7.4, and ionic strength 0.15, with protein concentrations of 500 nM for fraction I and 100–200 nM for fraction II. The values are means±S.E.M. of three to four measurements. $n_{app.}$, apparent stoichiometry of binding of high-affinity heparin to antithrombin; K_d, dissociation equilibrium constant for the complex formed; $\Delta F_{max.}$, maximal fluorescence enhancement on saturation of antithrombin with high-affinity heparin.

Antithrombin source	Fraction	$n_{app.}$	K_d (nM)	$\Delta F_{max.}$
BHK cells	I	0.61±0.05	175±13	0.30±0.01
	II	0.76±0.06	22±2	0.38±0.01
CHO cells	I	0.87±0.03	177±33	0.27±0.01
	II	0.92±0.05	21±3	0.42±0.02

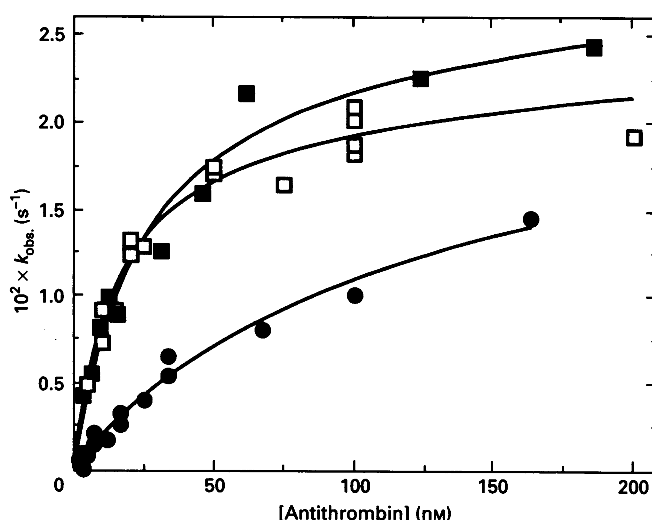

Fig. 3. Pseudo-first-order rate constant ($k_{obs.}$) for the heparin-catalysed inactivation of thrombin by plasma antithrombin or recombinant antithrombin fractions I and II from BHK cells, as a function of inhibitor concentration

□, Plasma antithrombin; ●, fraction I; ■, fraction II. The analyses were done as described in the Materials and methods section. The solid lines represent the nonlinear least-squares fits of the data to eqn. (1), giving the values for the parameters shown in Table 2.

material was not seen on re-chromatography of the fractions on heparin–Sepharose (see Fig. 4), although a small amount of non-binding protein would not have been detected.

Kinetic analyses of the inactivation of thrombin by recombinant antithrombin fractions I and II from BHK cells in the presence of heparin confirmed the different affinities of the two fractions for the polysaccharide. These analyses were based on previous evidence that the heparin-catalysed antithrombin–thrombin reaction is analogous to a rapid-equilibrium two-substrate enzyme reaction, in which heparin is the enzyme and antithrombin and thrombin are the substrates (Olson & Shore, 1986; Björk *et al.*, 1989*b*; see the Appendix). The experiments were done with a constant catalytic heparin concentration, thrombin concentrations well below the K_m of the catalyst (heparin) for the substrate (thrombin) (which is estimated to be ~ 100 nM under the conditions used; Olson & Shore, 1986; Olson & Björk, 1991), and increasing concentrations of antithrombin, which were at least 10-fold higher than the thrombin concentration. As derived in the Appendix, the inactivation of thrombin under these conditions is a pseudo-first-order process, with the following dependence of the observed pseudo-first-order rate constant ($k_{obs.}$) on the heparin and antithrombin concentrations:

$$k_{obs.} = (k_{cat.}/K^{T}_{m})\cdot[\mathrm{H}]_0\cdot[\mathrm{AT}]_0/(K_{AT,H}+[\mathrm{AT}]_0) + k_{uncat.}\cdot[\mathrm{AT}]_0 \quad (1)$$

