Conformational Changes Accompanying the Binding of Antithrombin III to Thrombin†

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ABSTRACT: The conformational aspects of the binding of antithrombin III to thrombin were investigated by difference spectroscopy, circular dichroism, and optical rotatory dispersion. The CD and ORD studies indicate an increase of 6–8% in α-helix content at the expense of the β structure, while the results from difference spectroscopy showed an increased exposure of approximately seven tyrosine residues. In the presence of heparin there is a slightly greater increase in helicity which is accompanied by exposure of an average of two tryptophan and one tyrosine residues. These spectral results indicate that the thrombin–antithrombin III complex formed in the presence of heparin differs in its conformation from that produced in its absence.

Antithrombin III is a plasma protein which inhibits a number of components of the blood coagulation cascade. These activities, as well as other effects of antithrombin III, were reviewed recently (Rosenberg, 1977). The inhibition of thrombin by antithrombin III was shown to be due to formation of a complex of the two proteins involving a serine residue of the thrombin and an arginine residue of the inhibitor (Rosenberg & Damus, 1973). This interaction is accelerated by heparin, and it was proposed that it produces this effect by altering the conformation of antithrombin III to one which is more favorable for binding with thrombin. In a previous report from this laboratory, evidence was presented that heparin does indeed produce an alteration in the conformation of antithrombin III (Villanueva & Danishefsky, 1977). It was shown by studies of circular dichroism and ultraviolet difference spectra that heparin causes an increase in the random conformation of the protein at the expense of the β structure and that it induces an exposure of tryptophan and tyrosine residues.

This paper describes investigations on the spectral changes which occur in the thrombin–antithrombin III reaction and the effect of heparin on this interaction. The results show that the complexes formed in the presence and absence of heparin have distinct differences in terms of the location of tyrosine and tryptophan residues, although they are similar with respect to polypeptide secondary structure. It appears, therefore, that the conformation of the two types of complexes are not identical.

Materials and Methods

Materials. Human antithrombin III was prepared from fresh plasma by procedures described previously (Danishefsky et al., 1978). This involved fractionation of plasma with polyethylene glycol (Thaler & Schmer, 1975), affinity chromatography on heparin–aminohexyl-Sepharose (Danishefsky et al., 1976) and gel filtration on Sephadex G-200. The product was homogeneous on the basis of results from sodium dodecyl sulfate gel electrophoresis. Human thrombin prepared from Cohn fraction III (Fenton et al., 1977) was a gift from Dr. John Fenton, II, of the New York State Department of Health, Albany, NY. Heparin from bovine lung was obtained from Upjohn International through the courtesy of Dr. L. L. Coleman. Chondroitin 6-sulfate is from Miles Laboratories. Antithrombin III concentration was determined spectrophotometrically by using $\epsilon_{1\%} = 6.5$ and a molecular weight of 58 000 (Nordeman et al., 1977). For thrombin, $\epsilon_{1\%} = 18.3$ and a molecular weight of 36 500 were used (Fenton et al., 1977). The molar extinction coefficient of the complex was calculated on a molar additivity basis. All absorbances were corrected for light scattering (Baughman & Waugh, 1967).

Experimental Design. In all experiments antithrombin III was incubated at room temperature with thrombin for various times in a defined molar excess of the inhibitor. The molar ratios of thrombin to inhibitor were usually between 1.0:2.0 and 1.0:2.3. Although it is known that the complex is formed in a 1:1 molar ratio (Abildgaaard, 1969; Rosenberg & Damus, 1973), the molar excess of the inhibitor was necessary to prevent rapid degradation of the primary complex. In studies involving the effect of heparin, antithrombin III and heparin were incubated for 3 min at 27 °C before adding thrombin. The first complete CD or UV difference spectrum was obtained within 3 min. The concentration of heparin used in CD and ORD ranged from 0.5 to 1.0 mg/mL. In difference spectroscopy, it ranged from 2 to 2.5 mg/mL. Since it is known that even in the presence of excess inhibitor the primary complex is still gradually degraded to secondary complexes (Chandra & Bang, 1977), only the spectra obtained within 3 min were used to evaluate the conformational aspects of the primary complex. Heparin has a CD spectrum with bands centered at 210 nm and 190 nm (Chung & Ellerton, 1976; Stone, 1971). At the concentration used in this study the heparin conbribution to the protein CD spectra amounts to 5% at 210 nm and 10% at 190 nm. This is cancelled out by manual subtraction from the observed CD spectra or the base line is obtained in the presence of heparin.

Difference Spectroscopy. The ultraviolet difference spectra were obtained in a Cary 15 recording spectrophotometer at a constant dynode setting of 3 and slit widths not exceeding 1.5 mm. Four semimicro cells with 1.000-cm path lengths were used. Solution compartments were numbered 1 to 4. Compartments 1 and 2 were in the sample beam, while compartments 3 and 4 were in the reference beam. In a typical experiment, 500 μL of antithrombin III (350 μg) was placed in compartments 1 and 3. In compartments 2 and 4 was placed 500 μL of thrombin (95 μg). After the base line was obtained, solutions in compartments 1 and 2 were mixed and replaced back into the cells. The difference spectra were obtained at various times for 24 h. In studies involving heparin, 10 μL of heparin solution was added to compartments 1 and 3 and 10 μL of buffer was placed into compartments 2 and 4.

