EVIDENCE FOR A HEPARIN-INDUCED CONFORMATIONAL CHANGE ON ANTITHROMBIN III*

German B. Villanueva and Isidore Danishefsky[1]

Department of Biochemistry, New York Medical College
Valhalla, NY 10595

Received December 8,1976

SUMMARY. The effect of heparin on the conformation of antithrombin III (AT-III) was investigated. Solvent perturbation difference spectroscopy shows that the binding of heparin to AT-III results in exposure of two tyrosine residues and a partial burial of a tryptophan residue. The occurrence of a conformational change suggested by this study is also substantiated by circular dichroism (CD) findings in the aromatic and peptide regions. The data in the peptide region show that heparin produces a decrease in the β-structure of AT-III, with a compensatory increase in random coil.

The principal mechanism whereby heparin exerts its anticoagulant effect, involves the enhancement of the proteinase-inhibitory actions of antithrombin III (AT-III) (1-5). The specific effect which heparin produces in AT-III is not known, although it has been presumed that it induces a conformational change (3,6). The present studies were initiated to determine the effect of heparin on the physical properties of AT-III. This report describes results obtained by investigations of ultraviolet difference spectra and circular dichroism. These give the first direct experimental evidence that heparin induces an alteration in the conformation of AT-III.

MATERIALS AND METHODS

Antithrombin III was prepared by fractionation of fresh plasma with polyethylene glycol (7) and affinity chromatography of the isolated material on heparin-aminohexylsepharose (8). The product was homogenous on the basis of results from SDS-gel electrophoresis and immunoelectrophoresis. Assays for antithrombin activity against thrombin (3) showed that the samples had activities of 690 units per mg. The units in this system are on the basis of defibrinated plasma, which is assigned a value of 100 units per ml. AT-III concentrations were determined spectrophotometrically using $E^{1\%}_{1cm} = 5.9$. This is an average of our own determination based on dry weight method and two other values reported elsewhere (9,10). The molecular weight used is 61,500, an average of two previously reported values (9,10). Absorbances were corrected according to Baughman and Waugh (11).

Heparin (146 USP units per mg) from beef lung was supplied by the Upjohn

*Supported by N.I.H. Grant HL-16955.

[1]To whom correspondence regarding this article should be addressed.

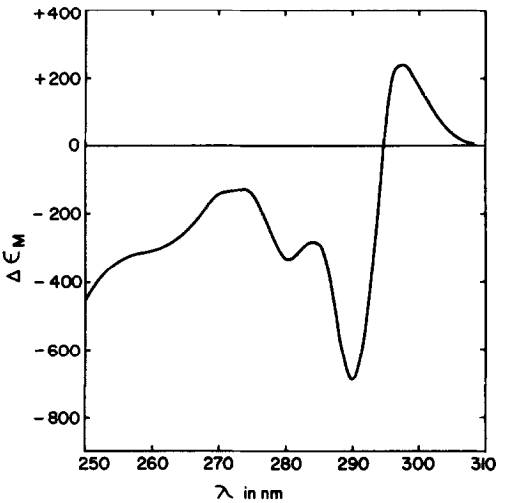

Figure 1. Ultraviolet Absorption Difference Spectrum of AT-III in the Presence of Heparin. The protein solution was placed in both cells and a baseline from 350 nm to 250 nm was obtained. A small aliquot (1-10 µl) of heparin was added to the sample cell and an equal amount of solvent was added to the reference cell. Concentrations used were 0.35 mg/ml for heparin and 1.3 mg/ml for AT-III. Solutions were buffered in 0.15 M NaCl, 0.01 M Tris, pH 7.5.

Corporation. Gel filtration on Sephadex G-75 provided fractions with over 150% of the original specific activity. The molecular weight of the heparin by sedimentation equilibrium (12) was 1.6×10^4.

N-Desulfated heparin and heparinylglycine methyl ester were prepared and characterized as described in previous publications (13,14). Chondroitin 4-sulfate was purchased from Miles Company. These three materials had no antithrombin-enhancing activities, when measured for their effect on the heparin-cofactor and progressive antithrombin assays (3).

