ANTICOAGULANT ACTIVITY OF HEPARIN: ISOLATION OF ANTITHROMBIN-BINDING SITES

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1. Introduction

The anticoagulant activity of heparin is due to its ability to specifically bind to antithrombin and thus strikingly accelerate the rate of inactivation of a number of the enzymes involved in the coagulation mechanism [1,2]. It was recently demonstrated that heparin may be separated into distinct fractions, having high and low affinity, respectively, for antithrombin [3,4]; the high-affinity fraction showed exceedingly high antigoagulant activity, whereas the low-affinity fraction was almost inactive. A preliminary structural characterization of the two heparin fractions failed to reveal any obvious dissimilarities [4]. In the present study heparin fragments capable of binding to antithrombin were isolated after digestion of heparin–antithrombin complexes with a bacterial heparinase. Fragments with high affinity for antithrombin were derived from high-affinity heparin only. They had a molecular weight of about $4–5 \times 10^3$. These findings suggest that binding of heparin to antithrombin may require a specific sequence of variously substituted sugar residues.

2. Materials

Radioactive heparin was prepared from mouse mastocytoma tissue, after labeling in vitro with [^{3}H] galactose (the procedure given in ref. [5] was followed, except that 2 mCi [^{3}H]galactose was substituted for the [^{14}C]glucose). The product was digested with chondroitinase ABC and was purified by ion-exchange chromatography on DEAE-cellulose [6], yielding heparin with a specific activity of 1.5×10^4 cpm/μg uronic acid.

Purified bovine antithrombin (generously donated by Dr I. Björk at this department) was coupled to Sepharose 4B as previously described [4]. Heparinase was isolated as previously described from *Flavobacterium heparinum* [7].

3. Methods

Methods for the determination of uronic acid, radioactivity and ratios of radioactive glucuronic acid/iduronic acid in heparin have been described [5,8].

Selective deamination of *N*-sulfated hexosamine residues was achieved by the method of Shively and Conrad [9].

Digestions with bacterial heparinase were carried out under conditions described previously [10].

4. Results and discussion

^{3}H-Labeled heparin (17×10^6 c.p.m.) was separated into low-, intermediate-, and high-affinity fractions by affinity chromatography on antithrombin-Sepharose (fig.1; the two latter fractions would both be included in the high-affinity heparin described in ref. [4]). Corresponding fractions derived from

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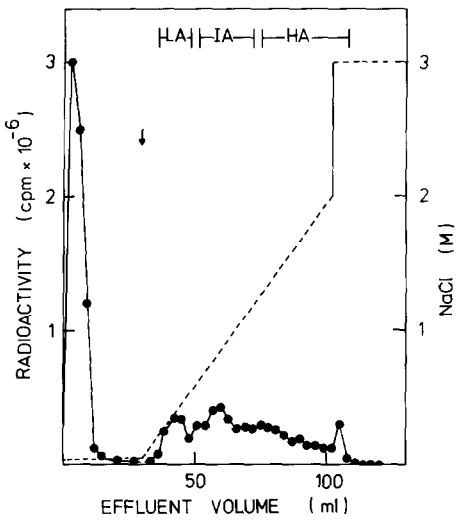

Fig.1. Fractionation of [^{3}H]heparin by affinity chromatography on antithrombin–Sepharose [4]. Gradient elution with sodium chloride (in 0.05 M Tris–HCl, pH 7.4) was started at the point indicated by the arrow. Effluent fractions of about 3 ml were collected, analyzed for radioactivity and pooled as indicated into low-affinity (LA), intermediate-affinity (IA) and high-affinity (HA), heparin respectively. The peak of break-through material was due to overloading, 17 X 10^6 cpm of [^{3}H]heparin being applied to a 3 ml-column. After two successive reapplications of the break-through fraction all the label was recovered in the three fractions LA, IA, HA; the final yield of these fractions amounted to 37%, 27% and 36%, respectively, of the starting material. The fractions were desalted by dialysis and stored at −20°. (- - -) NaCl concentration.

commercially available heparin showed anticoagulant activities of 20, 240, and 290 B.P. units/mg, respectively.

Samples of the three heparin fractions were mixed with antithrombin–Sepharose at low ionic strength; when all labeled polysaccharide had bound to the gel, heparinase was added and the samples were incubated as described in the legend to fig.2. Labeled oligosaccharides were released from the heparin-antithrombin complexes. After about two hours of incubation the amounts of liberated material had reached a plateau value that could not be increased further by addition of fresh enzyme. This value was inversely related to the antithrombin affinity of the starting material, suggesting that the heparin molecules had been subjected to selective digestion, affecting primarily those portions not bound to the antithrombin.

