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The interaction between heparin and antithrombin III: a comparison of two different heparin-dye conjugates

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The interactions of antithrombin III with two heparin–dye conjugates have been compared using their fluorescence anisotropy. The first, heparin labelled with 5-isothiocyanatofluorescein, where the dye was mostly bound to unsulphated glucosamine residues, exhibited binding which was characteristic of heparin with a low affinity for antithrombin III. The second, heparin labelled with a reactive naphthalene dye (DENMT), showed similar binding character. However, when the heparin was treated with an amino group blocking agent prior to labelling with DENMT, the resultant heparin–dye conjugate showed binding behaviour, the strength of which was consistent with heparin molecules having both high and low affinity for antithrombin III. Heparin molecules with a high affinity for antithrombin III did not possess free amino groups. The implications of these findings are discussed with regard to the reliability of the data obtained using heparin–fluorescein conjugates.

Introduction

The covalent attachment of fluorescent groups to heparin provides a method by which its interactions with other macromolecules can be studied. For example, changes in the steady-state fluorescence anisotropy of heparin–fluorescein, formed by the reaction between heparin and 5-isothiocyanatofluorescein [1], has been used to monitor the interaction of heparin with other macromolecules [2]. In our studies we have discovered certain difficulties in interpreting the data obtained using this particular heparin–dye conjugate, when investigating the interaction of heparin with antithrombin III. Moreover, we have shown that by first reversibly blocking the free amino groups of heparin and then labelling the free hydroxyl groups with a reactive naphthalene dye, a heparin–dye conjugate is obtained which provides a more reliable monitor of heparin interactions.

Materials and Methods

Heparin sodium (source: porcine mucosal, batch No. 49c-0496) which had an anticoagulant activity of 169 USP units/mg, dimethyl maleic anhydride, morpholine, 5-isothiocyanato fluorescein and trinitrobenzenesulphonic acid (TNBS) were obtained from Sigma Chemical Company. 1-Ethoxy-4-(dichloro-5-triazinyl)naphthalene and 1,4-diazobicyclo[2,2,2]octane (DABCO) were purchased from Aldrich Chemical Company. Human antithrombin III (1.8 IU/mg) was a generous gift from Dr. J.K. Smith of the Plasma Fractionation Laboratory, Oxford. The sample was heat-treated for reasons of safety and subsequently showed one major band and two very

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minor bands on polyacrylamide discontinuous gel electrophoresis. Cross-immunoelectrophoresis [3] of the sample showed that 85% of the antithrombin III possessed the ability to bind heparin. All other reagents were of analar grade.

High-affinity heparin and low-affinity heparin were prepared by eluting a sample of heparin (Sigma Batch No. 49c-0496, 169 units/mg) on an immobilised antithrombin III affinity chromatography column, as described elsewhere [4].

Heparin–fluorescein isothiocyanate (FITC) was prepared by the method of Nagasawa and Uchiyama [1], without first *N*-desulphating the heparin. Heparin (10 mg) in 0.5 ml of distilled water was added to 0.5 ml of 0.2 $mol \cdot l^{-1}$ carbonate/bicarbonate buffer (pH 9.2) containing 1 mg of 5-isothiocyanatofluorescein. The mixture was stirred for 4 h at room temperature (or for 16 h when amino-'blocked' heparin was to be labelled), the reaction mixture was then centrifuged and the supernatant was applied to a Sephadex G-25 column and eluted with distilled water. The elution of heparin–fluorescein was monitored at 493 nm. The degree of labelling of heparin–fluorescein was determined by freeze-drying a portion of the sample to obtain a dry weight and by comparison of the absorbance of heparin–fluorescein at 493 nm with a standard graph of *N*-fluoresceinyl thiocarbamate [1].