A Cary 60 recording spectropolarimeter, equipped with a Cary 6002 circular dichroism attachment, was used for CD measurements. Since heparin makes a small contribution to the CD spectra (3% at 210 nm and 15% at 195 nm), the baselines were run routinely with the solvent and the particular concentration of heparin used. Alternatively, the contribution of heparin was subtracted from protein curves. All CD spectra are averages of four to five determinations.

Measurements of difference spectra were made in a Cary 15 double beam recording spectrophotometer equipped with a 0-0.1 absorbance scale. Solvent perturbation experiments were carried out according to the methods of Herskovits and Sorensen (15), except that four matched 1-cm, 1-ml cuvettes were used. All difference spectra are averages of three to four determinations.

RESULTS

Absorption Difference Spectroscopy. The effect of heparin on the ultraviolet absorption spectrum of AT-III is shown by the difference spectrum described in Figure 1. The negative bands at 290 nm ($\Delta\varepsilon_M = -690$) and 280 nm

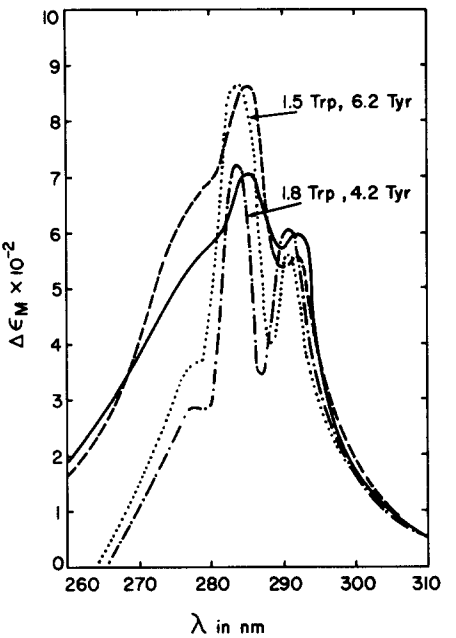

Figure 2. Solvent Perturbation Difference Spectra of AT-III in 20% Propylene Glycol. AT-III (——); AT-III in heparin (---). The dotted and dashed-dotted lines are theoretical curves representing the indicated number of exposed tryptophan and tyrosine residues based on the method of Herskovits and Sorensen (15).

($\Delta\epsilon_M$ = -340) indicate "blue shift" perturbation of the aromatic chromophores. The positive difference spectrum at 298 nm ($\Delta\epsilon_M$ = -240) can be ascribed to ^1L_a and ^1L_b transitions of tryptophan (16). On the basis of studies of several proteins (17), this is known to occur when the net negative charge around the tryptophan chromophore is increased. These spectral changes were not produced by N-desulfated heparin, heparinylglycine methyl ester and chrondroitin 4-sulfate.

Solvent Perturbation Studies. The solvent perturbation difference spectrum of AT-III is shown (solid line) in Figure 2. Two broad maxima were observed at 292 nm ($\Delta\epsilon_M$ = +600) and 285 nm ($\Delta\epsilon_M$ = +715). When fitted with the model compound data of Villanueva and Herskovits (18), the average number of exposed tryptophan and tyrosine residues in AT-III was found to be 1.8 and 4.2,