where $k_{cat.}/K^{T}_{m}$ is the specificity constant for thrombin as a substrate for heparin, $K_{AT,H}$ is the dissociation constant for the binary antithrombin–heparin complex, $k_{uncat.}$ is the second-order rate constant for the uncatalysed antithrombin–thrombin reaction, and a subscript zero indicates total concentrations. Rate constants for the inactivation of thrombin at different antithrombin concentrations were obtained from the slopes of semilogarithmic plots of thrombin activity versus time, which all were linear and showed no indications of lags, and were plotted against the concentration of the inhibitor (Fig. 3). Values for $K_{AT,H}$ and $k_{cat.}/K^{T}_{m}$ were obtained by non-linear least-squares fits of these data to eqn. (1) (Table 2). Values for $k_{uncat.}$, the rate constant for the inactivation of thrombin by antithrombin in the absence of heparin, were measured separately with 100 nM-antithrombin and 1 nM-thrombin (Table 2) and were used in these fits. Identical values of $k_{uncat.}$ were obtained in the presence and absence of polybrene (50 μg/ml), indicating no contamination by heparin of the antithrombin preparations. The analyses gave indistinguishable values for $k_{cat.}/K^{T}_{m}$ and $k_{uncat.}$ for plasma antithrombin and the two recombinant antithrombin fractions, but about a 5-fold higher value for $K_{AT,H}$ for fraction I than for either fraction II or plasma antithrombin, in good agreement with the fluorescence titrations. These results thus show that fraction I differs from fraction II and plasma antithrombin only in heparin affinity and not in the intrinsic rate constant of either the uncatalysed or the heparin-catalysed inactivation of thrombin.

Table 2. Parameters derived from kinetic analyses of the uncatalysed and heparin-catalysed inactivation of thrombin by plasma antithrombin or fractions I and II of recombinant antithrombin from BHK cells

The analyses were done at 25 °C, pH 7.4, and ionic strength 0.15, as described in the Materials and methods section. Values for $K_{AT,H}$, the dissociation constant for the binary antithrombin–heparin complex, and $k_{cat.}/K^{T}_{m}$, the specificity constant of heparin for thrombin, were obtained by non-linear least-squares fits of the data of Fig. 3 to eqn. (1). Values for $k_{uncat.}$, the second-order rate constant for the uncatalysed antithrombin–thrombin reaction, were obtained by separate measurements in the absence of heparin. All values are means±S.E.M.

Antithrombin	$K_{AT,H}$ (nM)	$10^{-7}\times k_{cat.}/K^{T}_{m}$ ($M^{-1}\cdot s^{-1}$)	$10^{-3}\times k_{uncat.}$ ($M^{-1}\cdot s^{-1}$)
Plasma	16±3	2.1±0.1	9.0±0.4
Fraction I	110±40	2.1±0.4	9.0±0.4
Fraction II	25±9	2.6±0.3	8.6±0.6

Carbohydrate differences between recombinant antithrombin fractions

In SDS/PAGE under reducing conditions, the bands given by fractions I and II from both BHK and CHO cells were more

Table 3. Relative proportions of different N-linked oligosaccharides and average degree of sialylation of these oligosaccharides from fractions I and II of recombinant antithrombin from BHK and CHO cells

Analyses were done by HPAE-PAD after the oligosaccharides had been released from the pooled glycopeptides and digested with neuraminidase, as described in the Materials and methods section. The proportions of the different oligosaccharides are expressed as percentages of the total amount of oligosaccharides recovered, as calculated from the peak areas of the chromatograms. Similarly, the average degree of sialylation is expressed as the amount of NeuAc obtained from the chromatograms as a percentage of the total amount of oligosaccharides recovered.

Antithrombin source	Fraction	Biantennary (%)	Triantennary (%)	Tetra-antennary (%)	Sialylation (%)
BHK cells	I	50	37	12	64
	II	52	37	11	64
CHO cells	I	64	26	9*	50
	II	74	25	1	98

* About one-third of these oligosaccharides contained one lactosamine repeat.

