Polymer Bulletin 24, 429–435 (1990)

Polymer Bulletin

Sedimentation study on the binding of fibrinogen or of antithrombin III with acidic polysaccharides including heparin

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Summary

The binding of fibrinogen with heparin and dextran sulfates, and of antithrombin Ⅲ with heparin and dextran sulfate were investigated by sedimentation velocity method. From the measurement of fibrinogen-acidic polysaccharide systems, it was confirmed that heparin and low molecular weight dextran sulfate(DSC) forms soluble complexes with fibrinogen, though the amount bound of the latter was rather small compared with high molecular weight dextran sulfate(DSD). Antithrombin Ⅲ was also found to be bound by heparin and DSC. The numbers of heparin molecules bound to one molecule of fibrinogen and antithrombin Ⅲ were estimated to be 4.2 and 0.3, respectively.

Introduction

Heparin, one of the acidic polysaccharides, is well-known to be a biologically multifunctional molecule, interacting not only with various cells but with such diverse proteins as are participated in coagulation, adhesion, lypolysis, and so on. Many acidic polysaccharides with high charge density and high molecular weight were observed to form complexes with proteins, such as albumin(1) and fibrinogen(2), who have negative net charges at neutral pH region. A complex formation leads to the retardation effect of acidic polysaccharides on the fibrinogen-fibrin conversion, which was analyzed according to the Freundlich -type adsorption isotherm(3). Moreover, the formation of a soluble complex for fibrinogen-heparin systems was confirmed by electrophoretic study(4).

The sedimentation velocity method affords efficient information about the size and shape of monodisperse polymers such as proteins. For fibrinogen , such paramaters as the sedimentation coefficient at infinite dilution , the diffusion coefficient , and friction coefficient were determined from sedimentation velocity measurement in water at 20℃ (5). LAGES and STIVALA (6) found that the sedimentation coefficient of fibrinogen in 0.1 M Tris buffer solution at pH 7.5 is remarkably increased by the addition of heparin, whereby heparin peak disappears at equimolar mixing of both components, and suggested the formation

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of fibrinogen-heparin complex.

On the other hand, antithrombin Ⅲ is well-known to be an important regulator of blood coagulation (7). In the presence of heparin, anticoagulant activity of antithrombin Ⅲ is remarkably accelerated (8), it has been suggested that this acceleration is due to a heparin-induced conformational change of antithrombin Ⅲ (9) and simultaneous effects on proteases and antithrombin Ⅲ (10) (11). And binding sites of heparin to antithrombin Ⅲ (12) and of antithrombin Ⅲ to heparin (13) were reported. It was also reported that antithrombin Ⅲ forms an equimolar complex with serine proteases (14). In this study, we examine quantitatively the binding amount of either heparin or dextran sulfate to fibrinogen or antithrombin Ⅲ by sedimentation velocity method.

Experimental

Bovine fibrinogen 95% clottable (Lot. 24, Code 82-022-4) was purchased from Miles Laboratories Inc. The molecular weight and the isoelectric point of fibrinogen are 340,000 and 5.5 (15), respectively. Bovine antithrombin Ⅲ (Lot. 78C-3953), whose specific activity is 400-600 units per mg protein, was purchased from Sigma Chemical Co. The molecular weight of the sample is 56,000 (16). Heparin-Na (Lot. M6G5365) was purchased from Nakarai Chemical Co. Dextran sulfate C-Na (Lot. 48C-0241: nominal molecular weight, 40,000) and dextran sulfate D-Na (Lot. M7E5064: nominal molecular weight, 500,000) were purchased from Sigma Chemical Co.

Fibrinogen stock powder was dissolved in 0.05 M phosphate buffer, and clarified filtration through a filter pad. The concentrations of fibrinogen solutions were determined by the optical density measurement at 280 nm with a Hitachi Spectrophotometer Model EPS-3T. The specific extinction of 0.1 % fibrinogen solution used was $E_{cm} = 1.506$. The pH and the ionic strength of the fibrinogen solution were 7.0 and 0.106, respectively.

Antithrombin Ⅲ, heparin and dextran sulfates solutions were prepared by dissolving the stock powder in 0.05 M phosphate buffer at pH 7.0 and 0.106 of ionic strength.

