Molecular Weight Analysis of Antithrombin III-Heparin and Antithrombin III-Thrombin-Heparin Complexes*

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The molecular interactions between components of the heparin-catalyzed antithrombin III-thrombin reaction were investigated by light scattering. When heparin was added to antithrombin III, the molecular weight increased to a maximum and then decreased to that of a 1:1 (antithrombin III-heparin) complex. The initial molecular weights at low heparin to antithrombin III ratios were consistent with the formation of a 2:1 (antithrombin III-heparin) complex in which only one antithrombin III molecule had undergone the conformational change measured by protein fluorescence enhancement. The peak molecular weight never reached that of a complete 2:1 complex. This behavior was observed for bovine and human antithrombin III in the presence of both unfractionated heparin and high molecular weight-high affinity heparin. Pentosane polysulfate also caused some multiple associations. Bovine antithrombin III and thrombin formed a 1:1 complex that underwent further aggregation within minutes, while the human proteins did not aggregate on this time scale after forming the 1:1 complex. In the presence of stoichiometric amounts of heparin, the bovine proteins formed an initial complex of $M_r = 230,000$ (corresponding to a dimer of heparin-antithrombin III-thrombin) which underwent further aggregation. The human proteins, however, formed a 1:1 (antithrombin III-thrombin) initial complex in the presence of heparin, followed by aggregation. These interactions of thrombin and antithrombin with heparin suggest complex interactions that could relate to heparin function.

Antithrombin III and heparin are involved in the rapid inactivation of several proteases of the blood coagulation cascade. Lundblad et al. (1) contains a number of studies on this topic. Heparin causes a conformational change in the antithrombin III molecule that can be monitored by a 40% increase in the intrinsic protein fluorescence of antithrombin III. Several studies have indicated that the stoichiometry needed for the antithrombin III fluorescence enhancement is about one heparin molecule per antithrombin III molecule regardless of the molecular weight of the heparin (2-4). In contrast, other reports claim that a 2:1 (antithrombin III-heparin) stoichiometry is possible (2, 5). These values have generally been determined by the amount of heparin needed to cause a spectroscopic change in antithrombin III. A more direct method of determining binding stoichiometry would be to measure the molecular weight of the complexes themselves. The studies reported here were initiated to examine the molecular species that exist when heparin, antithrombin III, and thrombin are mixed in solution. Heparin caused association of antithrombin III molecules to a partial formation of a 2:1 antithrombin III-heparin complex in which only one antithrombin III had undergone the fluorescence change; however, this complex did not proceed to a complete dimer of antithrombin III. The nondissociable antithrombin III-thrombin complexes formed high molecular weight complexes in the presence of heparin.

EXPERIMENTAL PROCEDURES

Human antithrombin III was obtained from the American Red Cross and was further purified by chromatography on a Bio-Gel A-0.5m (Bio-Rad Laboratories) column (2 x 40 cm) before use. Human antithrombin, with a reported specific clotting activity of 4000 NIH units/mg protein, was obtained from Sigma (Product No. T-3010). Bovine antithrombin III and bovine α-thrombin were purified to apparent homogeneity as previously described (6). The thrombin preparation had a specific clotting activity of 2800 NIH units/mg protein when compared to thrombin lot 3 (7). Extinction coefficients ($E_{19,540}$) for the determination of protein concentrations were 6.1 and 19.5 for antithrombin III (8) and thrombin (9), respectively. A $M_r = 56,000$ was assumed for antithrombin III (10) and 39,000 (11) for α-thrombin.

Unfractionated porcine heparin, with a reported specific activity of 161 units/mg, was donated by Riker Laboratories, 3M Co. (Northridge, CA). High affinity heparin was obtained by chromatography on an antithrombin III affinity column as previously described (12). The first material to bind to the antithrombin III matrix was designated “high affinity heparin.” The molar concentration of all heparin preparations was determined using the stoichiometric-fluorescence titration method already outlined (6), assuming that 1 mol of heparin is the amount of polysaccharide needed to saturate the fluorescence change for 1 mol of antithrombin III.

Pentosane polysulfate (sodium salt) was generously provided by Biosynth AG (Zurich), Lot No. 17068. The other polysaccharides, SP-54, was generously provided by Biochemie GMBH, Munchen.