2-(1,4-Diazobicyclo-2,2,2-octyl)-4-(1-ethoxy-4′-naphthyl)-6-(morpholinyl)-1,3,5-triazine (DENMT) [5] was prepared in the following manner. A portion (0.05 mol) of 1-ethoxy-4-(dichloro-5-triazinyl)naphthalene was dissolved in dry acetone (50 ml), and morpholine (10 g) was added dropwise over a period of 30 min, whilst the solution was stirred. Stirring was continued overnight, after which time the remaining acetone was removed with a stream of nitrogen gas and the reaction mixture was washed with distilled water. The product (2-chloro-4-(1-ethoxy-4′-naphthyl)-6-(morpholinyl)-1,3,5-triazine, CENMT) was filtered, dried and recrystallised three times from light petroleum (b.p. 60–80 °C). CENMT (0.05 mol) was dissolved in acetone (50 ml) and 5.6 g of DABCO (resublimed) were added with stirring. The reaction was allowed to proceed for 3 h at room temperature; the resultant product, DENMT, was first precipitated with a little distilled water then dried in vacuo over P_2O_5. This product showed a single band on TLC in an *n*-butanol/acetone/ammonia/water (8 : 6 : 2 : 1) system.

The heparin–ENMT dye-conjugates were prepared in the following manner. Heparin (10 mg) was dissolved in 1 ml of 0.1 $mol \cdot l^{-1}$ sodium phosphate buffer at pH 8. DENMT (10 mg) was added to the heparin solution and mixed rapidly for 1 h in the case of unblocked heparin and 16 h when the heparin was blocked at its amino groups. The insoluble dye was removed by centrifugation and the supernatant was applied to a Sephadex G-25 column and eluted with distilled water. The elution of the heparin-ENMT conjugate was monitored at 334 nm. The concentration of heparin (anionic sites) was determined by metachromatic titration with acridine orange [6]. The concentration of bound DENMT was determined from its absorbance at 340 nm using $\varepsilon_{340} = 5600\ l \cdot mol^{-1} \cdot cm^{-1}$. A glucosamine–ENMT complex, prepared from glucosamine in a similar manner as described for heparin, was used to determine the absorption coefficient of the bound dye.

The amino groups of heparin were reversibly 'blocked' by the method of Dixon et al. [7]. Heparin (10 mg) was dissolved in distilled water (1 ml) and the pH was adjusted to 8 with sodium hydroxide. Three portions of dimethyl maleic anhydride (5 mg) were added to the heparin solution with stirring, to avoid hydrolysis of the anhydride (which occurred if the whole 15 mg were added at once), and the pH of the reaction mixture was maintained at pH 8 using 1 $mol \cdot l^{-1}$ NaOH. The reaction was complete when the production of acid ceased. This 'blocking' agent was removed from the amino groups of heparin by titrating its solution to pH 3 with dilute HCl after the heparin-dye conjugate had been formed. Heparin samples treated in this way are referred to as 'blocked heparin–dye conjugates'.

The amino group content of heparin was assayed by reaction with trinitrobenzenesulphonic acid (TNBS) [8]. Heparin (10 mg) was dissolved in 0.1 $mol \cdot l^{-1}$ sodium phosphate buffer (pH 7.0) (1 ml) at 25 °C and an equal volume of a freshly prepared solution (0.1%) of TNBS was added. This solution was reacted in the dark for 3 h to ensure the maximum trinitrophenylation of the

heparin sample. The reaction mixture was then applied to a Sephadex G-25 desalting column, eluted with distilled water and monitored at 345 nm. Trinitrophenylated heparin was eluted in the void volume and freeze-dried. The concentration of amino groups was calculated from the absorbance at 345 nm using a molar decadic absorption coefficient of 12000 $l \cdot mol^{-1} \cdot cm^{-1}$ [8].

Absorption measurements and spectra were performed on a Perkin-Elmer Lambda 5 spectrophotometer. Fluorescence spectra and fluorescence anisotropies were recorded on a Schoeffel RRS 1000 spectrofluorimeter. For fluorescence anisotropy measurements, the 'L' format was used; the excitation beam passed through a rotatable Glan-Thomson polariser and the emission was monitored through a 'polarcote' polariser. A 'G'-factor was used to correct the anisotropy values for monochromator artifacts. For heparin–fluorescein, excitation and emission wavelengths were 480 and 530 nm, and for heparin–ENMT, 340 and 420 nm, respectively.

