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HIGHLY ACTIVE HEPARIN SPECIES WITH MULTIPLE BINDING SITES FOR ANTITHROMBIN

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SUMMARY

Porcine heparin has been fractionated by Sephadex G-100 gel filtration and affinity chromatography into mucopolysaccharide species with approximate molecular sizes of 20,000 daltons, and 7000 daltons, respectively. The larger component has a specific anticoagulant activity of 738 USP units/mg and contains two binding regions for antithrombin. The smaller component has a specific anticoagulant activity of 363 USP units/mg and possesses only a single interaction site for the inhibitor. These results provide the first demonstration that heparin molecules may bear multiple binding sites for antithrombin.

INTRODUCTION

Heparin acts as an anticoagulant by complexing with antithrombin and thereby accelerating the rate at which this protease inhibitor can neutralize enzymes of the hemostatic mechanism (1,2,3,4,5). Previous communications have examined the interactions of antithrombin with heparin species of molecular weight 6000-12,000 daltons and have concluded that this mucopolysaccharide binds to the inhibitor with a 1:1 molar stoichiometry. On this basis, it has been tacitly assumed that heparin can bear only a single interaction site for antithrombin. In this manuscript, we demonstrate that the mucopolysaccharide can possess multiple binding regions for the inhibitor.

MATERIALS AND METHODS

Human thrombin and human antithrombin were isolated in physically homogeneous form by methods previously reported from our laboratory (1). Heparin of porcine origin was obtained from the Wilson Chemical Co. at an early stage in the manufacturing process and prior to treatment with oxidizing agents. This crude mucopolysaccharide preparation was subsequently purified by cetylpyridinium chloride precipitation (6).

The anticoagulant potency of mucopolysaccharide fractions was estimated by quantitating their ability to accelerate the interaction of antithrombin with thrombin and comparing the extent of enzyme neutralization to that attained with a heparin standard of known U.S.P. potency (7).

The concentration of antithrombin was determined by absorbance measurements at 280 nm assuming an extinction coefficient of 6.5. No appreciable alteration in this parameter was noted when various levels of heparin were added to samples of inhibitor. Mucopolysaccharide concentrations were

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estimated colorimetrically by assay of uronic acid at 530 nm according to the carbazole method of Bitter and Muir (8). The relationship between this parameter and the dry weight of heparin fractions were experimentally determined. When antithrombin was present in heparin samples, it resulted in an apparent decreased absorbance of the mucopolysaccharide of ~3% at the above wavelength. To compensate for this effect, solutions containing heparin as well as inhibitor were compared to a standard mucopolysaccharide curve constructed by employing a level of antithrombin identical to that present in the relevant samples.

Affinity chromatography was conducted by mixing limiting amounts of antithrombin with various heparin fractions at molar ratios of 0.03 to 1.0. The environmental conditions utilized during complex formation were 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5 and 24°. Thereafter, heparin bound to inhibitor was separated from uncomplexed mucopolysaccharide and subsequently isolated free of antithrombin by techniques analogous to those reported previously (9). The resultant products attained maximal anticoagulant potency when molar ratios of inhibitor to mucopolysaccharide were set at <0.10. Further reductions in this parameter to 0.03 or less did not result in the isolation of heparin species with higher biologic activities. Therefore,the molar ratios of antithrombin to mucopolysaccharide were maintained at 0.08 to 0.10 for all subsequent separations.

RESULTS AND DISCUSSION

Approximately 4 g of porcine heparin fractionated by cetylpyridinium chloride precipitation were filtered at flow rates of 40 ml/hr through a column of Sephadex G-100 (5 x 190 cm) equilibrated with 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5. Fractions of high molecular weight and low molecular weight were pooled as indicated in Fig 1. Based upon three separate experiments, the specific anticoagulant potencies of these products were 250 units/mg and 125 units/mg, respectively. Each of these preparations was subjected to affinity fractionation utilizing limiting amounts of antithrombin as described in the methods section. This procedure allowed us to obtain highly active fractions with the greatest affinities for the inhibitor and the highest anticoagulant potencies. The resultant products constituted ~10% of the mass of the high molecular weight and low molecular weight pools, respectively. Neither species could be subfractionated into components with higher anticoagulant potencies by employing smaller amounts of antithrombin. Therefore, the two fractions must be quite homogeneous with respect to their interactions with this inhibitor. The specific anticoagulant activities of highly active preparations derived from the high molecular weight and low