Quasielastic light scattering and total intensity light scattering were performed using the apparatus and procedure already described in full (13). The bovine proteins and human antithrombin III samples were prepared for light scattering by chromatography on a Bio-Gel A-0.5m column to eliminate dust. The protein samples prepared by gel filtration were monodisperse, as indicated by the molecular weight of the protein samples. Contamination or aggregation would provide molecular weights that were higher than those previously obtained by other measurements. All light scattering experiments were performed in 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl at 20 °C.

Sedimentation coefficients were calculated from single determinations of the sedimentation velocities measured in a Beckman Model I ultracentrifuge by standard procedures under the conditions given in the text. A UV absorption scanning system was used to measure protein concentrations. Centrifugation experiments were performed in 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl.

The hydrodynamic properties (diffusion constant and hydrodynamic radius) of antithrombin III and heparin-antithrombin III com-

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plexes were determined by dynamic light scattering measurements as described previously (13).

The time course for thrombin inactivation in the presence of antithrombin III was determined by adding thrombin to a solution of antithrombin III in 50 mM Tris-HCl (pH 8.4), 0.10 mM NaCl, 0.1% polyethylene glycol, 8000 (Sigma). The final reaction mixture contained 10 μM thrombin and 10 μM antithrombin III. At various time intervals, an aliquot of this mixture was diluted into a solution containing chromogenic substrate S-2288, H-b-isouleucyl-t-prolyl-L-arginine-p-nitroanilide dihydrochloride (Helena Laboratories), and the absorbance of the diluted mixture was continuously monitored at 405 nm. The thrombin activity was proportional to the change in absorbance at 405 nm per unit time (velocity of p-nitroaniline liberation from S-2288). The kₜₐₜ for bovine thrombin acting on S-2288 was 100 s⁻¹ (6).

RESULTS

Molecular Weight of Antithrombin III-Heparin Complexes—Fig. 1 shows the molecular weight of a solution of bovine antithrombin III as a function of added heparin. Initially, antithrombin III had a Mₚ = 59,000 which was approximately the reported molecular weight of antithrombin III (56,000, Ref. 11). This latter result indicated that the protein itself was monomeric and that contaminants had been eliminated from the sample. As heparin was added, the molecular weight increased as did the heparin-dependent change in protein fluorescence intensity (Fig. 1).

The molecular weight reached a maximum and then decreased to a final value that corresponded to the molecular weight for a 1:1 complex of antithrombin III-heparin (heparin Mₚ = 13,000). The intermediate aggregation behavior was not a function of the method of heparin addition as the same values were obtained by approaching the peak molecular weight from either side of the maximum. This was shown by a back titration where antithrombin III was added to a solution containing a 5:1 ratio of heparin to antithrombin III (●—●, Fig. 1) or by a single large addition of heparin to the antithrombin III preparation ( ■—■, Fig. 1) or by the addition of varying amounts of heparin to four different protein samples that generated the titration curve (○—○, Fig. 1).

At low heparin to antithrombin III ratios (<0.4 heparin/antithrombin III), the molecular weight increase and the fluorescence change did not correspond. That is, the molecular weight increased with a steeper slope than the fluorescence change. The behavior was complex but could correspond to a specific stoichiometry. If the fluorescence of the protein is assumed to be a two-state phenomenon where the protein molecules show either normal fluorescence or fully enhanced fluorescence with no intermediate intensity levels, the initial portions of the molecular weight and fluorescence titration curves correlated approximately with the binding of two antithrombin III molecules to a single heparin molecule in a complex where only one of the antithrombin III molecules had undergone the fluorescence change. The theoretical line for such a relationship is given by the dashed line in Fig. 1. This curve would pass through the molecular weight for a 2:1 (antithrombin III:heparin) complex (Mₚ = 124,000) at exactly half of the fluorescence enhancement. The maximum theoretical molecular weight was never reached (Fig. 1). This behavior could be due to an equilibrium association with insufficient affinity to form the full dimer at this protein concentration. In this case, the maximum of the molecular weight should change for titrations carried out at different protein concentrations. However, the maximum molecular weight was approximately the same as that shown in Fig. 1 (titration at 5 mg/ml) for other titrations carried out at 0.56, 0.75, and 1.03 mg of protein per ml. In each case, the initial part of the curve corresponded closely to the 2:1 (antithrombin III:heparin) ratio described above.