Limiting salt concentrations, using sodium chloride to disrupt macromolecular interaction, were determined as previously reported [9]. Briefly, the antithrombin III–heparin–dye conjugate complex was formed by the addition of antithrombin III (0.1 $mmol \cdot l^{-1}$) to the particular heparin–dye conjugate ($3.0 \cdot 10^{-3}$ mg/ml in 20 $mmol \cdot l^{-1}$ Tris-HCl buffer at pH 7.3), the fully formed complex being indicated by a maximisation of the fluorescence anisotropy (see Figs. 3 and 4), which was given the value of 100%. A small aliquot of a solution of sodium chloride (4 $mol \cdot l^{-1}$) was then added to the solution of the complex and, after a period of equilibration (20 min), the fluorescence anisotropy was measured. This process was repeated until the anisotropy indicated the complete dissociation of the antithrombin III–heparin–dye conjugate complex. The percentage change in fluorescence anisotropy was plotted against the concentration of sodium chloride in solution to give a plot which clearly showed the progressive dissociation of the complex with increasing salt concentration. A linear least-squares procedure was used to fit the linear part of the salt dissociation curve to a point of intersection with the x-axis, which was the concentration of salt required for the complete dissociation of the complex, i.e., the limiting salt concentration. This was repeated at least three times, and the mean and standard deivation were calculated.

The chemical nature and clinical potency of the parent sample of heparin and its high- and low-affinity fractions are characterised in Table I of Ref. 9.

Results and Discussion

Table I lists the degrees of labelling of heparin–dye conjugates, assuming a weight average molecular weight for heparin of 16000 (as determined by its elution behaviour on LKB Ultrogel AcA-44, compared with that of heparin fractions of known molecular weights). The assay of heparin for amino groups, with TNBS (Fig. 1), showed approximately one free amino group to three molecules of heparin in this sample (about

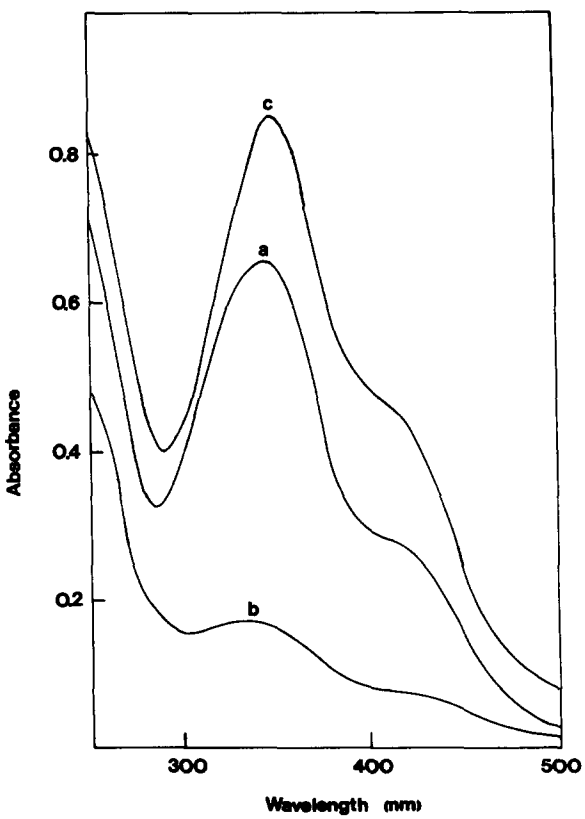

Fig. 1. Absorption spectra of: (a) heparin–TNBS (3 mg/ml), (b) blocked heparin–TNBS (3 mg/ml), and (c) glucosamine–TNBS (10 mg/ml).

TABLE I

DEGREE OF LABELLING FOR HEPARIN–DYE CONJUGATES AND THE LIMITING SALT CONCENTRATION VALUES FOR THEIR COMPLEXES WITH ANTITHROMBIN III

Heparin–dye conjugate	Degree of labelling	Limiting salt concentration [a] ($mol \cdot l^{-1}$)
Heparin–TNBS	0.3	–
Blocked heparin–TNBS	0.06	–
High-affinity heparin–TNBS	> 0.01	–
Heparin–fluorescein	0.2	n.d. [b]
Blocked heparin–fluorescein	0.02	0.09 ± 0.02
Heparin-ENMT	0.63	0.08 ± 0.03
Blocked heparin–ENMT	0.12	0.69 ± 0.05
High-affinity heparin–ENMT	0.12	0.72 ± 0.03
High-affinity blocked heparin–ENMT	0.13	0.69 ± 0.04
Low-affinity heparin–ENMT	0.5	0.11 ± 0.03
Low-affinity blocked heparin–ENMT	0.15	0.08 ± 0.02

[a] Values quoted are the means of three measurements.
[b] n.d., not detectable.

