# Permanent activation of antithrombin by covalent attachment of heparin oligosaccharides

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## 1. INTRODUCTION

Heparin exerts its anticoagulant action by accelerating the rate at which the plasma protease inhibitor antithrombin inactivates coagulation proteases [1]. The accelerating effect depends on the tight binding ($K \sim 10^7$ $M^{-1}$) of heparin molecules to antithrombin, which results in a conformational change of the inhibitor [2–5]. Only ~ 1/3rd of the molecules in heparin preparations bind with high affinity to antithrombin [6–8]. These molecules contain a specific, antithrombin-binding, pentasaccharide sequence that includes a unique, 3-*O*-sulphated glucosamine residue [9,10]. Oligosaccharides (octasaccharides and larger) containing this antithrombin-binding sequence have been isolated by affinity chromatography following partial depolymerization of the polysaccharide with nitrous acid [10,11]. The ability of these oligosaccharides to potentiate the inhibition of individual coagulation enzymes varies with molecular size; species composed of < 16 sugar units are essentially unable to accelerate the inhibition of thrombin by antithrombin, but greatly enhance the rate of inhibition of factor $X_a$ [12,13]. This finding suggests that the acceleration of the inactivation of thrombin, but not that of factor $X_a$, involves a direct interaction between heparin and the enzyme [12–15].

Here, we present a method for covalently attaching a high-affinity heparin oligosaccharide to antithrombin and show that this binding results in similar reactivity of the inhibitor against factor $X_a$ as that caused by only non-covalently bound oligosaccharide. Therefore, we conclude that the oligosaccharide is covalently bound at the heparin binding site of antithrombin and permanently activates the inhibitor.

## 2. MATERIALS AND METHODS

Bovine antithrombin was isolated by affinity chromatography on heparin–Sepharose [16]. Concentrations of the protein were determined by absorbance measurements at 280 nm with the use of an absorption coefficient of 0.67 $l \cdot g^{-1} \cdot cm^{-1}$ [17]. An $M_r$ = 56 000 [17] was used for calculation of molar ratios.

Heparin oligosaccharides were produced by partial deaminative cleavage of commercial heparin (Kabi-Vitrum, Stockholm), with nitrous acid, essentially as in [10]. Oligosaccharides with high affinity for antithrombin were isolated from this preparation by affinity chromatography on the matrix-linked protein [7,10] and were used without further size fractionation. Alternatively, a tetradecasaccharide ($M_r$ ~ 3700) was isolated from a similar preparation by gel chromatography on Sephadex G-50 [18].

Purified commercial heparin was coupled to CNBr-activated agarose [19] with a resulting heparin content of 2.2 mg/ml wet gel.

Heparin concentrations were determined by the carbazole method [20]. In analyses of the polysaccharide in the presence of antithrombin, the absorbance given by the same amount of the protein alone was subtracted.

Antithrombin activity against thrombin or factor



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$X_a$ was meaured by spectrophotometric assays with chromogenic oligopeptide substrates [21,22].

## 3. RESULTS AND DISCUSSION

Reaction of a 2-amino-2-deoxy-D-glucopyranosyl residue with nitrous acid results in the formation of a 2,5-anhydro-D-mannose unit, the aglycon being released [23]. Minor amounts of 2-deoxy-2-C-(formyl)-D-*arabino*-pentofuranoside are also formed [24]. Treatment of heparin with nitrous acid thus leads to the formation of fragments having 2,5-D-anhydromannose residues, with reactive aldehydo functions, at their reducing terminals [10,25]. Oligosaccharides obtained in this manner, with high affinity for antithrombin, were covalently attached to the protein by a procedure based on the formation of a labile Schiff base between the anhydromannose residue of an oligosaccharide non-covalently bound to the inhibitor and the amino group of any neighbouring lysine residue of the protein. Such a Schiff base can be reduced to a secondary amine with $NaCNBH_3$ in aqueous solution at neutral pH, which results in formation of a stable covalent bond [26]. The high-affinity oligosaccharides, either unfractionated with regard to molecular size (3.8 mg) or isolated tetradecasaccharide (3.5 mg), were mixed with antithrombin (20–25 mg) in 10 ml 0.1 M sodium phosphate (pH 7.0). The amount of tetradecasaccharide corresponds to a molar ratio relative to the protein of ~2.7; 15 mg $NaCNBH_3$ was added, and the reaction was allowed to proceed at room temparature for 24 h. Antithrombin (free and conjugated) was then separated from unreacted oligosaccharides and excess reagent on a column of Sephadex G-100 (2.5 × 95 cm) in 1.5 M NaCl, 0.02 M sodium phosphate (pH 7.0). The high salt concentration was used to dissociate any non-covalently bound oligosaccharide from the protein.