Sedimentation velocity experiments were carried out with either Mom model 3170/b ultracentrifuge or Spinco model E ultracentrifuge, both equipped with the Schlieren optical system at 20 ± 0.2℃. In the former apparatus, samples were centrifuged at 60,000 rev/min, and in the latter apparatus, samples were centrifuged at 59,780 rev/min. The schlieren photographs were taken at 6-minute intervals. Measurements of the sedimentation coefficients and the areas of the sedimentation spectra were made with enlarged tracing(X20) of the negative films (Mom) and plates (Spinco) by using a Nikon Profile Projector 6C-2.

Results and Discussion

Some example of the sedimentation pattern of fibrinogen, heparin, and dextran sulfate D (DSD) are shown in Figure 1. In the sedimentation pattern of fibrinogen, a small faster peak appears besides the main peak. This peak occupying about 6 % of the total area may be assigned to the dimer as suggested

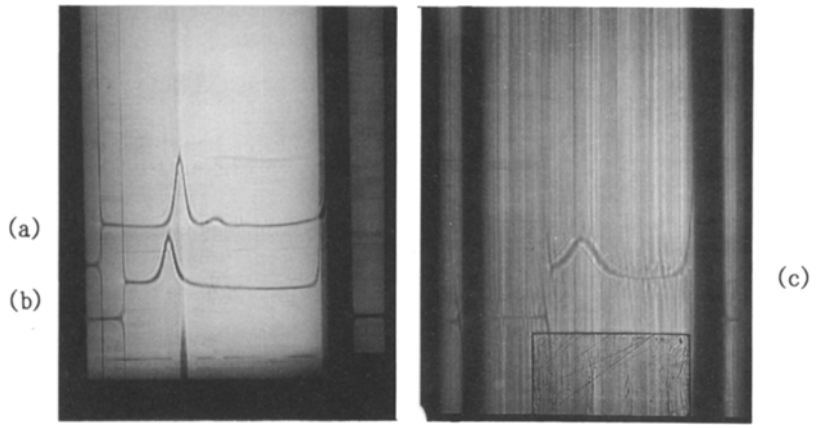


Figure 1. Sedimentation patterns of some samples in 0.05 M phosphate buffer, pH 7.0, at 20℃: (a) fibrinogen (0.8 g/dl, 12 min.), (b) dextran sulfate D (0.8 g/dl, 12 min.), and (c) heparin (0.7 g/dl, 66 min.)

by MULLER et al. (17). The observed sedimentation coefficient, S_{OBS}, of solute at a given temperature and in a given solvent is converted to the sedimentation coefficient $S_{20,w}$ at 20℃ in water by equation [1].

$$S_{20,w} = S_{OBS} \frac{\eta_{T,solv}(1 - v\rho_{20,w})}{\eta_{20,w}(1 - v\rho_{T,solv})} \qquad [1]$$

Where, v is the partial specific volume of the solute, and $\eta_{T,solv}$ and $\rho_{T,solv}$, respectively, are the solvent viscosity and solvent density at T℃. The subscript 20,W indicates "at 20℃ in water". Plotting $1/S_{20,w}$ against the concentration of solute and extrapolating to zero concentration, we obtain the sedimentation coefficient, $S^{\circ}_{20,w}$, of solute at zero concentration. The experimental results are shown in Figure 2. The numerical values of v used are: 0.71 for fibrinogen (18), 0.72 for antithrombin Ⅲ (19), 0.47 for heparin (20), and 0.60 for dextran sulfate (21). Table I summarizes the results. These values of $S^{\circ}_{20,w}$ are in accord fairly well with literature values (5), (7), (20).

The weight average molecular weight Mw of heparin was estimated by using the equation.

$$S^{\circ}_{20,w} = 0.0350\ Mw^{0.44} \qquad [2]$$

derived by the author from the experimental data by LASKER (20). The calculated molecular weight 11,000 is comparable with the value 9,000 estimated by the author intrinsic viscosity. With respect to dextran sulfate, the equation