diffuse than that obtained with plasma antithrombin (Fig. 2), consistent with a greater size heterogeneity of the two recombinant fractions. Moreover, in all experiments, fraction I reproducibly migrated more slowly than fraction II (Fig. 2), indicative of a slightly higher M_r. To clarify the structural background for this apparent molecular size difference, we digested the two fractions with a mixture of endoglycosidase F and *N*-glycosidase F, known to remove N-linked (but not O-linked) carbohydrate side chains from glycoproteins (Elder & Alexander, 1982; Plummer *et al.*, 1984; Tarentino *et al.*, 1985; Chu, 1986). The digestion was done after denaturation of the proteins, which greatly facilitates removal of the side chains by making them more accessible to the glycosidases (Tarentino *et al.*, 1985). A moderate concentration of enzyme was sufficient to deglycosylate plasma antithrombin and recombinant antithrombin fractions I and II from BHK cells (Fig. 2). The digestion markedly decreased the apparent size heterogeneity of the two fractions and also abolished their apparent M_r difference. The two deglycosylated proteins thus migrated in SDS/PAGE under reducing conditions as essentially single sharp bands with no discernible difference in mobility from each other or from the band given by deglycosylated plasma antithrombin (Fig. 2). The apparent M_r of the digested proteins was $\sim$ 45000, similar to that of unglycosylated recombinant antithrombin produced by CHO cells in the presence of tunicamycin (Zettlmeissl *et al.*, 1989), and also in approximate agreement with the size of the polypeptide chain of antithrombin (Petersen *et al.*, 1979; Bock *et al.*, 1982), indicating complete removal of the carbohydrate side chains. The antithrombin fractions from CHO cells were found to be considerably more difficult to deglycosylate than those from BHK cells, as is evident from the only partial deglycosylation occurring under the conditions used for the latter material (Fig. 2). However, full deglycosylation could be achieved with about 50-fold more enzyme, in agreement with earlier studies on unfractionated recombinant antithrombin from CHO cells (Zettlmeissl *et al.*, 1989). Under these conditions the results were similar to those obtained with the two fractions from BHK cells (not shown). Taken together, these observations thus indicate that fractions I and II from both BHK and CHO cells are heterogeneously glycosylated and that fraction I has a higher total carbohydrate content than fraction II.

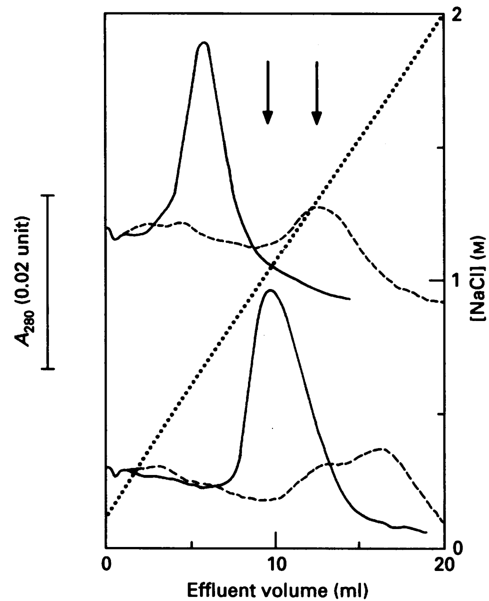

Fig. 4. Affinity chromatography on matrix-linked heparin of recombinant antithrombin fractions I and II from BHK cells before and after digestion with endoglycosidase F/*N*-glycosidase F under non-denaturing conditions

Upper panel, fraction I; lower panel, fraction II. ——, A_{280} of undigested protein; ------, A_{280} of protein digested with endoglycosidase F/*N*-glycosidase F; ··········, NaCl concentration. The arrows denote the elution positions of plasma antithrombin before (left) and after (right) digestion with endoglycosidase F/*N*-glycosidase F.

The relative proportions of different N-linked oligosaccharides in fractions I and II and the average degree of sialylation of these oligosaccharides were analysed by HPAE-PAD after release of the oligosaccharides from the total glycopeptide pool, followed by desialylation (Table 3). All oligosaccharides recovered were regular biantennary, triantennary and tetra-antennary oligosaccharides, fucosylated at the proximal GlcNAc (Zettlmeissl *et al.*, 1989; Nimtz & Conradt, 1991). Fraction I from CHO cells contained a higher proportion of tetra-antennary oligosaccharides and a lower proportion of biantennary oligosaccharides than fraction II, in agreement with the differences in glycosylation suggested by the deglycosylation experiments presented above. Fraction I from CHO cells also had a lower content of NeuAc than fraction II, a result that was verified by analyses of NeuAc released by acid hydrolysis from the undigested proteins. However, no corresponding differences in oligosaccharide composition could be ascertained for the two fractions from BHK cells. Analyses of the glycosylation pattern at individual glycosylation sites were not feasible, because of the limited amounts of material available.