After dialysis, antithrombin containing covalently bound oligosaccharide was separated from residual unreacted protein by affinity chromatography on heparin–agarose (fig.1). The rationale of this experiment was that the bound oligosaccharide would be expected to block the heparin binding site of the inhibitor and thereby prevent the antithrombin–oligosaccharide conjugate from binding to the matrix-linked heparin. The chromatogram shown is that obtained with antithrombin reacted with the

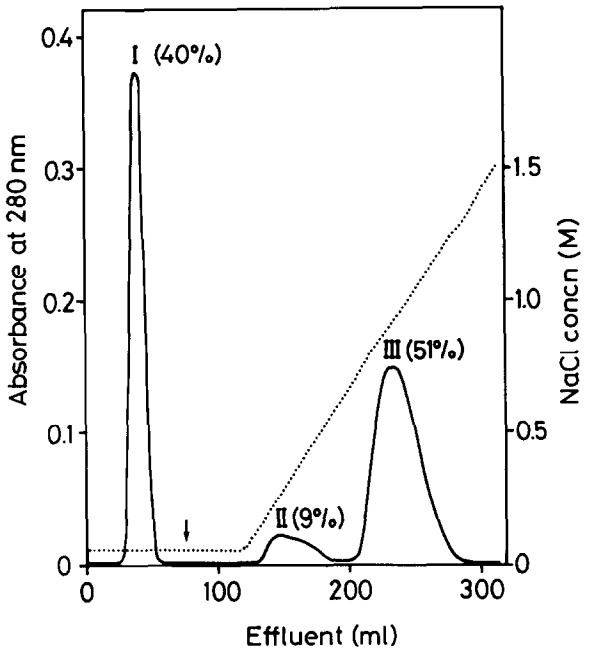


Fig.1. Separation of antithrombin containing covalently bound, high affinity heparin oligosaccharide from unreacted antithrombin by affinity chromatography on heparin–agarose. An amount of 18 mg antithrombin, reacted with unfractionated, high-affinity oligosaccharides and separated from excess reagents on Sephadex G-100, was applied in 6 ml at 30 ml/h to a 2.6 × 6 cm column of heparin–agarose in 0.05 M NaCl, 0.05 M Tris–HCl (pH 7.5) after dialysis against this buffer. Elution was performed at 60 ml/h with a linear gradient (total vol. 200 ml) to 1.5 M NaCl in the same Tris buffer. The arrow marks the start of the gradient: (——) $A_{280}$; (• • •) [NaCl] measured by conductimetry. The numbers give the amount of protein in each fraction in % of the total amount recovered, which was 88% of that applied to the column.

unfractionated high-affinity oligosaccharide preparation. As anticipated, a large peak (40% of the total protein recovered) without affinity for the immobilised heparin (fraction I) was obtained. This peak was found to contain heparin and to have considerable activity against factor $X_a$ without heparin added, as expected for the conjugate. The small peak with low affinity for the matrix-linked heparin (fraction II) also contained some heparin and had activity; however, due to the small amount of material in this peak it was not investigated further. Fraction III eluted from the immobilised heparin at the same ionic strength as untreated

antithrombin and was found to contain no heparin by carbazole analysis. Moreover, this fraction showed the same low activity against thrombin or factor $X_a$ in the absence of added heparin as untreated antithrombin and also the same increase in activity against both enzymes after addition of the polysaccharide. It was therefore concluded that fraction III consisted of antithrombin that had not reacted with any oligosaccharide.

Similar results as these were obtained for the isolated tetradecasaccharide, reacted with the protein under the same conditions. Reducing the oligosaccharide/protein ratio to ~50% in an experiment performed only with unfractionated oligosaccharides resulted in a proportional decrease in the yield of fraction I. Treatment with $NaCNBH_3$ only did not affect the elution behaviour of antithrombin on heparin–agarose, nor the activity of the protein.

