Binding of Low-Affinity and High-Affinity Heparin to Antithrombin. Ultraviolet Difference Spectroscopy and Circular Dichroism Studies†

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ABSTRACT: Heparin acts as an anticoagulant by binding to the plasma protease inhibitor antithrombin, thereby increasing the rate at which the inhibitor inactivates a number of coagulation serine proteases. Two forms of heparin, differing in their affinity for matrix-linked antithrombin, may be separated by affinity chromatography. The high-affinity heparin fraction has a high anticoagulant activity, while the low-affinity fraction is virtually devoid of such activity. The binding of these two fractions to antithrombin in solution has been characterized by ultraviolet difference spectroscopy and circular dichroism. Neither heparin fraction affected the far-UV CD spectrum of antithrombin, indicating that heparin binding does not lead to significant changes of the secondary structure of the protein. In contrast, both fractions changed the near-UV absorption and CD spectra of antithrombin, suggesting local perturbations of the environment of some aromatic amino acids of the protein on heparin binding. However, the highly active, high-affinity heparin fraction caused much larger spectral changes than the relatively inactive, low-affinity fraction. This is compatible with the concept that the high-affinity fraction induces a conformational change of antithrombin, related to the activation of the inhibitor. Quantitative studies showed the two heparin fractions to bind to antithrombin in a molar ratio of 1:1, possibly to the same site on the protein, but their affinities differed widely. The binding constant of the high-affinity fraction at physiological ionic strength thus was found to be of the order of 10^7 M^{-1}, while that of the low-affinity fraction was about 5×10^4 M^{-1}.

The potent anticoagulant activity of heparin is largely due to the interaction of the polysaccharide with antithrombin III (also called heparin cofactor), a plasma protease inhibitor with the ability to inhibit most, if not all, of the serine proteases participating in the coagulation process (for reviews, see Rosenberg 1977a,b). In the absence of heparin the inhibitor slowly forms an inactive, extremely stable, equimolar complex with the different enzymes. Heparin exerts its effect by binding to antithrombin and dramatically accelerating the rate of inhibition without affecting the stoichiometry or stability of the enzyme–inhibitor complex. This accelerating effect may be due to a heparin-induced conformational change of the antithrombin molecule (Rosenberg & Damus, 1973; Li et al., 1976; Villanueva & Danishefsky, 1977; Einarsson & Andersson, 1977).

It has recently been shown that about one-third of all heparin molecules in typical commercial preparations accounts for the major part of the anticoagulant activity of the polysaccharide (Lam et al., 1976; Höök et al., 1976; Andersson et al., 1976). These active molecules can be isolated as a high-affinity heparin fraction by affinity chromatography of commercial heparin on matrix-bound antithrombin. The remainder of the heparin preparation, which shows low affinity for immobilized antithrombin in this procedure, is almost devoid of anticoagulant activity. We have initiated a series of investigations of the binding of these two heparin fractions to human and bovine antithrombin in order to elucidate those changes of the antithrombin molecule that may be related to its activation by the high-affinity heparin fraction. In this paper we present the results of such studies by ultraviolet difference spectroscopy and circular dichroism. The major results are that both low-affinity and high-affinity heparin bind to antithrombin in solution with a stoichiometry of 1:1, but with binding constants differing by about two orders of magnitude. The binding of the high-affinity fraction causes marked changes of the ultraviolet absorption and circular dichroism spectra of antithrombin, while the low-affinity fraction affects these spectra to a much lower extent. This is in agreement with, but does not prove conclusively, the hypothesis that the active, high-affinity heparin fraction induces in antithrombin a conformational change which is involved in the activation of the inhibitor.

Materials and Methods

Human and bovine antithrombin were prepared from plasma by the method developed by Miller-Andersson et al. (1974). The purity of the preparations was comparable to that of material used in an earlier study (Nordenman et al., 1977).