Heparin affinity of deglycosylated recombinant antithrombin fractions

To investigate whether the decreased heparin affinity of fraction I was due to the different glycosylation pattern of this fraction, we digested fractions I and II, as well as plasma antithrombin, with endoglycosidase F/*N*-glycosidase F under non-denaturing conditions. These experiments could be done only with the two fractions from BHK cells, since the protein produced by CHO cells was difficult to deglycosylate even under denaturing conditions. SDS/PAGE under reducing conditions showed that 80–90 % of the deglycosylated recombinant fractions and plasma antithrombin migrated as a band of M_r $\sim$ 45000, indicating that most, although not all, of the carbohydrate side

chains could be removed from the proteins also in their native state (results not shown). This removal resulted in the major part of plasma antithrombin eluting at a higher ionic strength in affinity chromatography on immobilized heparin (Fig. 4), consistent with earlier observations that one or more carbohydrate chains of the protein interfere with heparin binding (Peterson & Blackburn, 1985; Brennan *et al.*, 1987). Notably, deglycosylated fraction I eluted at the same position as deglycosylated plasma antithrombin (Fig. 4). A substantial amount of deglycosylated protein from fraction II also eluted at this position, although a large part of the material eluted at a slightly higher ionic strength (Fig. 4), possibly because of a more complete removal of critical carbohydrate residues in this fraction. The results thus indicate that the decreased heparin affinity of glycosylated fraction I, compared with that of glycosylated fraction II and plasma antithrombin, is primarily due to its higher carbohydrate content.

Digestion of non-denatured fraction I from BHK cells with endoglycosidase H did not affect the mobility of the protein in SDS/PAGE under reducing conditions, nor its elution position in heparin affinity chromatography (results not shown). These findings indicate that the decreased heparin affinity of fraction I is not due to a carbohydrate component of the high-mannose type (Tarentino & Maley, 1974; Tarentino *et al.*, 1974; Trimble & Maley, 1984), consistent with no such component being detected in the analyses of N-linked oligosaccharides by HPAE-PAD.

The two fractions of recombinant antithrombin produced by BHK cells were also digested with neuraminidase. The untreated fractions migrated somewhat more slowly towards the anode and showed more bands than plasma antithrombin in PAGE under native conditions, reflecting lower and more heterogeneous sialylation (Zettlmeissl *et al.*, 1989). Digestion with neuraminidase decreased the mobility of the two fractions appreciably and also caused them to migrate indistinguishably from neuraminidase-treated plasma antithrombin, indicating that all or most of the NeuAc residues were removed by the enzyme from all three proteins (Zettlmeissl *et al.*, 1989). However, this removal did not alter the elution position of either fraction I or II (nor of plasma antithrombin) in heparin affinity chromatography (results not shown), suggesting that the different heparin affinities of the two fractions are not due to different contents of NeuAc.

DISCUSSION

Previous studies have shown that recombinant antithrombin produced by mammalian cell lines is heterogeneously glycosylated (Zettlmeissl *et al.*, 1987, 1988, 1989). In the present work we provide evidence that this heterogeneity affects the biological properties of the inhibitor. A substantially decreased affinity for heparin of a large proportion of the recombinant antithrombin molecules produced by two different cell lines thus was found to be due to a higher carbohydrate content of these molecules. Variations in the affinity of antithrombin for matrix-linked heparin, most likely involving differences in glycosylation, have previously been reported for antithrombin expressed in transformed African green monkey kidney (COS) cells (Stephens *et al.*, 1987) and BHK cells (Li, 1991). A difference in carbohydrate structure affecting biological activity in an analogous manner as for antithrombin has been shown for tissue plasminogen activator, expressed in CHO cells (Spellman *et al.*, 1989). A fully glycosylated form of this enzyme having three oligosaccharide side chains thus had only about two-thirds of the activity of a form lacking one of these chains (Einarsson *et al.*, 1985). Moreover, lowered *N*-glycosylation of the enzyme following site-specific mutagenesis of two or three carbohydrate attachment sites resulted in a 2–3-fold increase in specific activity (Zettlmeissl *et al.*, 1991). Another protein in which decreased glycosylation leads to increased biological activity is erythropoetin, for which removal of one of three oligosaccharide side chains by site-specific mutagenesis was shown to almost double the activity (Yamaguchi *et al.*, 1991). In contrast, glycosylation variants of a plasma proteinase inhibitor related to antithrombin, α_1-proteinase inhibitor, had indistingishable inhibitory activities (Guzdek *et al.*, 1990), consistent with the observations in the present work that the differences in glycosylation of antithrombin did not affect the intrinsic rate constant of the uncatalysed or heparin-catalysed inhibition of thrombin.