The heparinase-digested samples were transferred in toto to glass columns and the liberated ^{3}H-labeled oligosaccharides were washed off the gels at low ionic strength (fig.3); the heparin fragments that had remained bound to the antithrombin-Sepharose gels were eluted with sodium chloride (linear gradient). The radioactivity recovered in the wash fractions corresponded to 39%, 78%, and 89%, of the original high-affinity, intermediate-affinity, and low-affinity heparin, respectively.

Gradient elution produced peaks at low salt concentration from all the three samples (fig.3; the products of intermediate-affinity heparin are not included). In addition, the intermediate- and high-affinity (fig.3B) heparins yielded more retarded com-

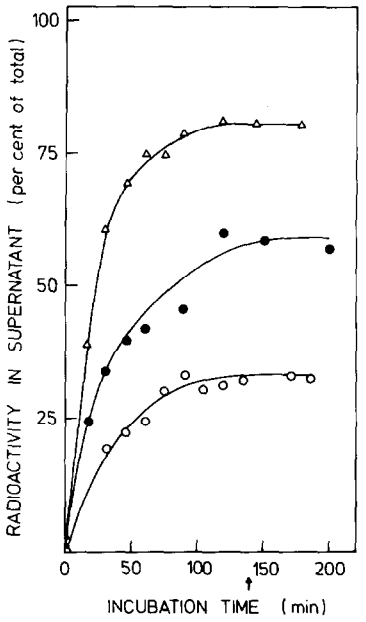

Fig.2. Digestion of heparin-antithrombin complex with heparinase. Samples of (○) high-affinity (2 X 10^6 c.p.m.); (●) intermediate-affinity (1.8 X 10^6 c.p.m.); and (△) low-affinity (1.1 X 10^6 c.p.m.) heparin were mixed with 1.5 ml of antithrombin–Sepharose in 0.1 M sodium acetate buffer pH 6.8 (final volume, 3 ml) and were then incubated at 21°C until supernatants obtained by centrifugation at 1000 X *g* for 5 min were essentially free of radioactivity. The samples were then mixed with 0.2 mg of heparinase and incubated at 29°C. Samples of 0.02 ml were withdrawn from the supernatant of the reaction mixture as indicated and analyzed for released radioactivity. More enzyme (0.1 mg) was added at the time indicated by the arrow.

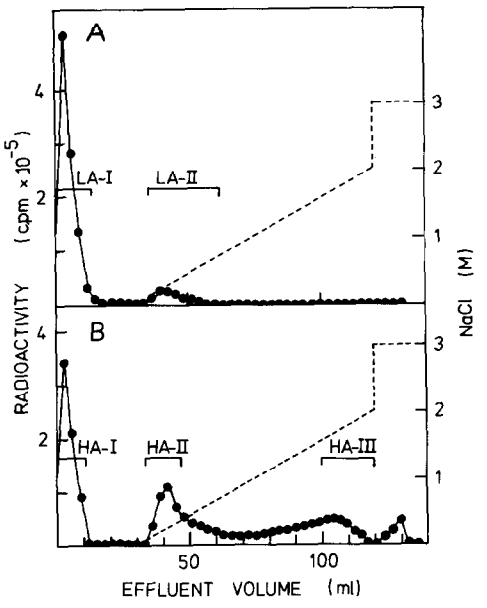

Fig.3. Salt elution of products obtained by digestion of [^{3}H] heparin-antithrombin–Sepharose complex with heparinase. (A), Low-affinity heparin; (B), high-affinity heparin. The digests, prepared as described in the legend to fig.2, were poured into glass columns (0.6 × 4 cm) and the gels were washed with 0.1 M sodium acetate, pH 6.8. Elution with a linear gradient of NaCl (- - - -) in 0.1 M sodium acetate, pH 6.8, was started as indicated by the arrow. Fractions were pooled as shown by the horizontal bars, concentrated and desalted by passage through columns of Sephadex G-15.

ponents, having approximately the same elution positions as the corresponding intact polysaccharides. Such retarded components were absent from the low-affinity heparin digest (fig.3A). On reapplication to the column the high-affinity fraction, HA-III, was quantitatively retained by the gel and required the same high salt concentration for elution as in the initial preparative experiment.