Heparin, isolated from pig intestinal mucosa (stage 14, Inolex Pharmaceutical Div., Park Forest South, Ill.), was purified by repeated precipitations with cetylpyridinium chloride from 1.2 M sodium chloride (Lindahl et al., 1965). A sample with a narrow molecular weight distribution was obtained by chromatography of this preparation on a column of Sephadex G-100 (Pharmacia Ltd., Uppsala, Sweden). Fractions eluting slightly before the center of the peak, i.e., with K_{av} values between 0.25 and 0.31, were pooled. In this manner low-molecular-weight material was excluded. The fractions pooled represented about 15% of the total amount of heparin applied to the column.

Low-affinity and high-affinity heparin fractions were prepared from this heparin sample by affinity chromatography on antithrombin–Sepharose, essentially as described by Höök et al. (1976). However, the column size was increased, and the separation conditions were modified as stated in the legend to Figure 1. The low- and high-affinity peaks were pooled and the fractions were precipitated from 1 M sodium chloride with 2%

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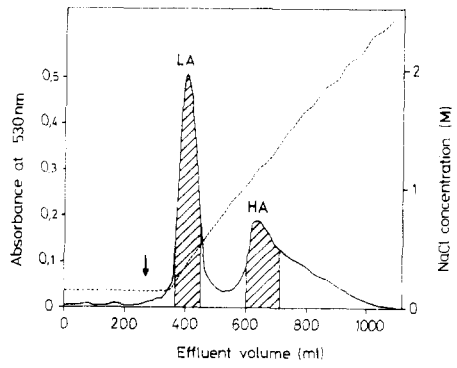

FIGURE 1: Affinity chromatography of heparin on antithrombin-Sepharose. An amount of 50 mg of heparin was applied to a column (2.6 × 14 cm) in 0.05 M Tris buffer, pH 7.4, + 0.05 M NaCl. The column was washed with buffer and a linear gradient (total volume 1000 mL) to 0.05 M Tris buffer, pH 7.4, + 3 M NaCl was then started at the position marked by the arrow. The flow rate was 50 mL/h and 8-mL fractions were collected. A sample of 250 μL was taken for the carbazole reaction. (—) Absorbance at 530 nm; (• • •) ionic strength, measured by conductance. The materials represented by the hatched areas of the peaks were used for further studies. LA, low-affinity heparin; HA, high-affinity heparin.

cetylpyridinium chloride. Solutions of known concentrations of the two fractions were prepared by dissolving weighed amounts of dry, salt-free polysaccharide in the appropriate solvent.

Molecular weights were determined by sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, Calif.), using the rapid long column meniscus depletion method (Chervenka, 1970). A value of 0.43 mL/g (Laurent, 1961) for the partial specific volume of heparin was used in the calculations.

Ultraviolet (UV)[1] absorption difference spectra, as well as absorption differences at a constant wavelength, were measured on the ±0.05 absorbance difference scale of a Shimadzu MPS-5000 recording spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan). The experiments were performed at room temperature (22 ± 2 °C), with tandem cells having 0.438-cm or 1-cm pathlengths per compartment and protein concentrations ranging from 22 to 55 μM (i.e., about 1.2–3.1 g/L). The data were expressed as differences in molar absorbancy between sample and reference.

Circular dichroism (CD) measurements were made with a Jasco J41 A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). All experiments were performed at room temperature (22 ± 2 °C) in cells having pathlengths from 0.05 to 1 cm with protein concentrations varying from 7.8 to 38 μM (i.e., about 0.45–2.2 g/L). The results were expressed as mean residue ellipticities in the far-UV (190–250 nm) region and as molar ellipticities in the near-UV (250–310 nm) region. A mean residue weight of 112 was used for the peptide moiety of antithrombin (Nordenman et al., 1977).