The observation that an increased glycosylation of recombinant antithrombin leads to a decreased affinity for heparin is in agreement with previous work indicating that the carbohydrate side chains of antithrombin affect its heparin-binding properties. A normal variant of antithrombin (antithrombin β) found in small amounts in plasma thus has about 3-fold higher heparin affinity than the major fraction of the inhibitor, presumably due to the absence of an oligosaccharide side chain at Asn-135 (Peterson & Blackburn, 1985; Brennan *et al.*, 1987). When present, this side chain apparently interferes with heparin binding, most likely by steric hindrance (Peterson & Blackburn, 1985). Repulsion from the negative charge of the NeuAc residues is less probable, in view of the negligible effect of removal of these residues on heparin affinity shown in this work. An effect of carbohydrate side chains on heparin binding is also indicated by the identification of a mutant antithrombin with decreased heparin affinity, in which Ile-7 is replaced by Asn, resulting in an Asn-Cys-Thr glycosylation sequence not present in normal antithrombin (Brennan *et al.*, 1988). The lower heparin affinity of the mutant inhibitor appears to be at least partly due to the presence of an extra carbohydrate side chain at this site.

The nature of the carbohydrate side chains that cause a decrease in the heparin affinity of a large proportion of the recombinant antithrombin molecules could not be identified in this work, because of the limited amounts of material available. The presence of O-linked oligosaccharides interfering with heparin binding is unlikely because of the specificity of the enzymes used for deglycosylation and because no O-glycosylation could previously be demonstrated in either plasma antithrombin or the unfractionated recombinant protein (Franzén *et al.*, 1980; Mizuochi *et al.*, 1980; Zettlmeissl *et al.*, 1989). Moreover, all classical *N*-glycosylation sites having the sequence Asn-Xaa-Ser/Thr (Nakai & Kanehisa, 1988) are normally glycosylated in the major fraction of plasma antithrombin with which comparisons were made (Petersen *et al.*, 1979; Bock *et al.*, 1982), making the presence of an N-linked oligosaccharide at a site not used in the plasma inhibitor also less likely. However, a potential other *N*-glycosylation site, Asn-Xaa-Cys, which carries carbohydrate side chains in some proteins (Nakai & Kanehisa, 1988), occurs close to the *C*-terminus in the antithrombin sequence (Petersen *et al.*, 1979; Bock *et al.*, 1982) and may have been glycosylated by the cell lines producing the recombinant proteins. An alternative, and perhaps more likely, explanation is that the carbohydrate side chains at one or more critical, normal, *N*-glycosylation sites are appreciably larger in the low-affinity recombinant antithrombin fraction than in the high-affinity fraction or the plasma protein. By analogy with previous results, a bulkier than normal oligosaccharide side chain at Asn-135, and probably also at Asn-155 and Asn-192, thus would be expected to decrease the heparin affinity of the inhibitor (Peterson & Blackburn, 1985; Brennan *et al.*, 1987; Zettlmeissl *et al.*, 1991). This alternative is supported by the demonstration in the present and previous work (Zettlmeissl *et al.*, 1989) that the oligosaccharides of recombinant antithrombin produced by mammalian cells have a considerable proportion of tri- and tetra-antennary chains, fucosylated at the

proximal GlcNac, which are not detected in normal plasma antithrombin. This explanation is also in agreement with the finding that the weakly binding fraction produced by CHO cells has a higher overall proportion of tetra-antennary side chains than the more tightly binding fraction. The fact that a corresponding difference in overall oligosaccharide composition could not be demonstrated for the two fractions from BHK cells may be due to the presence of bulky oligosaccharides at sites affecting heparin affinity being compensated by the attachment of smaller chains to other sites in the low-affinity fraction from this cell line. A further possibility for the different heparin affinities of the two fractions consistent with the data is that a site at which glycosylation normally interferes with heparin binding, e.g. Asn-135, is not glycosylated in the fraction with high affinity for heparin. In this case, the presence of bulkier than normal carbohydrate side chains at the other carbohydrate attachment sites of this fraction may account for the protein still having an apparent molecular size and heparin affinity comparable with those of plasma antithrombin. However, glycosylation of all sites with bulky oligosaccharides in the low-affinity fraction presumably leads to a further increase in apparent molecular size and a decrease in heparin affinity.