Heparin fractions eluted from the antithrombin–Sepharose after heparinase digestion were subjected to gel chromatography on Sephadex G-100. Contrary to the undegraded parent polysaccharides the digestion products were quantitatively retarded on the column (fig.4). Fractions HA-II and HA-III both yielded distinct peaks of highly retarded material (peak elution volumes, 62 ml and 52 ml, respectively). As similarly retarded fragments were obtained from the intermediate-affinity heparin but not from the low-affinity heparin (fig.4) these components would seem to include the antithrombin-binding site(s) of heparin. In addition, all fractions having affinity for antithrombin contained a portion of less retarded molecules, emerging closely after the excluded volume of the column (peak elution volume, 30 ml). Analysis (by paper chromatography after acid hydrolysis and

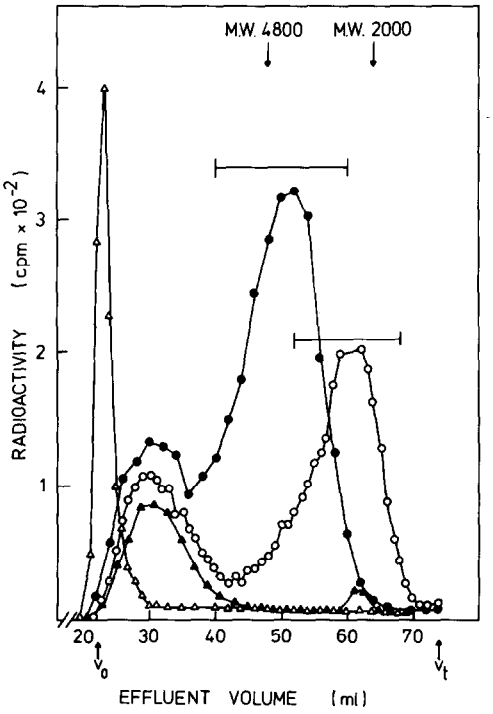

Fig.4. Gel chromatography on Sephadex G-100 of fractions HA-II (○) and HA-III (●) derived from high-affinity heparin, and fraction LA-II (▲) from low-affinity heparin (see fig.3). A sample of the intact parent polysaccharide (△) is included for comparison. The samples were applied to a 1.2 × 95 cm gel column, which was then eluted with 1.0 M NaCl in Tris–HCl, pH 7.4 at a rate of about 2 ml/h. Fractions of 1.0 ml were analyzed for radioactivity and pooled as indicated; the pools were concentrated and desalted by passage through Sephadex G-15. The two arrows indicate the peak elution positions of heparin fragments obtained by partial deamination with nitrous acid. The molecular weight of the larger fragment was determined by sedimentation equilibrium ultracentrifugation; the smaller fragment was a hexasaccharide, mol. wt. about 2000. Fragments of high-affinity heparin, less retarded than fraction HA-III on affinity chromatography (between 70 and 100 ml effluent volume; see fig.3B) behaved like fraction HA-III on Sephadex G-100. Furthermore, corresponding fragments of intermediate-affinity and high-affinity heparin appeared alike on gel chromatography.

ion-exchange treatment) of these larger-sized molecules showed the occurrence of labeled galactose and xylose; these components may thus represent polysaccharide-protein linkage regions joined by a core structure [11].

Judging from the elution positions of appropriate markers (fig.4) the more retarded components of fractions HA-III and HA-II would have average molecular weights in the order of $4–5 \times 10^3$ and $2–3 \times 10^3$, corresponding to approximately 12–16 and 6–10 monosaccharide units, respectively. The molecular size of these fragments, remaining bound to the antithrombin after heparinase digestion, thus considerably exceeds that of the liberated digestion products. Gel chromatography of such products (fraction HA-I in fig.3) on Sephadex G-25 showed that more than 80% of the material was retarded, disaccharides being the major product. In contrast, fraction HA-III was completely excluded from Sephadex G-25. This resistance of portions of the heparin molecule towards heparinase must be at least partly due to a protective effect of the antithrombin present during digestion, as no significant amounts of HA-III material could be detected after heparinase digestion of high-affinity heparin in the absence of antithrombin-Sepharose.

The low-molecular weight components of fractions HA-II and HA-III did not contain any detectable amounts of labeled neutral sugar. Analysis with regard to uronic acid composition showed [^{3}H]iduronic/total [^{3}H]uronic acid ratios of 51% and 55%, respectively; the ratio for the high-affinity heparin starting material was 54%. Treatment of the same components with nitrous acid resulted in degradation to di- and tetra-saccharides, as shown by gel chromatography on Sephadex G-25; hence, a major portion of the hexosamine residues must have been *N*-sulfated [9]. The gross composition of the isolated fragments thus conforms to that of 'heparin'. A detailed investigation of these products will hopefully reveal the structure of the antithrombin-binding site in the heparin molecule.

Acknowledgements

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