Titrations of antithrombin with low-affinity or high-affinity heparin were performed by adding successive volumes of a concentrated (10–20 g/L) polysaccharide solution to the same solution of antithrombin. An identical protein solution, to which only buffer was added, served as reference. An equal volume of heparin solution was added to the buffer compartment of the reference tandem cell in the difference spectroscopy measurements, and the absorbance difference between sample and reference was recorded directly in these experiments. In the circular dichroism studies, however, the ellipticity difference was calculated from separate measurements of sample and reference; the contribution of heparin to the ellipticity at the wavelength used was found to be negligible. All data were corrected for the dilution of the antithrombin solution by the titrant. Titration curves were plotted as the observed change of molar absorbancy or ellipticity at constant wavelength vs. the ratio between the total molar concentrations of heparin and antithrombin.

Stoichiometries and binding constants were evaluated by a three-parameter computer fit of the titration data to a theoretical equation derived from the fundamental expression for the equilibrium constant of the protein–ligand reaction (Krause et al., 1974). The derivation is based on two assumptions, namely, that the change of the physical quantity measured is proportional to the amount of protein–ligand complex formed, and that all binding sites are equivalent and independent. Evaluation of the data by this equation thus neglects the possibility of weak secondary binding of ligand to the protein. The computer fitting procedure selected that combination of the three parameters stoichiometry (n), binding constant (K), and maximal change of absorbance or ellipticity at ligand saturation (ΔQ_{max}), which gave the best least-squares fit to the experimental data. No weighting of the data was made in this analysis. The least-squares curve and the experimental data were plotted on a digital plotter to facilitate detection of systematic deviations of the data from the theoretical curve (indicative of, for example, additional binding sites with lower association constants).

In some experiments stoichiometries and binding constants were obtained by plotting the data according to an equation suggested by Gutfreund (1972), or according to the Scatchard equation (Scatchard, 1949). Stoichiometries could not be determined by the latter procedure, as calculations of free ligand concentrations from physicochemical measurements, such as the ones used, require previous knowledge of the number of binding sites on the macromolecule. The value of ΔQ_{max} required for the use of these equations was obtained by the method of computer fitting the data as described earlier, since the customary double-reciprocal plot may result in a large error in ΔQ_{max} (Krause et al., 1974).

Protein concentrations were measured spectrophotometrically in a Zeiss PMQ II spectrophotometer (Carl Zeiss, Oberkochen, West Germany) and were calculated from absorption coefficients ($A_{280}^{1\%,1cm}$) of 6.5 for human and 6.7 for bovine antithrombin (Nordenman et al., 1977). Molecular weights of 58 000 and 56 000 for the human and bovine protein, respectively, were used in all calculations of molar ratios (Nordenman et al., 1977).

Results

Heparin Preparation. A heparin sample with a reduced molecular weight heterogeneity was isolated by gel chromatography. The high- and low-affinity heparin species of this material were then separated by affinity chromatography on matrix-linked antithrombin (Figure 1). The high-affinity heparin eluting at very high ionic strengths was not included in the material used for further study, as a preparation relatively homogeneous with respect to its affinity for antithrombin was desired. The low- and high-affinity fractions had anticoagulant activities of 20 and 285 BP units/mg, respectively.

The molecular weights of the purified heparin preparations were determined by sedimentation equilibrium. In these analyses all plots of the logarithm of fringe displacement vs. the square of the distance from the center of rotation were linear from fringe displacements of about 75 μm to the cell bottom. This indicates apparent molecular size homogeneity of all samples during the conditions of the experiments (i.e.,

[1] Abbreviations used: UV, ultraviolet; CD, circular dichroism.