$$S^{\circ}_{20,w} = 0.0245\ Mw^{0.44} \qquad [3]$$

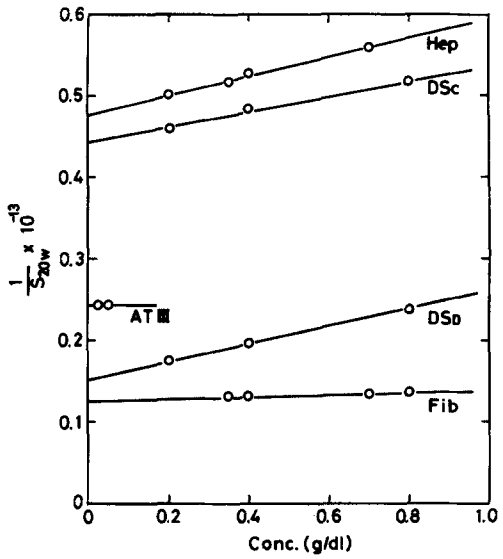


Table I $S°_{20,w}$ and M_w of proteins and polysaccharides

	$S°_{20,w}$	M_w
Fib	7.9	340,000
ATⅢ	4.1	56,000
Hep	2.1	11,000
DSC	2.3	30,000
DSD	6.5	322,000

Figure 2. $1/S_{20,w}$ plotted against the concentrations of proteins and acidic polysaccharides

proposed by SENTI et al. (21) was used to estimate the molecular weight . The numerical value obtained Mw = 30,000 for DSC and Mw = 322,000 for DSD , respectively , are compared with the nominal values 40,000 and 500,000.

In general, for globular proteins, the sedimentation coefficient of at zero concentration S° is related to Mw by the following equation (22).

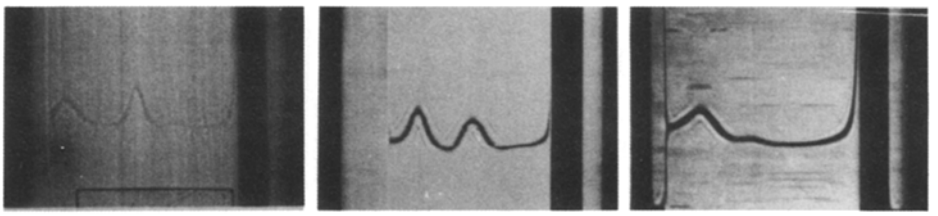
$$\frac{S°[\eta]^{1/3}}{M^{2/3}} = 2.5 \times 10^{6} \frac{(1 - v\rho)}{\eta_{o} N_A} \qquad [4]$$

where $[\eta]$ is the intrinsic viscosity of the solute , M is the molecular weight of the solute , N_A is the Avogadro's number , and η_o and ρ are viscosity and density of the solvent, respectively. HALSALL (23) has pointed out that equation [4] is simplified, in a good approximation to

$$\log S° = \log k + 2/3 \log M \qquad [5]$$

where k is a constant.

The sedimentation patterns and $S_{20,w}$ for protein-acidic polysaccharide mixtures are shown in Figure 3 and Table II, respectively, from which limiting sedimentation coefficient S° was evaluated. As is obvious in Figure 3, two peaks are distinguished in the sedimentation spectra. The observed sedimentation coefficients of acidic polysaccharides are expected to increase by the dilution effect owing to the complex formation. However, the sedimentation coefficients of the slower peak were shifted to decrease in the protein-heparin systems.



(a) (b) (c)

Figure 3. Sedimentation patterns for protein-polysaccharide mixtures in phosphate buffer, pH 7.0 at 20℃: (a) fibrinogen-heparin (0.35 g/dl-0.35 g/dl, 40 min.), (b) fibrinogen-DSD (0.40 g/dl-0.40 g/dl, 24 min.), and (c) antithrombin Ⅲ-heparin (0.05 g/dl-0.40 g/dl, 66 min.)

These results indicate that high molecular weight fractions of heparin are dominantly bound to proteins.

If protein molecules adsorb polysaccharide molecules and form a complex, we can estimate the "molecular weight" of the complex from the S° value of the complex by using equation [5]. For fibrinogen, $S^\circ = 7.9$ and $M = 340,000$ were used as the reference(5), and $S^\circ = 4.1$ and $M = 56,000$ for antithrombin Ⅲ were used (7). From the "molecular weight" of the complex, we can calculate the amount of adsorbed polysaccharide in the complex.