Results obtained by light scattering are weight-averaged values that do not identify the distribution of molecular weights. Diffusion constants, however, were also obtained for the samples shown in Fig. 1. At the maximum weight averaged molecular weight, the Z-averaged diffusion constant was 4.2 × 10⁻⁷ cm²/s. This was only a 25% decrease from monomeric antithrombin III and was nearly the expected change for association of two spherical particles of similar mass. Because the diffusion constant is Z-averaged, it is extremely sensitive to formation of larger molecular weight aggregates. This result provided strong evidence that the association shown in Fig. 1 consisted of complexes containing no more than 2 antithrombin III molecules (a monomer-dimer relationship).

To test the molecular weight distribution further, sedimentation velocity studies were conducted. Table I shows the results for various combinations of antithrombin III and heparin. The S value for antithrombin III and the antithrombin III-excess heparin mixtures were lower than those for intermediate amounts of heparin as predicted from the molecular weight data in Fig. 1. This result also indicated that the increase in molecular weight in Fig. 1 was not due to a few large aggregates which would sediment very rapidly and

![Fig. 1. Weight averaged molecular weight of bovine antithrombin III-heparin mixtures as a function of unfractionated heparin:antithrombin III molar ratio. Four antithrombin III samples were adjusted to the ratios shown by adding successive aliquots of heparin (●). Another sample was used for addition of one large amount of heparin (Δ). In addition, a back titration was performed by adding antithrombin III to a mixture of antithrombin III that was saturated (6:1 mole ratio) with heparin (○). The antithrombin III fluorescence titration is also shown (■). The concentration of antithrombin III in all samples was 5.0 mg/ml. The dashed line shows the expected curve for the formation of a heparin-(antithrombin III)₂ complex where only one antithrombin III molecule had undergone fluorescence enhancement.](image-url)
not contribute to the principal sedimentation pattern.

The sedimentation patterns at intermediate levels of heparin were very broad (Fig. 2), suggesting the presence of two sedimenting species that were not in equilibrium with each other on the time scale of the experiment (1–2 h). The molecular weight change indicated that only 40% of the antithrombin III molecules participated in the dimerization process (Fig. 1). Approximate separation of the sedimentation pattern (Fig. 2) into two populations of 40% fast sedimenting species and 60% slow sedimenting species produced the two midpoints indicated in Fig. 2. These midpoints corresponded to sedimentation values of 5.17 and 3.31. The latter agreed closely with that of pure monomeric antithrombin III (Table I) and the former was approximately that expected for a dimer of antithrombin III (sedimentation velocity should change about 1.6-fold). The sedimentation results therefore corroborate with the light scattering data and indicate that a partial dimerization of antithrombin III occurs in the presence of heparin.

The heparin used in Figs. 1 and 2 was commercially prepared. It is known that heparins of higher specific activity and molecular weight can be obtained by affinity chromatography on antithrombin III-Sepharose. This purification results in 3–5-fold higher specific activity (see Ref. 12 and references therein). If this is carried out under conditions where the affinity column was overloaded with heparin, the purified material displays a much higher molecular weight than commercial heparin. The actual preparation used in these studies was that reported by Pletcher et al. (12) and displayed high molecular weight on gel filtration. This high molecular weight/high affinity heparin was tested for its ability to cause the observed aggregation of antithrombin III. The results of a titration curve are shown in Fig. 3. The qualitative shape of the curve was very similar to that obtained with commercial heparin. The initial parts of the curve correlated with about 2:1 (antithrombin III-heparin) complex where only one protein molecule had undergone the conformational change. The maximum molecular weight was less than that expected for a full dimer and the peak molecular weight was similar to that obtained with commercial heparin (Fig. 1). At high heparin concentrations, the molecular weight decreased to that of a 1:1 complex of antithrombin III to heparin (heparin $M_r = 30,000$).

The high affinity heparin also showed a rapid decline in molecular weight after the peak so that, at maximum fluorescence, the molecular weight corresponded to about a 1:1 (antithrombin III-heparin) complex. If each heparin molecule contained two binding sites for antithrombin III and both functioned in causing the conformational change, the molecular weight at maximum fluorescence change should be 140,000. The observed molecular weight at the point of maximum fluorescence change is much below this value and there appeared to be few heparin molecules in this preparation that functioned in the conformational change. The value calculated from the graph is less than 15% of the heparin molecules.