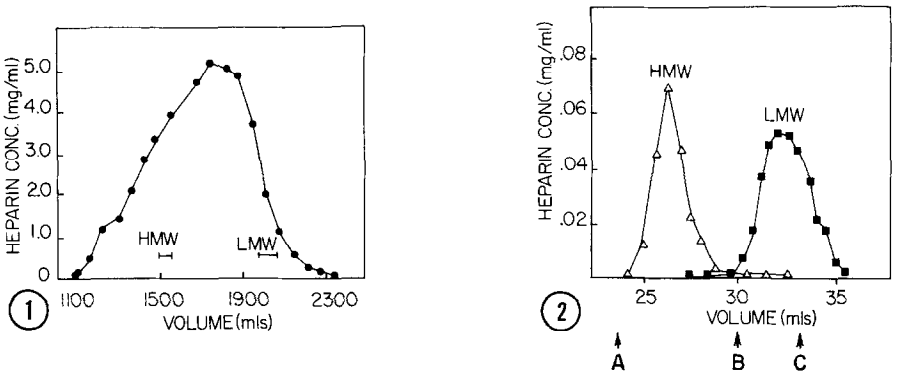

Fig. 1 Sephadex G-100 Chromatography of Porcine Heparin.

Fig. 2 Gel Filtration Chromatography of Highly Active Heparin Fractions. Approximately 0.5 mg of highly active heparin obtained from high molecular weight and low molecular weight mucopolysaccharide pools were chromatographed on columns of Sephadex G-100. The arrows indicate the peak elution volumes of heparin standards of 23,000 daltons (a), 10,000 daltons (b), and 6000 daltons (c), respectively. Molecular weights of the latter mucopolysaccharides were established by analytic ultracentrifugation.

molecular weight pools averaged 738 units/mg and 363 units/mg, respectively. The above data was obtained during three separate fractionations.

These products were rechromatographed on columns of Sephadex G-100 (0.55 x 180 cm) at flow rates of 4 ml/hr. The column matrix was previously equilibrated with 0.5 M NaCl in 0.01 M Tris-HCl, pH 7.5 and calibrated with heparin standards of known molecular size. As shown in Fig 2, the high molecular weight and low molecular weight preparations are reasonably homogeneous and exhibit approximate molecular weights of ~20,000 daltons and ~7000 daltons, respectively.

The stoichiometrics of interaction between antithrombin and high molecular weight as well as low molecular weight heparin species were subsequently investigated. We have previously demonstrated that the heparin-antithrombin complex is a stable entity during gel filtration (10). Therefore, we added antithrombin to either form of heparin such that excess quantities of inhibitor or mucopolysaccharide were present in the reaction mixtures. The environmental conditions employed were 0.2 M NaCl in 0.01 M Tris-HCl, pH 7.5

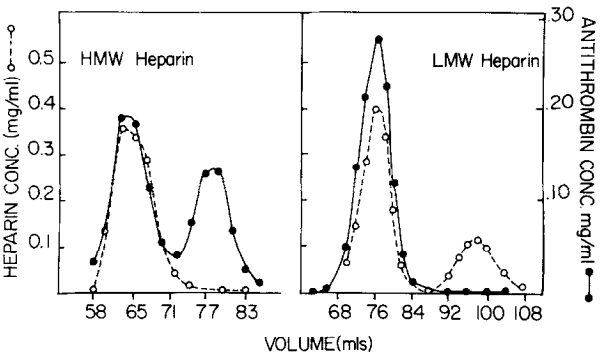

Fig. 3 Gel Filtration Chromatography of Mixture of Heparin and Antithrombin.
A, Approximately 0.34 mg of highly active heparin derived from the high molecular weight mucopolysaccharide pool were mixed with 2.5 mg of antithrombin and subsequently chromatographed on a column of Sephadex G-100.
B, Approximately 0.5 mg of highly active heparin isolated from the low molecular weight mucopolysaccharide pool were added to 2.0 mg of inhibitor and then filtered on a column of Sephadex G-100.