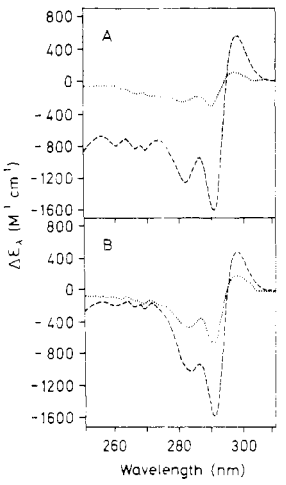

FIGURE 2: Ultraviolet absorption difference spectra of human (A) and bovine (B) antithrombin due to low-affinity heparin (• • •) or high-affinity heparin (- - -). Protein concentrations were 22–26 μM, and the solvent was 0.05 M Tris buffer, pH 7.4, + 0.1 M NaCl. The molar ratios between heparin and antithrombin were 4.5 for low-affinity heparin and 2.8 for high-affinity heparin. Tandem cells with 1-cm compartments were used, and heparin or buffer was added to the appropriate reference compartments. The unit on the ordinate is the difference between the molar absorptivities of heparin–antithrombin complex and free protein.

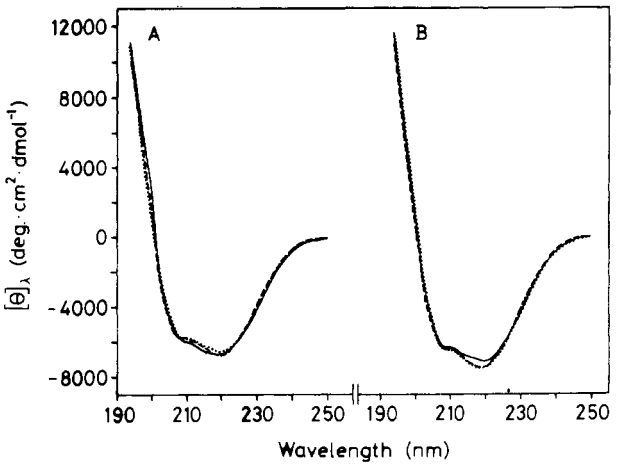

FIGURE 3: Far-UV circular dichroism spectra of human (A) and bovine (B) antithrombin alone (—) and in the presence of either low-affinity heparin (• • •) or high-affinity heparin (- - -). Cells with 0.05-cm pathlengths and protein concentrations of 7.8–8.1 μM were used. The molar ratios between heparin and antithrombin were 4.8 for low-affinity heparin and 1.6 for high-affinity heparin. The solvent was 0.05 M Tris buffer, pH 7.4, + 0.1 M NaCl. Heparin of the same concentration as in the sample was included in the blanks. The unit on the ordinate is mean residue ellipticity.

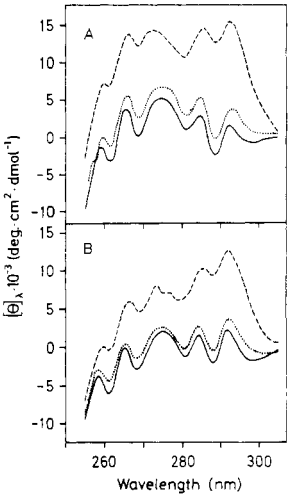

FIGURE 4: Near-UV circular dichroism spectra of human (A) and bovine (B) antithrombin alone (—) or in the presence of either low-affinity heparin (• • •) or high-affinity heparin (- - -). Cells with 1-cm pathlengths and protein concentrations of 21–34 μm were used. The molar ratios between heparin and antithrombin were 4.6 for low-affinity heparin and 1.5 for high-affinity heparin. The solvent was 0.05 M Tris buffer, pH 7.4, + 0.1 M NaCl. No heparin was included in the blanks, as the polysaccharide had no ellipticity in the near-UV region. The unit on the ordinate is molar ellipticity.

narrow molecular weight distributions), and a negligible concentration dependence of the apparent molecular weights within the concentration range covered (about 0.1 to 1.5 g/L). Apparent molecular weights of 15 200 and 15 700 were obtained for low-affinity heparin in two experiments at different rotor speeds, while three experiments, also at different speeds, gave an average value of 15 300 ± 300 for high-affinity heparin. These molecular weights may be slightly in error in view of the uncertainty associated with the partial specific volume of heparin (Laurent, 1961; Lasker & Stivala, 1966). Moreover, the preparations certainly must be somewhat polydisperse in spite of their apparent size homogeneity. Therefore a value of 15 000 was used in all calculations of molar concentrations of either heparin fraction.