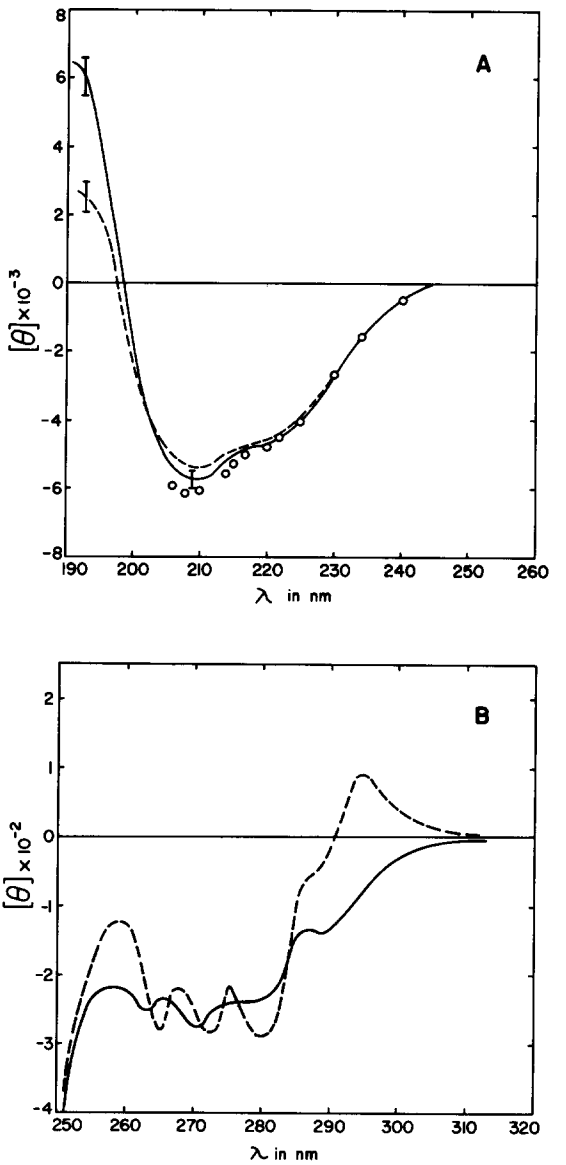

Figure 3. Circular Dichroism Spectra of AT-III. (A) Far-ultraviolet region; (B) Near-ultraviolet region, AT-III (——); AT-III in heparin (---). The open circles are the fit of the polylysine data based on Greenfield and Fasman (21), representing 7% α-helix, 31% β-structure and 62% random coil. Concentrations used: AT-III, 0.017% in the far-ultraviolet region and 0.085% in the near-ultraviolet region; heparin, 0.04 mg/ml and 0.22 mg/ml, respectively.

respectively. The calculated theoretical curve for this number of tryptophan and tyrosine residues is shown in the Figure. The perturbation difference spectrum of AT-III in the presence of heparin is shown (dashed line) in

Figure 2. Two similar broad maxima of relatively different intensities were observed. The maxima at 292 nm and 285 nm have $\Delta\varepsilon_M$ values of 560 and 870, respectively. Curve fitting of the experimental data show that 1.5 tryptophan and 6.2 tyrosine residues are exposed to the perturbing influence of the solvent. Thus, when heparin binds to AT-III there is an exposure of two tyrosine residues and partial burial of a tryptophan residue.

Circular Dichroism Studies. The CD spectra of AT-III in the far-ultraviolet shown in Figure 3A, exhibit relatively small ellipticities with bands centering at 222 nm, 209 nm and 193 nm. These bands have mean residue ellipticities, $[\theta]_\lambda$, of -4500±500, -5800±600 and +6300±900 degrees·cm^2·dmole, respectively. On the basis of the polylysine data of Greenfield and Fasman (19), the fit of our antithrombin curve between 208 nm and 240 nm corresponds to 7% α-helix, 31% β-structure and 62% random conformation. The fit with the model parameters of Martinez et al. (20), using the CD and X-ray data of reference proteins shows 7% α-helix, 11% β-structure and 82% random structure. The presence of heparin results in a decreased ellipticity at 193 nm to 2400±900 degrees·cm^2·dmole and a small reduction in amplitude at the 208-210 nm region, but did not produce any significant change on the Cotton effect at 222 nm. The decrease in amplitude at 193 nm indicates that the β-conformation is reduced in the presence of heparin and that the random structure is increased. It should be pointed out, however, that a quantitative estimation in the region below 208 nm cannot be obtained. The limitations and experimental error involved in this region are discussed elsewhere (19,20).

The CD spectra of AT-III in the near-ultraviolet region (Figure 3B) show poorly resolved multiple negative bands at 280 nm, 271 nm, 263 nm and a shoulder at 289 nm. The addition of heparin is accompanied by the appearance of a well-defined positive peak at 295 nm and the bands below 289 nm become more distinct and clearly defined.