and 24°. The resultant solutions were filtered at flow rates of ~10 ml/hr through a column of Sephadex G-100 (0.55 x 200 cm) equilibrated with 0.2 M NaCl in 0.01 M Tris-HCl, pH 7.5. The column effluents were monitored for protein by absorbance measurements at 280 nm and for mucopolysaccharide by the carbazole reaction. These assays revealed two distinct peaks. The first peak contained protein as well as mucopolysaccharide and represented a stable heparin-antithrombin complex, Fig 3A and 3B. The second peak consisted of free antithrombin (Fig 3A) or free heparin (Fig 3B), respectively. When highly active heparin derived from the high molecular weight pool was investigated, an excess of antithrombin was employed. When highly active heparin isolated from the low molecular weight pool was studied, an excess of mucopolysaccharide was utilized. Of course, an excess of either reactant favored complex formation and minimized the effects of dissociation during the process of gel filtration. Thus, this set of conditions insured full occupancy of all available binding regions within the heparin-antithrombin complex zone of the chromatogram. Therefore, comparison of mucopolysaccharide and protein levels in the first peak should provide an unambiguous estimate of the number of interaction sites present on either heparin species.

Data obtained during two separation filtrations of antithrombin and highly active high molecular weight heparin under conditions identical to Fig 3A demonstrate that the stoichiometric molar ratio of inhibitor to mucopolysaccharide in column effluents between 60 ml and 70 ml average 1.85 ± 0.06 (S.E.M.). In other experiments (not shown) we have added incremental amounts of antithrombin to fixed levels of mucopolysaccharide until free inhibitor was observed. The maximal stoichiometric ratio was attained within the complex region when the aforementioned molar ratio of inhibitor to mucopolysaccharide was reached. After this value was achieved, addition of larger amounts of antithrombin had no effect upon this characteristic parameter but resulted in the appearance of increased levels of free inhibitor.

We have also studied the interactions of antithrombin with highly active low molecular weight heparin species under conditions identical to Fig 3B. Results derived from two separate filtrations reveal that the stoichiometric molar ratio of inhibitor to mucopolysaccharide in column effluents between 70 ml and 84 ml averages 0.96 ± .03 (S.E.M.). This stoichiometric relationship was further analyzed (not shown) by admixing increasing levels of mucopolysaccharides with a fixed quantity of inhibitor and examining the reaction mixtures by gel filtration chromatography as described above. The stoichiometric ratio of inhibitor to mucopolysaccharide was maximal when the molar ratio of antithrombin to heparin cited above was employed. Thereafter, further addition of mucopolysaccharide had no effect upon this characteristic parameter but resulted in the appearance of greater quantities of free heparin in the column chromatograms.

In summary, we have demonstrated that porcine heparin species of molecular weight ~20,000 daltons can bear two binding regions for antithrombin. Mucopolysaccharide of similar origin but with a molecular weight of ~7000 daltons possesses only a single interaction site for the inhibitor. It is of great interest that the specific anticoagulant activity of the higher molecular weight component is approximately twice that of the low molecular weight

species. This represents the highest specific anticoagulant activity ever reported for a heparin fraction. If the two binding regions present on heparin of molecular weight ~20,000 daltons were functionally equivalent to the single interaction site found on mucopolysaccharide of molecular weight ~7000 daltons, one would expect the potency per mg of these two fractions to be identical. Our findings indicate that this is not the case, Indeed, additional experiments (not presented) suggest that either the binding of antithrombin to highly active high molecular weight heparin and/or the kinetics of interaction between the resultant mucopolysaccharide-inhibitor complex and thrombin must exhibit cooperative behavior. This would introduce a new order of complexity into the functional analyses of heparin species. Furthermore, it may represent an important mechanism for controlling antithrombin action that is dependent upon the extent of site occupany for heparins of larger molecular size.

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