The amount of adsorbed polysaccharide is also estimated from the area of the peak corresponding to the complex. According to TRAUTMAN et al.(24), the concentration of the slow component C_S° is given by

$$C_S^\circ = \frac{(x_S^{obs}/x_m)^2 \, C_S^{obs}}{r} \qquad [6]$$

with

$$r = \frac{(x_F^{obs}/x_m)^{2(1-\sigma)} - 1}{(x_F^{obs}/x_S^{obs})^2 - 1}, \quad \sigma = \frac{S_S^\circ}{S_F^\circ}$$

Table Ⅱ $S_{20,w}$ for fibrinogen-polysaccharide and antithrombin Ⅲ-polysaccharide mixtures

g/dl	g/dl	$S_{20,w}$ fast	$S_{20,w}$ slow
Fib - Hep			
0.35	-	7.62	
-	0.35		1.93
0.35	0.35	8.01	1.81
Fib - DSC			
0.40	-	7.58	
-	0.40		2.08
0.40	0.40	8.52	2.20
Fib - DSD			
0.40	-	7.58	
-	0.40		5.13
0.40	0.40	13.09	5.42
ATⅢ - Hep			
0.05	-	4.12	
-	0.40		1.90
0.05	0.40	4.40	1.85
ATⅢ - DSC			
0.05	-	4.12	
-	0.40		2.08
0.05	0.40	4.38	2.08

Where, acutual concentration C° is given by

$$C^{\circ} = (x/x_m)^2 C^{obs} \qquad [7]$$

Subscripts $_S$ and $_F$ denote slower component and faster component, respectively, x is the distance from the center to the peak , x_m is the distance from the center to the meniscus , C^{obs} is the concentration determined from the area under the curve. The amounts of polysaccharides bound, estimated from equation [5] and [6] are summarized in Table III . The amount bound calculated from equation [5] by using sedimentation coefficients are slightly higher than these from equation [6] by using concentration. This seems to be due to the overestimation of sedimentation coefficients. The sedimentation coefficients in the mixtute of two solutes tend to be larger than true values. The values obtained from equation [6] are highly accurate, because Johnston-Ogston effects are avoided by correction of the concentration.

On the fibrinogen-acidic polysaccharide systems, the complex formation of fibrinogen with heparin and DSC could not be detected on turbidimetry and conformational study (25). But in this study the formation of soluble complexes were found in reversible binding of polysaccharides to proteins, although both have negative net charges in phosphate buffer at pH 7.0. And the result for fibrinogen-heparin is in good agreement with that estimated from electrophoresis result by us (4). The amounts bound seem to be dependent on molecular weight of polysaccharide, but less dependent on charge density.

In the antithrombin Ⅲ-polysaccharide systems, antithrombin Ⅲ forms soluble complexes with heparin and DSC. The amounts adsorbed of DSC to antithrombin Ⅲ is nearly equal to that of heparin. In our kinetic study (10), it was found that the main role of heparin is to accelerate the antithrombin Ⅲ-thrombin reaction,

Table Ⅲ Amount of acidic polysaccharides bound to proteins

Protein g/dl	P.S* g/dl	Amount bound From eq.(5) M of complex	P.S/protein g/g	mol/mol	r	From eq.(6) P.S/protein g/g	mol/mol
Fib - Hep							
0.35	0.35	386,000	0.14	4.2	0.98	0.14	4.2
Fib - DSC							
0.40	0.40	422,000	0.24	2.7	0.96	0.17	1.9
Fib - DSD							
0.40	0.40	789,000	1.32	1.4	0.47	0.56	0.6
ATⅢ - Hep							
0.05	0.40	61,000	0.09	0.5	0.89	0.05	0.3
ATⅢ - DSC							
0.05	0.40	61,000	0.09	0.2	0.64	0.07	0.1

* P.S; polysaccharide

but DSC does not accelerate this reaction. These results suggest that DSC can not induce conformation change of antithrombin Ⅲ because some binding sites for antithrombin Ⅲ are probably different from heparin. Moreover, it was reported that antithrombin Ⅲ forms an equimolar complex with serine proteases (14). In this case, it is noted only 0.3 mole of heparin bind to one mole of antithrombin Ⅲ, despite a large excess of heparin in the mixture. It was shown that commercial heparin used in this study, which is polydisperse and unfractionated, contained low affinity fractions with antithrombin Ⅲ (26). However, fractionated heparin should have higher affinity with antithrombin Ⅲ by taking into account both results that lysyl residue having positive charge in antithrombin Ⅲ was essential for heparin binding (13) and the dissociation constant, k_d, of antithrombin Ⅲ-heparin complex (26) was smaller than those of fibrinogen-heparin complex (4), which may result from unique molecular conformation of heparin.

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Accepted June 21, 1990 S