Studies with Human Antithrombin III—The aggregation behavior observed above for bovine antithrombin III appeared to correspond to a partial dimerization between one antithrombin III molecule that had undergone the conformational change and one that had not. The basis and implications of this interaction are not known. Aggregation could be due to a property of the heparin or the protein. It is possible that the protein preparation contained molecules that were incapable of participating in the association reaction. The generality of this property was examined by comparing the behavior of the human and bovine proteins.

Fig. 4 shows a heparin titration of human antithrombin III. Once again, the initial molecular weight indicated a monodisperse protein. The initial molecular weight changes as heparin was added correlated with formation of a 2:1 antithrombin III to heparin complex in which only one of the antithrombin III molecules had undergone the conformation change detected by the fluorescence change (the dashed line, Fig. 4). The maximum molecular weight change was less than that of an antithrombin III-heparin (2:1) complex. High heparin concentrations caused a decrease in molecular weight. Consequently, the behavior of the human protein appeared indistinguishable from that of the bovine protein.

Fig. 4 also shows a titration of human antithrombin III with pentosane polysulfate, a substitute heparin that functions in factor $X_a$ inhibition but not in thrombin inhibition (see Ref. 12 and references therein). This polysaccharide also showed antithrombin III aggregation behavior but the maximum molecular weight was considerably lower than that for heparin. Pentosane polysulfate also causes much less antithrombin III fluorescence change than heparin (12). It is not known if these two properties are related. Qualitatively similar
plexes—Antithrombin III and thrombin form a titrated with pentosane polysulfate or observations were made when bovine antithrombin III was heparin to a solution of antithrombin after adding either thrombin or both thrombin and unfractionated heparin. To a solution of human antithrombin III, thrombin activity was determined in a separate assay (Fig. 4). This indicated the formation of a dissociable antithrombin III-thrombin complex followed by the rate-limiting inactivation of thrombin. Olson and Shore (15) have shown that the antithrombin III-thrombin inactivation reaction for the human proteins is a two-step process with rapid formation of a dissociable complex followed by the irreversible inactivation reaction.

Inclusion of stoichiometric amounts of heparin in the reaction enhanced the velocity of the inactivation reaction so it could not be monitored on this time scale. The molecular species in solution were aggregated extensively in the time of the first assay (about 4 s). The weight averaged molecular weight obtained by extrapolation of molecular weight to zero time was 230,000. This corresponded empirically to about a dimer of heparin-antithrombin III-thrombin. Following the initial increase in molecular weight there was a slower increase for both the heparin and heparin-free samples.

Molecular weight studies were also carried out for mixtures of the human proteins in both the absence and presence of heparin. In the absence of heparin, the molecular weight increased rapidly to 95,000 and remained at this value over a significant period of time (Fig. 6). This differed from the bovine proteins, which slowly aggregated in the absence of heparin (Fig. 5).

In the presence of stoichiometric amounts of heparin, the molecular weight increased rapidly followed by a slow increase and, finally, a curvilinear aggregation (Fig. 6). Linear extrapolation of the initial molecular weights to zero time gave a molecular weight corresponding to approximately a monomer of antithrombin III-thrombin. This behavior differed significantly from the bovine proteins. The aggregation behavior was also seen after adding heparin to a solution of antithrombin III-thrombin complexes formed in the absence of heparin (Fig. 6). The bovine and human proteins seem to interact differently in both the absence and presence of heparin.

Hydrodynamic Measurements—The hydrodynamic properties of antithrombin III-heparin complexes are shown in Table II. This method measures $Z$-averaged diffusion con-

**Fig. 4.** Molecular weight of human antithrombin III in the presence of varying amounts of heparin or pentosane polysulfate. Light scattering measurements were initiated after adding either thrombin or both thrombin and unfractionated heparin to a solution of antithrombin III. The final thrombin, antithrombin III, and heparin concentrations were identical (24 µM). The disappearance of thrombin activity in the absence of heparin is also shown (O). The procedure for measuring thrombin activity is described under “Experimental Procedures.”

**Fig. 5.** Molecular weight of bovine antithrombin III-thrombin mixtures in the absence (●) and presence (△) of heparin as a function of time. Light scattering measurements were initiated after adding either thrombin or both thrombin and unfractionated heparin to a solution of antithrombin III. The final thrombin, antithrombin III, and heparin concentrations were identical (24 µM). The disappearance of thrombin activity in the absence of heparin is also shown (O). The procedure for measuring thrombin activity is described under “Experimental Procedures.”

