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HEPARIN BINDING TO ANTITHROMBIN III: VARIATION IN BINDING SITES AND AFFINITY

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SUMMARY

Heparin fractions of different molecular weights and anticoagulant activities were prepared by chromatography on protamine-Sepharose, and the association constants and stoichiometry for binding to antithrombin III were determined by measurement of enhancement of tryptophan fluorescence. A 7,900 molecular weight heparin preparation bound to antithrombin III with a stoichiometry of close to 2:1, whereas 14,300 and 21,600 molecular weight fractions bound at approximately 1:1 with the protein. Apparent association constants were 0.66 x 10^6 M^{-1} for the low molecular weight preparation and 2.89 x 10^6 M^{-1} for the high molecular weight material. Maximal fluorescence enhancement was greater with the higher molecular weight heparin. These results suggest a model of heparin-antithrombin III binding in which two sites on antithrombin III can accommodate one large heparin molecule with high affinity or two smaller molecules with low affinity.

INTRODUCTION

Considerable evidence supports the proposal that heparin acts as an anticoagulant by virtue of its capacity to bind to and activate antithrombin III. Heparin is polydisperse and a variety of procedures have been applied to the separation of heparin molecules with varying anticoagulant activity. Beside providing heparin of high specific activities for the potential clinical application of coating vascular prostheses, the efforts at fractionating heparin offer an opportunity to further knowledge of the details of the interaction of heparin and antithrombin III.

We have developed a preparative method for the fractionation of heparin by ion-exchange chromatography on either DEAE-Sephadex or protamine-Sepharose (1). We have found as have others previously that, in general, increasing anticoagulant activity is associated with increasing molecular weight of heparin. We report in this paper preliminary studies of the binding to antithrombin III of several fractions of heparin with differing molecular weight

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and anticoagulant activity.

MATERIALS AND METHODS

Heparin of porcine mucosal origin (Sigma Chemical Company, lot number 96C-0093; specific anticoagulant activity specified by the manufacturer was 158.7 units/mg by the U.S.P. J-A standard) was fractionated on protamine-Sepharose as previously described (1). The carbazole reaction of Bitter and Muir (2) was used to assay heparin concentration. Anticoagulant activity in USP units/mg was assayed by the amidolytic thrombin assay (3) with a synthetic chromogenic substrate (S-2160, Ortho Diagnostics, Inc., Raritan, New Jersey 08869). Heparin molecular weights were measured by high-pressure liquid chromatography (performed by Dr. F. J. Petracek, Riker Laboratories, St. Paul, Minnesota (4)). Antithrombin III of bovine origin was isolated as previously described (5), and the protein concentration was determined by absorbance measurements, with $A^{1\%}_{280\ nm} = 6.0$ (6).

Antithrombin III binding affinity and stoichiometry of three heparin fractions were determined by tryptophan fluorescence based on the method of Einarsson and Andersson (7) using an initial reaction volume of 2.0 ml of antithrombin III in buffer (0.05 M Tris, 0.2 M glycine, 0.03 M NaCl, pH 7.4) to which the heparin in the same buffer was added in successive increments. The samples were excited at the apparent maximum of 290 nm in a Baird-Atomic SF-100 fluorescence spectrophotometer; emission spectra were scanned between 300 and 400 nm. Binding stoichiometry and affinity experiments were performed separately: antithrombin III concentrations greater than 10 times the apparent dissociation constant K_d for the former and in the vicinity of K_d for the latter. Binding stoichiometry was obtained from a plot of $F-F_0/F_{max}-F_0$ versus [heparin (mol/L)]/[antithrombin III (mol/L)], where F is the fluorescence of the heparin-protein complex, F_0 is the fluorescence of the uncomplexed protein, and F_{max} is the maximum observed fluorescence in two-fold ligand excess (8). Binding affinity was determined from Hill plots of log $(F-F_0/F_\infty-F)$ versus log free heparin (mol/L), where F and F_0 are defined as above and F_∞ is the fluorescence intensity of protein saturated with heparin, determined by extrapolation of the double-reciprocal plot of $(F-F_0)^{-1}$ versus $[\text{heparin (mol/L)}]^{-1}$. The affinity constant for binding, Ka, was obtained from the linear regression determined by least-squares, where Ka = 1/[free heparin (mol/L)] at log $[(F-F_0)/(F_\infty-F)] = 0$ (9).

RESULTS

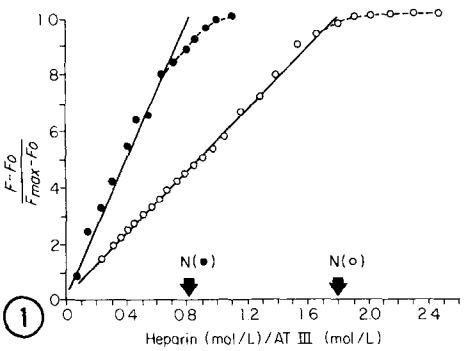
The observed stoichiometries, affinities, and maximal increases in fluorescence of antithrombin III with the three protamine-Sepharose fractions examined are presented in Table I. Typical plots for the stoichiometry of binding by heparin fractions I and III are given in Fig. 1; Hill plots for the same fractions are presented in Fig. 2. There is nearly a 1:1 binding stoichiometry of heparin fraction III to antithrombin III, whereas for fraction I nearly 2 molecules of heparin bind per molecule of protein. Additionally, the apparent Ka of the heparin-protein complex is 4-5 fold larger for fraction III than for fraction I. Heparin fraction II has a Ka