Ultraviolet Difference and Circular Dichroism Spectra of Heparin–Antithrombin Complexes. Low-affinity or high-affinity heparin was added in excess to human or bovine antithrombin, and the resulting aromatic ultraviolet absorption difference spectra were measured (Figure 2). Experiments to be presented will show that the two antithrombins were completely saturated with high-affinity heparin and about 85% saturated with low-affinity heparin under the conditions used. All spectra measured on the addition of either heparin fraction to either protein had similar shapes, resulting mainly from a red shift accompanied by an overall decrease in absorption. In spite of this general similarity some differences between the four spectra are evident from Figure 2. The most striking feature is the markedly lower magnitude of the spectra given by low-affinity heparin compared with those measured for high-affinity heparin. The antithrombins from the two species gave similar, although not identical, results. The main difference was that the spectrum resulting from the binding of low-affinity heparin to human antithrombin was of much lower magnitude than the same spectrum measured for the bovine protein.

Far-UV and near-UV circular dichroism spectra of human or bovine antithrombin in the absence or presence of excess low-affinity or high-affinity heparin were also measured. The conditions were such that both proteins were essentially completely saturated with high-affinity heparin, but were saturated with low-affinity heparin to only about 70% in the experiments in the far-UV region and to 85–90% in the measurements in the near-UV region. No change of the far-UV spectra could be detected when either heparin fraction was added to either protein (Figure 3). However, the presence of high-affinity heparin was found to increase markedly the ellipticity of all near-UV CD bands of both human and bovine antithrombin, although the wavelength positions of the bands were not appreciably altered (Figure 4). In contrast, the addition of the low-affinity fraction to either antithrombin produced aromatic CD spectra only insignificantly different from the spectra of the free proteins. The difference CD spectra calculated from the curves for high-affinity heparin in Figure 4 are given in Figure 5. The two spectra for human and bovine

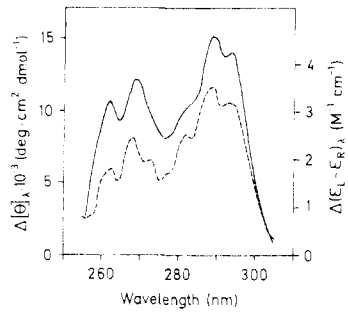

FIGURE 5: CD difference spectra calculated from the spectra given in Figure 4 of antithrombin in the presence of excess high-affinity heparin and of free antithrombin. (--) Human antithrombin; (- - -) bovine antithrombin. The difference CD is expressed both as molar ellipticity [θ] and as the difference between the molar absorbancies for left and right circularly polarized light ($\epsilon_L - \epsilon_R$).

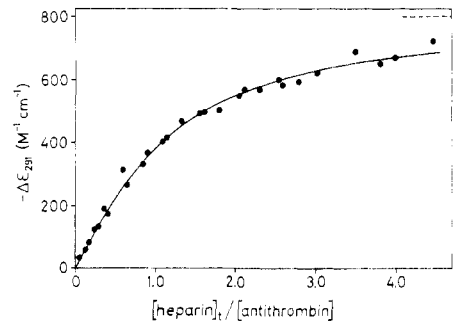

FIGURE 7: Titration of bovine antithrombin with low-affinity heparin, monitored by difference absorption measurements, in 0.05 M Tris buffer, pH 7.4, + 0.1 M NaCl. A tandem cell with 1-cm compartments and a protein concentration of 28 μM were used. The data were obtained by two individual titrations. The units on the axes and the symbols are as in Figure 6.