80% of these groups were 'blocked' with dimethyl maleic anhydride). Heparin, which has a high affinity to antithrombin III, showed no significant reaction with TNBS, indicating an absence of free amino groups. Gel filtration on Sephadex G-10 showed that a reaction occurred between TNBS and glucosamine, but not with glucose or maltose, confirming that this reagent was specific to amino groups. Amino acid analysis on the 12-, 24- and 48-h hydrolysates of the heparin sample confirmed the absence of serine, or any other amino acid residues which could have provided reactive groups at the concentrations found with TNBS.

Reaction of FITC with heparin gave a degree of labelling (Table I) which was similar to that with TNBS and therefore mostly occurred at the amino groups of glucosamine residues. Amino-blocked heparin (blocked heparin) gave a poor degree of labelling with FITC and labelling was found not to occur with high-affinity heparin, this being consistent with the absence of amino groups. However, reaction of heparin, blocked heparin and high-affinity heparin with DENMT gave higher degrees of labelling. Reaction of the amino blocking agent prior to reaction of high-affinity heparin with DENMT had little effect on the degree of labelling, the value of which was consistent with the absence of amino groups. In contrast, blocking the amino groups of low-affinity heparin significantly reduced its degree of labelling on reaction with DENMT.

Fig. 2 shows the excitation and fluorescence emission spectra of heparin–ENMT and blocked heparin–ENMT (both at a concentration of 1 mg/ml in distilled water at pH 6.2). These heparin–dye conjugates both have excitation maxima at 338 nm and emission maxima at 420 nm. Fig. 3 shows the change in the fluorescence anisotropy of heparin–fluorescein and blocked heparin–fluorescein ($1 \cdot 10^{-3}$ mg/ml in 2 mmol ·

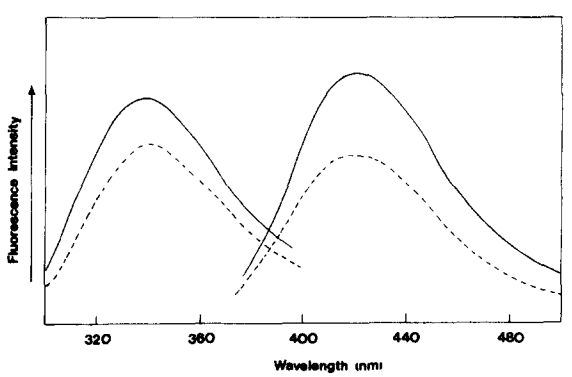

Fig. 2. Fluorescence excitation and emission spectra of 1 mg/ml heparin–ENMT (solid line) and blocked heparin–ENMT (dashed line).

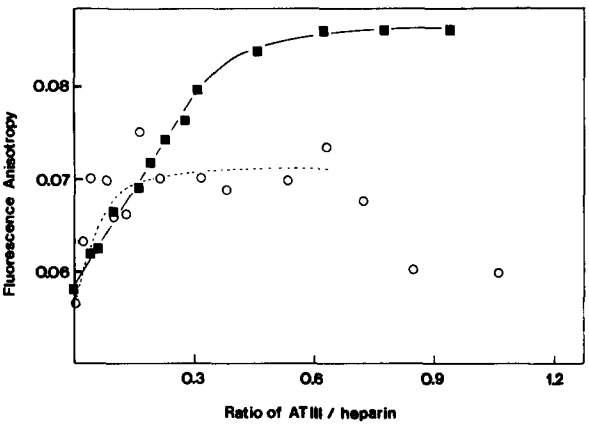

Fig. 3. Changes in the fluorescence anisotropy of heparin–fluorescein (O) and blocked heparin–fluorescein (■) following the addition of antithrombin III, (ATIII).