Table I

Heparin Fractionated on Protamine-Sepharose

Fraction	Eluant molarity	Specific anticoagulant activity (USP units/mg)	Molecular weight (number averaged)	Antithrombin III binding Ka (M^{-1})	Heparin/ Antithrombin III[4]	Maximal fluorescence enhancement[5]
I	1.3	132	7,900	0.66×10^{6}[1]	1.85	2.3[1]
II	1.6	258	14,300	1.58×10^{6}[2]	1.03	3.5[2]
III	2.0	282	21,600	2.89×10^{6}[3]	0.84	7.5[3]

[1]Mean of four determinations.
[2]Mean of three determinations.
[3]Mean of two determinations.
[4]Mean of two determinations for each fraction.
[5]Antithrombin III concentration was 1.15 μmol/L.



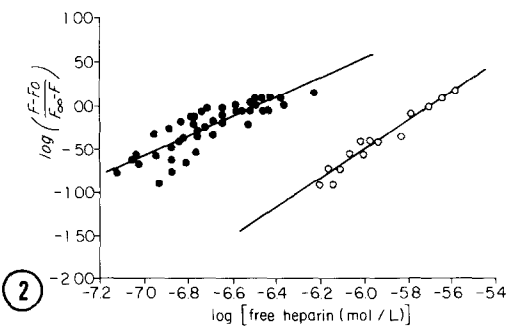


Fig. 1. Plots for determining binding stoichiometry of heparin fractions I (o) and III (•). Antithrombin III concentrations were 19.7 μmol/L and 7.66 μmol/L, respectively. N↓ indicates the estimated ratio of heparin to antithrombin III.

Fig. 2. Hill plots for binding of heparin to antithrombin III. Data points from four experiments with heparin fraction III (•) and from a single representative experiment with fraction I (o) are illustrated. Antithrombin III concentration in all experiments was 1.15 μmol/L.

intermediate between the other two fractions; its binding stoichiometry is similar to that of fraction III. Maximal fluorescence enhancement of antithrombin III parelleled the binding affinities.

DISCUSSION

Einarsson and Andersson utilizing tryptophan fluorescence to quantitate heparin interaction with antithrombin III demonstrated differences in binding

affinity among several heparin preparations (7). High-affinity heparin prepared by chromatography on matrix-immobilized antithrombin III bound to antithrombin III at one site with an apparent Ka of 2.3×10^6 M^{-1} (7). Two other samples of heparin yielded Scatchard plots which were interpreted as indicating two sites with apparent Ka's of 0.6×10^6 and 0.2×10^6 for one sample and 0.7×10^6 and 0.1×10^6 for the other. Einarsson and Andersson concluded that their high activity heparin purified by complexing with antithrombin III bound to the sites on antithrombin III with the higher Ka.

We have previously shown that for fractions prepared by ion-exchange chromatography increasing molecular weight is associated with increasing anticoagulant activity (1). The results presented in Table I demonstrate that a high-activity, high-molecular weight heparin fraction exhibits a larger apparent association constant for binding to antithrombin III than do smaller heparin molecules with less anticoagulant activity. Binding stoichiometry is different for large and small molecular weight fractions with nearly two molecules of low molecular weight heparin but only one molecule of the higher molecular weight heparin binding per molecule of antithrombin III.

We propose a model of heparin interaction with antithrombin III based on the differences in heparin binding affinity and stoichiometry. The model entails the presence of two binding sites on the antithrombin III molecule which can react with one sufficiently large heparin molecule or two smaller molecules; in either case, a conformational change is induced in antithrombin III that allows accelerated binding to and inactivation of the antithrombin III-sensitive coagulation factors. Our model differs from that of Einarsson and Andersson in that we propose that high affinities of heparin for antithrombin III depend on simultaneous binding to two sites with lower intrinsic affinities rather than on the high intrinsic affinity of a single site. The difference in magnitude of change in fluorescence of antithrombin III we have observed between low and high molecular weight heparin at considerable ligand

excess indicates a smaller conformational change in the protein when two small heparin molecules bind.

Hopwood et al. (10) have obtained a fragment of heparin by digesting heparin bound to antithrombin III with heparinase. The fragment has a molecular weight of 4,800 and binds with an affinity similar to that of "high affinity" intact heparin as indicated by the salt concentration required for elution from immobilized antithrombin III. Laurent and co-workers (11) assuming a single binding locus in heparin of the size of the fragment obtained by Hopwood et al. have calculated that the molecular weight dependence of the anticoagulant activity of heparin can be explained on probabilistic grounds. More recently Rosenberg et al. have fractionated a low molecular weight (6,000) preparation of commercial heparin by antithrombin III affinity chromatography and find consistent structural differences between the fractions with varying anticoagulant activity (12). These results suggest the existence of a specific sequence for optimum binding to antithrombin III. The observations of Hopwood et al. (10) and Rosenberg et al. (12) would be reconcilable with our two-site model if the specific sequence in high affinity heparin consists of two separate loci. The upper limit on the separation of the two loci would be set by the end to end length of the 4,800 molecular weight fragment of Hopwood et al. (10).

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