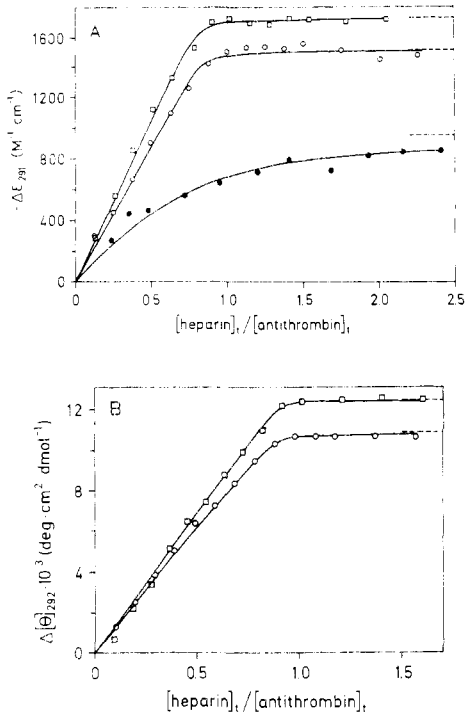

FIGURE 6: Titrations of human antithrombin with high-affinity heparin (□), and of bovine antithrombin with low-affinity (●) or high-affinity heparin (○) in 0.05 M Tris buffer, pH 7.4, + 0.1 M NaCl. The analyses were monitored by difference absorption measurements (A) in tandem cells with 0.44-cm compartments and with protein concentrations of 52–55 μM, or by circular dichroism measurements (B) in cells with 1-cm pathlengths and with protein concentrations of 34–38 μM. The units on the ordinate are the differences in molar absorptivity or molar ellipticity between heparin–antithrombin complex and free protein, and the unit on the abscissa is the ratio between the total concentrations of heparin and antithrombin. The solid lines represent the least-squares computer fit of the data to the theoretical binding equation. The broken lines are the ΔQ_{max} values obtained by this procedure.

antithrombin are remarkably alike, which lends considerable credibility to their fine structure.

Titration of Antithrombin with Heparin. The changes of the ultraviolet absorption and ellipticity of antithrombin on the addition of low-affinity or high-affinity heparin were used to monitor titrations of the protein with the two heparin fractions in attempts to determine binding stoichiometries and equilibrium constants. Both human and bovine antithrombin were titrated with high-affinity heparin, and these titrations could be followed by either difference absorption or circular dichroism measurements. However, the binding of low-affinity heparin could be studied only with bovine antithrombin and only by difference absorption measurements, as the changes caused by this heparin fraction of the CD of either protein and of the UV absorption of human antithrombin were too small to give useful data.

The results of experiments at protein concentrations chosen to give optimal performance of the instruments are presented in Figure 6. The shape of the titration curves obtained for high-affinity heparin shows that the protein concentrations used were high compared with the dissociation constants of the complexes. The curves thus were suitable for the determination of stoichiometries, but could only give approximate estimates of the binding constants. The titration curves for both human and bovine antithrombin were computer fitted with stoichiometries of 0.85–0.9 and binding constants of about 10^7 M^{-1}; the latter value, however, may be considerably in error. Attempts to decrease the protein concentration sufficiently to allow a more accurate determination of the binding constants with either of the two methods used were futile due to the increased error of the measurements.

In the case of the binding of low-affinity heparin to bovine antithrombin, the situation was different. The protein concentration used was too low to give optimal precision in the determination of the stoichiometry, but was more suitable for the evaluation of the binding constant. The data presented in Figure 6A were computer fitted with a stoichiometry of 0.7 and a binding constant of 9×10^4 M^{-1}. It was not possible to improve the precision of the estimate of the stoichiometry by increasing the protein concentration, due to the concomitant increase of the noise level of the spectrophotometer. However, a more accurate value of the binding constant for low-affinity heparin could be obtained by measurements at a lower protein concentration and in a longer cell (Figure 7). The experimental curve was computer fitted with a stoichiometry of 0.9 and a binding constant of 6×10^4 M^{-1}. These values were confirmed by plotting the data according to the equation suggested by Gutfreund (1972), which gave a stoichiometry of 0.7 and a binding constant of 5×10^4 M^{-1}. An additional evaluation by the Scatchard equation (Scatchard, 1949), which was done by assuming a stoichiometry of one, gave a binding constant of 6×10^4 M^{-1}. It should be noted that the data of Figure 7 were obtained at room temperature (22 ± 2 °C), due to the lack of a constant-temperature cell holder for the tandem cell used, and the binding constant measured for low-affinity heparin may therefore be somewhat inaccurate.