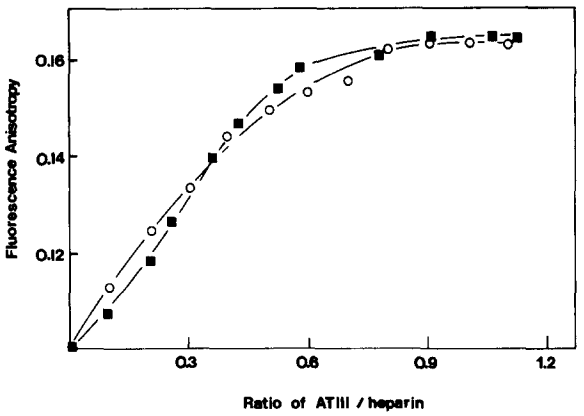

Fig. 4. Changes in the fluorescence anisotropy of heparin–ENMT (O) and blocked heparin–ENMT (■) following the addition of antithrombin III (ATIII).

l^{-1} Tris-HCl buffer (pH 7.3)) when titrated with antithrombin III. For the antithrombin III/heparin–fluorescein interaction the small increase in the anisotropy indicated poor binding, a situation which was improved when the heparin was treated with the amino group blocking agent prior to reaction with FITC. Comparison of the limiting salt concentration values (Fig. 5), which indicates the strength of binding for this interaction with previously reported values (Table I and Ref. 9), are, however, consistent with an interaction formed between antithrombin III and heparin which had a low affinity for this protein. Therefore, reaction with FITC, without *N*-desulphating the heparin prior to reaction, primarily occurs at sites on the low-affinity heparin molecules. This is true even after attempting to block the free amino groups of heparin.

As FITC shows little tendency to label blocked heparin and high-affinity heparin, a more reactive dye, DENMT, was used. This day labels the hydroxyl groups of heparin and produces heparin–dye conjugates representative of the whole sample. DENMT has been shown to have a high reactivity towards hydroxyl groups of saccharides [5], and with heparin reaction is most likely to occur at the terminal hydroxyl groups, although reaction with free amino groups would be expected. Fig. 4 shows that heparin–ENMT and blocked heparin–ENMT (under the same condition as in Fig. 3) gave similar increases in their fluorescence anisotropy when titrated with antithrombin III. There was no significant increase in either the quantum yield or the fluorescence lifetime of probe (approx. 4 ns) during these titrations; the increase in anisotropy therefore reflects the increase in the size of the rotor which occurs when antithrombin III binds to the heparin–ENMT conjugate. The limiting salt concentration values for these interactions are quite different (Fig. 5 and Table I). When the unblocked heparin–DENMT–antithrombin III was disrupted with sodium chloride, the limiting salt concentration was typical of a complex formed with

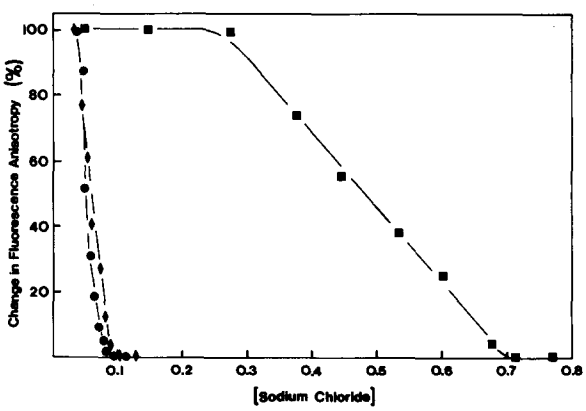

Fig. 5. Effect of added sodium chloride on the fluorescence anisotropy of antithrombin III/heparin–dye conjugate complexes: blocked heparin–fluorescein (◆), heparin–ENMT (●), and blocked heparin–ENMT (■).

low-affinity heparin (Table I and Ref. 9). However, when the amino groups of heparin were blocked the limiting salt concentration was typical of a complex formed between high-affinity heparin and antithrombin III. Blocking the amino groups of high-affinity heparin and low-affinity heparin had little effect on the limiting salt concentrations of their complexes with antithrombin III.

The data presented here show that the fluorescent labelling of the free amino groups of heparin does not provide a reliable monitor of its interaction with antithrombin III. This is of importance when investigating all heparin interactions, since in general low-affinity molecules have lower charge densities than high-affinity heparin molecules [9]. However, the reaction of a fluorescent probe with free hydroxyl groups results in a heparin–dye conjugate which is representative of all heparin species present in the sample.

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