**Fig. 6.** The interaction of the human proteins in the absence and presence of heparin. To a solution of human antithrombin III was added either human thrombin (C) or both thrombin and unfractionated heparin (●), and the average molecular weight was determined over time. The effect of heparin on the preformed antithrombin III-thrombin complex (△) was also investigated by adding unfractionated heparin to the heparin minus sample at the time shown by the arrow. The molar concentrations of thrombin, antithrombin III, and heparin were identical (12 µM).
thrombin and heparin (heparin/antithrombin III) mixtures saturated with heparin (2.92 mg/ml) resulted in protein precipitation (data not shown). Therefore, the methods used to study the molecular weights and hydrodynamic properties of heparin-antithrombin III mixtures could not be used for mixtures of heparin and thrombin.

**D**

**U**

**S**

**I**

**C**

**O**

DISCUSSION

Upon addition of heparin to antithrombin III, the molecular weight increased in a manner consistent with the formation of a trimeric complex consisting of one heparin and two antithrombin III molecules. The fluorescence enhancement of antithrombin III did not increase in parallel with the aggregation. If a two-state model for the fluorescence enhancement is assumed, the early stages of the aggregation behavior correlated with formation of a complex with one antithrombin III molecule that had undergone the conformational change and one antithrombin III molecule that had not undergone the conformational change. The two-state assumption requires that there are no antithrombin III molecules with intermediate fluorescence intensity. However, this aggregation behavior did not proceed to a full 2:1 (antithrombin III:heparin) ratio and the failure to reach this level was not related to an equilibrium with low affinity; variation of the protein concentration over nearly a 10-fold range gave approximately the same extent of aggregation. The curves also could not be fit to any simple interaction model consisting of a single binding constant between an antithrombin III molecule and an antithrombin III-heparin complex. Because the aggregation did not exceed the theoretical formation of a dimer at any of the protein concentrations used, the aggregation probably involved no more than two protein molecules per heparin molecule rather than a larger order aggregation. Z-averaged diffusion constants for the complexes, as well as sedimentation patterns, supported this conclusion.

The aggregation was observed by light scattering techniques which required high concentrations of protein. Ten μM antithrombin III was the lowest concentration used in this study. Consequently, the binding affinity for the aggregation can only be stated to be less than 10 μM, the lowest concentration of antithrombin III used to measure aggregation in this study.

**TABLE II**

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<tr>
<th>Hydrodynamic properties of antithrombin III and antithrombin III saturated with heparin</th>
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<tr>
<td>Molecular species</td>
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<td>Antithrombin III</td>
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<td>Antithrombin III plus excess heparin</td>
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*The D\(_{20w}\) values were obtained from quasielastic light scattering measurements. The hydrodynamic radii were calculated from the Stokes-Einstein equation \( R = kT/6\pi\eta D \). The D\(_{20w}\) value for the antithrombin III-heparin complex is the average of different anti-thrombin III mixtures saturated with heparin (heparin/antithrombin III \( \geq 4.0 \)). The variation between the samples was less than 7%. The protein concentration in all samples was 5.0 mg/ml.

Light scattering studies of antithrombin III-thrombin mixtures indicated that the human and bovine proteins behaved very differently. The results showed that the bovine proteins formed an initial dissociable complex followed by formation of the inhibited complex, in agreement with previous results (15). The bovine antithrombin III-thrombin complex then underwent a slow aggregation, whereas the human antithrombin III-thrombin complex did not aggregate on the time scale used here.

In the presence of heparin, the first molecular species observed for the bovine proteins was an aggregate with a molecular weight consistent with at least two molecules each of heparin, antithrombin III, and thrombin. This aggregate then underwent slow association to even larger complexes. The first complex formed for the human antithrombin III-thrombin plus heparin, however, had a molecular weight corresponding to a 1:1 antithrombin III-thrombin complex. This complex then underwent complex associations to very large aggregates. These results indicated that the human and bovine antithrombin III-thrombin complexes interacted differently in both the absence and presence of heparin. The higher order aggregates present immediately for bovine product preparations suggest tight binding to heparin, while the initial monomeric human protein complex may suggest a lower heparin affinity.

These studies have shown complex behavior for antithrombin and for antithrombin III-thrombin in their interaction with heparin. These interactions were observed at greater than 10 μM protein. Determination of the relevance of these several interactions to the role of heparin in enhancing the reaction between antithrombin III and thrombin will require further studies.

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**REFERENCES**