Discussion

The aim of this investigation was to study how the two

heparin forms obtained by affinity chromatography bind to human or bovine antithrombin in solution. Most of the analyses were carried out with both proteins, although the use of bovine antithrombin was found to be necessary in the quantitative analyses of the binding of low-affinity heparin. The discussion therefore will be centered around the binding of the two heparin forms to bovine antithrombin. However, in those instances where both proteins were studied, they were found to behave in a highly similar manner. It is therefore reasonable to assume that conclusions drawn from studies on the interactions between heparin and bovine antithrombin are at least approximately valid also for the corresponding interactions with human antithrombin.

A major result of this investigation is that the difference in the affinity of matrix-bound antithrombin for the two heparin forms, which is seen in affinity chromatography, is shown also by antithrombin in solution. Our quantitative studies thus indicate that the binding constant for the highly active, high-affinity heparin fraction at physiological pH and ionic strength is about 100 times higher than the binding constant for the relatively inactive, low-affinity fraction. This result strongly supports the contention that tight binding to antithrombin is a prerequisite for heparin anticoagulant activity (Lam et al., 1976; Höök et al., 1976; Andersson et al., 1976). However, the magnitude of the difference between the binding constants of the two fractions must be considered only approximate on the basis of the results of this study. A more precise evaluation of the association constant for high-affinity heparin thus will have to be made by methods allowing measurements at lower protein concentrations, such as fluorescence. A binding constant of about 2×10^6 M^{-1} for the binding of one high-affinity heparin preparation to human antithrombin has already been obtained by Einarsson & Andersson (1977) using this method. A further uncertainty associated with the difference in binding constants is the possibility that both low-affinity and high-affinity heparin may contain molecules differing somewhat in their affinity for antithrombin. Other factors, such as heparin molecular weight, ionic strength, and pH, may also influence the binding of the two heparin forms to the protein.

A stoichiometry of 0.7–0.9 was determined for the binding of both high-affinity and low-affinity heparin to antithrombin. The accuracy of this value is not sufficient to distinguish it from a stoichiometry of one, in view of the errors in the determinations of both the molecular weights and the concentrations of the macromolecules involved. The residual polydispersity of the heparin preparation, although slight, introduces a further uncertainty. The most likely conclusion, therefore, is that antithrombin can bind equimolar amounts of low-affinity or high-affinity heparin. Our experiments do not permit a definite decision whether the two heparin forms bind to the same site or to different sites on the protein. An indication that the binding may occur to the same site is the general similarity of both the ultraviolet difference and circular dichroism spectra of antithrombin in the presence of low-affinity heparin to the corresponding spectra in the presence of high-affinity heparin. However, the differences between the spectra suggest, at the same time, that the mode of binding of the two heparin forms to the protein is distinctly different, a conclusion further supported by the difference between their binding constants. Low-affinity and high-affinity heparin may thus bind to the same site on the antithrombin molecule, but low-affinity heparin may lack some structural features necessary for tight binding to this site, or alternatively may possess some features incompatible with such binding.