DISCUSSION

The data based on solvent perturbation difference spectra show that 1.8 tryptophan and 4.2 tyrosine residues are exposed in AT-III, while 1.5

tryptophan and 6.2 tyrosine residues are exposed in the presence of heparin. The exposure of about two tyrosine residues and a small burial of tryptophan indicate a conformational change in AT-III polypeptide chain in the vicinity of these amino acid residues. The occurrence of a conformational change is substantiated by circular dichroism and ultraviolet difference spectral studies. The CD data of AT-III in the far-ultraviolet region show that the helical structure is not changed by heparin. However, there is a decrease in the β-conformation, with a compensating change in random structure. The fact that the dichroic bands in the near ultraviolet region appear to be more distinct and clearly resolved in the presence of heparin suggest variations in the microenvironments of the aromatic chromophores. The "blue shift" perturbation indicated by the negative difference spectra at 280 nm and 290 nm are also in agreement with increased exposure of tyrosine residues to the aqueous environment.

It is interesting to note that although the change in exposure of tryptophan is relatively small, there are considerable changes in orientations and charge environment of this chromophore. These are indicated by the positive ultraviolet difference spectrum at 298 nm (17) and the appearance of a positive CD band at 295 nm (21). Conceivably, a tryptophan residue may be located in the vicinity of the heparin binding site and the charge effect is induced by the bound acidic mucopolysaccharide.

In summary, we conclude from this study that when heparin binds to antithrombin III a conformational change of the polypeptide chain occurs in the vicinity of tryptophan and tyrosine residues. This involves a decrease in β-conformation with a compensatory change in random structure. The specific characteristics of antithrombin-heparin binding and the possible involvement of other amino acid residues are presently being investigated in this laboratory.

Acknowledgements. We are grateful to Professor Theodore T. Herskovits of Fordham University for his valuable suggestions and discussions.

References:

1. Abildgaard, U. (1968) Scand. J. Clin. Lab. Invest. 21, 89-91.
2. Yin, E.T., Wessler, S. and Stoll, P.J. (1971) J. Biol. Chem. 246, 3712-3719.
3. Rosenberg, R.D. and Damus, P.S. (1973) J. Biol. Chem. 248, 6490-6505.
4. Damus, P.S., Hicks, M. and Rosenberg, R.D. (1973) Nature 246, 355-357.
5. Stead, N., Kaplan, A.P., and Rosenberg R.D. (1976) J. Biol. Chem. 251, 6481-6488.
6. Li. E.H., Fenton II, J.W. and Feinman, R.D. (1976) Arch. Biochem. Biophys. 175, 153-159.
7. Thaler, E. and Schmer, G. (1975) Brit. J. Haemat. 31, 233-240.
8. Danishefsky, I., Tzeng, F., Ahrens, M. and Klein, S. (1976) Thromb. Res. 8, 131-140.
9. Miller-Anderson, M., Borg, H. and Anderson, L. (1974) Thromb. Res. 5, 439-452.
10. Kurachi, K., Schmer, G., Hermodson, M., Teller, D. and Davie, E. (1976) Biochemistry 15, 368-372.
11. Baughman, D.J. and Waugh, D.F. (1967) J. Biol. Chem. 242, 5252-5259.
12. Iphantis, D.S. (1964) Biochemistry 3, 297-317.
13. Danishefsky, I., Eiber, H.B. and Carr, J.J. (1960) Arch. Biochem. Biophys. 90, 114-121.
14. Danishefsky, I. and Siskovic, E. (1971) Carbohyd. Res. 16, 199-205.
15. Herskovits, T.T. and Sorensen, M. (1968) Biochemistry 7, 2533-2542.
16. Andrews, L.J. and Forster, L.S. (1972) Biochemistry 11, 1875-1879.
17. Ananthanarayanan, V.S. and Bigelow, C.C. (1969) Biochemistry 8, 3723-3728.
18. Villanueva, G.B. and Herskovits, T.T. (1971) Biochemistry 10, 3358-3365.
19. Greenfield, N. and Fasman, G. (1969) Biochemistry 8, 4108-4115.
20. Chen, H., Yang, J., and Martinez, H. (1972) Biochemistry 11, 4120-4131.
21. Bewley, T.A. and Li, C.H. (1972) Biochemistry 11, 884-888.