Our findings that the glycosylation state of recombinant antithrombin affects the heparin affinity of the inhibitor are of particular relevance for studies aimed at characterizing the structure and localization of the heparin-binding site of antithrombin by site-directed mutagenesis. Valid conclusions regarding the effect of a particular amino acid substitution on heparin affinity thus require that wild-type and mutant recombinant proteins with equivalent glycosylation patterns be compared. Alternatively, comparisons of heparin affinity can also be made between fully deglycosylated forms of the proteins.

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APPENDIX

Derivation of the rate equation for the heparin-accelerated inactivation of thrombin by antithrombin at catalytic heparin concentrations, thrombin concentrations below the K_m of heparin for thrombin, and antithrombin concentrations much larger than those of thrombin

Evidence has been presented to show that the heparin-catalysed antithrombin–thrombin reaction can be described kinetically by the formalism of a rapid-equilibrium two-substrate enzyme reaction, in which antithrombin and thrombin are the substrates and heparin is the 'enzyme' (Olson & Shore, 1986; Björk *et al.*, 1989). For such a reaction model, the rate of thrombin inactivation is given by the differential equation (Dixon & Webb, 1979),

$$-\frac{d[T]}{dt}=\frac{k_{cat.}\cdot[H]_0\cdot[T]\cdot[AT]}{K_{AT,H}\cdot K_m^T+K_m^T\cdot[AT]+K_m^{AT}\cdot[T]+[T]\cdot[AT]}+k_{uncat.}\cdot[AT]\cdot[T] \quad (A1)$$

where $k_{cat.}$ is the first-order catalytic rate constant for conversion of the intermediate heparin–antithrombin–thrombin ternary complex to the product antithrombin–thrombin complex and free heparin, K_m^T and K_m^{AT} are the Michaelis constants of heparin for thrombin and antithrombin respectively, $K_{AT,H}$ is the binary complex dissociation constant for the antithrombin–heparin interaction, $k_{uncat.}$ is the second-order rate constant for the uncatalysed antithrombin–thrombin reaction, $[H]_0$ is the total heparin concentration, and [T] and [AT] are the time-dependent thrombin and antithrombin concentrations respectively. Under conditions where the antithrombin concentration is in large molar excess over the thrombin concentration, the inhibitor concentration remains essentially constant during the reaction (i.e. $[AT]\approx[AT]_0$, where the subscript denotes zero time), thereby reducing this two-substrate reaction to an apparent one-substrate reaction with thrombin as the substrate, the concentration of which changes with time. Rearrangement of eqn. (A1) to standard Michaelis–Menten form with thrombin as the substrate gives:

$$-\frac{d[T]}{dt}=\frac{k_{cat.,app.}\cdot[T]}{K_{m,app.}+[T]}+k_{uncat.}\cdot[AT]_0\cdot[T] \quad (A2)$$

$$\text{where } k_{cat.,app.}=\frac{k_{cat.}\cdot[H]_0\cdot[AT]_0}{K_m^{AT}+[AT]_0}$$

$$\text{and } K_{m,app.}=\frac{K_{AT,H}+[AT]_0}{K_m^{AT}+[AT]_0}\cdot K_m^T$$

Under conditions where the thrombin concentration is much less than $K_{m,app.}$ eqn. (A2) reduces to:

$$-\frac{d[T]}{dt}=\frac{k_{cat.}}{K_m^T}\cdot[H]_0\cdot\frac{[AT]_0}{K_{AT,H}+[AT]_0}\cdot[T]+k_{uncat.}\cdot[AT]_0\cdot[T] \quad (A3)$$

where the ratio $\frac{k_{cat.}}{K_m^T}$ represents the specificity constant for thrombin as a substrate for heparin at saturating antithrombin concentrations. Integration of eqn. (A3) yields:

$$[T]_t=[T]_0\cdot e^{-k_{obs.}\cdot t} \quad (A4)$$

where $[T]_t$ and $[T]_0$ are thrombin concentrations at time t and zero time respectively and:

$$k_{obs.}=\frac{k_{cat.}}{K_m^T}\cdot[H]_0\cdot\frac{[AT]_0}{K_{AT,H}+[AT]_0}+k_{uncat.}\cdot[AT]_0 \quad (A5)$$

Eqns. (A 4) and (A 5) indicate that thrombin inactivation should be described by a pseudo-first-order exponential decay with the observed pseudo-first-order rate constant, $k_{obs.}$, being the sum of hyperbolic and linear terms with respect to the antithrombin concentration and linearly dependent on the heparin concentration.

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