Determinations of stoichiometry by spectroscopic methods are complicated by the fact that additional, weak binding sites under certain circumstances may escape detection (Greenfield, 1975). Computer simulation, using the equations published by Roosdorp & Sjöholm (1976), indicated that binding of high-affinity heparin to a secondary site, giving no contribution to the absorption or ellipticity measured (i.e., with $\Delta Q_{max} = 0$) and having a binding constant lower than about 10^5 M^{-1}, would not have been noticed in our analyses. A similar analysis for the binding of low-affinity heparin suggested that in this case an additional "silent" site with a binding constant less than about 5×10^3 M^{-1} might not have been detected. Antithrombin may thus weakly bind additional molecules of low-affinity or high-affinity heparin. However, it is reasonable to assume that binding of the two forms to the high-affinity site or sites, which were detected, is responsible for the major effects on the antithrombin molecule.

It has been suggested earlier that heparin acts as an anticoagulant by converting the conformation of antithrombin into a more active form (Rosenberg & Damus, 1973; Villanueva & Danishefsky, 1977). Our results are compatible with such a conformational change but, unfortunately, do not prove conclusively that it does occur. Thus, we have not been able to reproduce with either low-affinity or high-affinity heparin the far-UV circular dichroism changes observed for unfractionated heparin by Villanueva & Danishefsky (1977). Furthermore, the effects of heparin on the aromatic ultraviolet absorption and circular dichroism spectra of antithrombin seen in this investigation, and also by others in studies with unfractionated heparin (Villanueva & Danishefsky, 1977; Einarsson, 1976), are difficult to interpret unequivocally. These spectral perturbations are caused to their full extent only by the highly active high-affinity heparin fraction and therefore presumably are related to the activation of antithrombin. They may thus reflect a local conformational change of the protein, which affects the environment around one or more of its aromatic amino acids. Such a change could involve the transfer of one or more chromophores from the interior of the protein into the solvent, which would be in accord with the negative absorption difference around 290 nm (Donovan, 1969, 1973). Another possibility of this nature, which is in agreement primarily with the increased intensity of the circular dichroism bands (Strickland, 1974), is that the binding of high-affinity heparin may decrease the motility of the region of the antithrombin molecule containing its active site, thereby stabilizing this region in its active conformation. Motility of the functional part of antithrombin may, in fact, explain the slow inactivation of serine proteases by the inhibitor in the absence of heparin. However, the spectral changes observed may also originate from interactions between some chromophoric groups of the protein and the bound high-affinity heparin molecule without a conformational change being involved (Donovan, 1969, 1973; Strickland, 1974; Greenfield, 1975). One such type of interaction is a local change of the electrostatic field around a chromophoric group caused by the highly charged polysaccharide chain. Another possibility is a coupling between an electronic transition of a chromophoric group of the protein and one of the far-UV transitions of heparin (Stone, 1971, 1977). However, the data are not compatible with merely an exclusion of solvent from a chromophore at the binding site, since this would have resulted in a positive UV-absorption difference spectrum over the whole wavelength range studied (Donovan, 1969).

The ultraviolet absorption and circular dichroism changes permit some conclusions on the nature of the chromophoric groups affected by the interaction between high-affinity heparin and antithrombin. The appearance of both types of difference spectra at wavelengths around 290 nm, with difference

absorption peaks at 298 and 291 nm and difference CD peaks at 293 and 288 nm, strongly suggests that tryptophan is involved (Wetlaufer, 1962; Donovan, 1969; Strickland, 1974). The magnitudes of the differences are rather large (viz., an $\epsilon_L - \epsilon_R$ of about 4 M^{-1} cm^{-1} and a $\Delta\epsilon$ of about -1700 M^{-1} cm^{-1}), but not sufficiently large to necessarily indicate contributions from more than one tryptophan residue (Donovan, 1969; Strickland, 1974). The difference spectra at lower wavelengths may indicate perturbations also of tyrosine and phenylalanine residues. However, this cannot be decided conclusively, because of the complex absorption and CD spectra of tryptophan. A further complication arises from the fact that disulfide bonds, of which there are three in antithrombin (Kurachi et al., 1976; Nordenman et al., 1977), also contribute to the circular dichroism in this region.

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