Before final analyses, the protein in fraction I was separated from possibly remaining unconjugated oligosaccharide, or from heparin that might have leaked from the heparin–agarose, by gel chromatography on a column of Sephadex G-200 (1.6 × 90 cm) in 1.5 M NaCl, 0.02 M sodium phosphate (pH 7.0). The amount of heparin associated with the protein was then analysed by the carbazole method, and the activity of the protein against factor $X_a$ in the absence of added heparin was determined (table 1). The conjugates isolated after reaction of antithrombin with either the unfractionated oligosaccharide preparation or with the tetradecasaccharide contained ~5% heparin, on a weight basis. This value corresponds to a molar ratio of tetradecasaccharide/antithrombin that is not

Table 1

Heparin oligosaccharide content and anti-factor $X_a$ activity of covalent oligosaccharide–antithrombin complexes

| High-affinity oligosaccharide bound to antithrombin | Saccharide/antithrombin | | Activity[a] (%) | | |
|---|---|---|---|---|---|
| | weight | molar | Ionic strength = 0.15[b] | Ionic strength = 1.0[c] | + Polybrene[c] |
| Unfractionated | 0.05 | | 98 | 87 | 76 |
| Tetradecasaccharide | 0.05 | 0.7 | 98 | 85 | 80 |
| Control[d] | 0.5 | | 100 | 0 | n.d. |
| Control[d] | 0.1 | | 100 | n.d. | 0 |

n.d., not determined

[a] Expressed as the amount of factor $X_a$ inactivated by the sample (without heparin added) in % of the amount of enzyme inactivated by the controls (assayed at ionic strength = 0.15 and without polybrene)

[b] In the standard assay, 50 μl sample or untreated antithrombin (15–18 mg/l in 0.05 M Tris–HCl (pH 8.2) ionic strength = 0.15) was incubated at 37°C for 7 min with 50 μl buffer or oligosaccharide solution in buffer; 50 μl factor $X_a$ (13 ICTH units/ml in the Tris–HCl buffer) was added, and the incubation was continued for 20 s. A 100 μl portion was then transferred to a photometric cuvette at 37°C, containing 400 μl 1 mM Bz-Ile–Glu–Gly–Arg-pNA (S-2222; AB Kabi, Stockholm) in the Tris–HCl buffer. The rate of release of *p*-nitroaniline from the substrate was monitored at 405 nm. The proportions of antithrombin and factor $X_a$ were such that nearly all the enzyme was inactivated in the presence of an excess of heparin. The inactivation of factor $X_a$ by antithrombin alone was negligible

[c] The reactions between the samples or controls and factor $X_a$ were performed at ionic strength = 1.0 or in the presence of 3.5 mg/l polybrene (at ionic strength = 0.15)

[d] The controls were untreated antithrombin in the presence of noncovalently bound, $M_r$-unfractionated, high-affinity oligosaccharide at the weight ratios given. The two controls gave the same anti-factor $X_a$ activity at ionic strength = 0.15 in the absence of polybrene. Substitution of comparable amounts (on a weight basis) of high-affinity heparin for oligosaccharide also gave no activity at ionic strength = 1.0 or in the presence of polybrene

experimentally distinguishable from one, in view of the errors involved in the heparin determination (table 1). Moreover, the two putative antithrombin–oligosaccharide conjugates had about the same activity against factor $X_a$ at physiological ionic strength and without heparin added as untreated antithrombin in the presence of an excess of oligosaccharide (table 1).

To further ensure that the activity of the samples was not due to contaminating, non-covalently bound heparin, we also assayed the activity at an ionic strength of 1.0 or in the presence of the heparin-binding polycation polybrene (table 1). Under these conditions, untreated antithrombin in the presence of added oligosaccharide or heparin had no detectable activity. In contrast, the anti-factor $X_a$ activities of the 2 conjugate samples were only moderately decreased with either assay modification. This decrease may indicate either the presence of small amounts of non-covalently bound heparin or oligosaccharide in the two samples, or, alternatively, that both a high salt concentration and the presence of polybrene to some extent influence the interaction also between covalently bound oligosaccharide and antithrombin. However, the analyses strongly support the conclusion that the major part of the heparin is covalently linked to the inhibitor.

Together, these results show that high-affinity heparin oligosaccharides can be covalently attached to antithrombin by the method used and that these covalently bound oligosaccharides activate the inhibitor. The oligosaccharides therefore must be bound at the heparin binding site of the protein. Most likely, the covalent linkage is established while the oligosaccharides are reversibly associated with this site, and the noncovalent interactions are preserved also after formation of the covalent bond. Since the covalent binding presumably occurs between the reducing terminal of the oligosaccharide and available lysine residues at or near the heparin binding site of the protein, the efficiency of the coupling should depend on the length of the oligosaccharide. Apparently a tetradecasaccharide satisfies the requirements for covalent binding; however, more detailed studies of this problem are in progress with the use of radioactively labelled oligosaccharides of various lengths. Determination of the site(s) of covalent attachment in the protein sequence should contribute to the localization of amino-acid residues involved in the heparin binding state.

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