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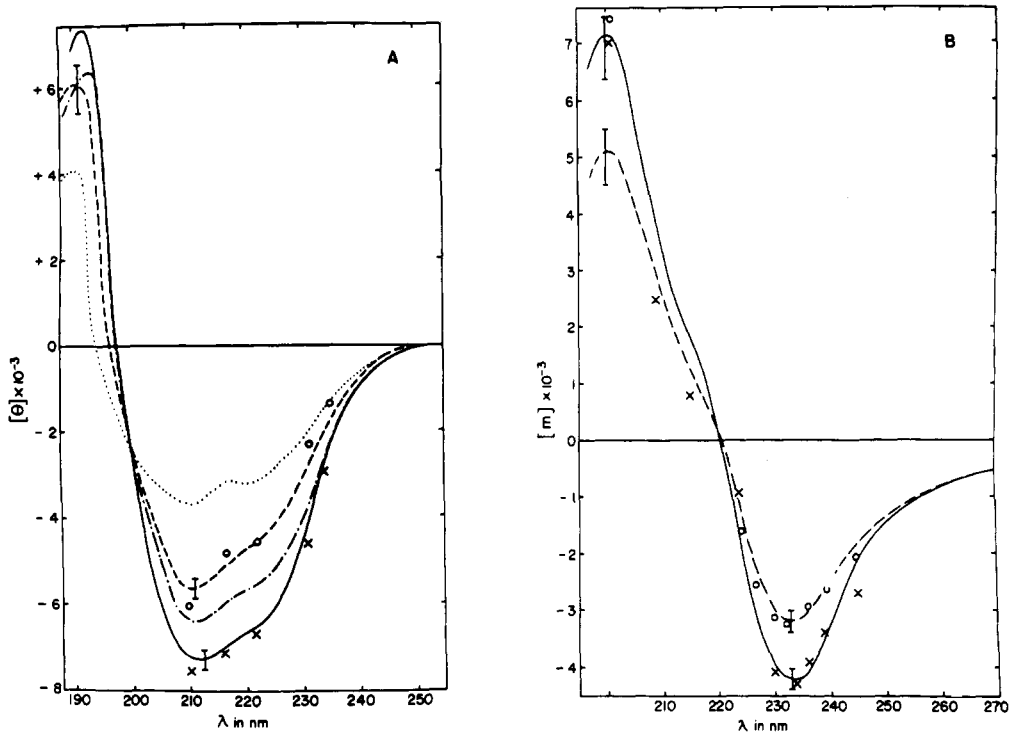

FIGURE 1: (A) Circular dichroism spectra of thrombin (···), antithrombin III (-----), and thrombin–antithrombin III complex (—) in 0.15 M NaCl, 0.01 M Tris, pH 7.5. The dashed line represents the CD spectra of the complex based on additivity. Points designated by O and X are the calculated CD spectra based on reference proteins (Chen et al., 1972) corresponding to 6% α helix, 7% β structure, and 87% random conformation and 14% α helix, 0% β structure, and 86% random conformation, respectively. (B) ORD spectra of the thrombin–antithrombin III complex. Experimental curve (—); calculated curve based on additivity (- - -). The O and X are calculated spectra based on reference proteins corresponding to 5% α helix, 6% β structure, 89% random conformation and 13% α helix, 0% β structure, and 87% random conformation, respectively.

Solvent perturbation difference spectra were obtained according to the method of Herskovits & Laskowski (1962). All the data were analyzed by the procedure of Herskovits & Sorensen (1968a,b). Estimates of the apparent number of exposed tryptophan and tyrosine residues were obtained by the best fit of the protein curves using the relationship

$$\Delta\epsilon_\lambda(\text{protein}) = a\Delta\epsilon_\lambda(\text{Trp}) + b\Delta\epsilon_\lambda(\text{Tyr}) \qquad (1)$$

where a represents the number of exposed tryptophan residues and b the number of exposed tyrosine residues. $\Delta\epsilon_\lambda$(Trp) and $\Delta\epsilon_\lambda$(Tyr) are the molar absorbance difference values of free tyrosine and tryptophan as a function of wavelength, λ. The tabulated model data of *N*-acetyl esters of tyrosine and tryptophan were used (Herskovits & Sorensen, 1968a).

Circular Dichroism and Optical Rotatory Dispersion. Circular dichroism measurements were peformed at 25 °C on a Cary 60 recording spectropolarimeter equipped with a Cary 6001 circular dichroism attachment. Cylindrical cells with optical path lengths of 0.1 and 1.0 cm were used to measure the circular dichroism in the 190–250- and 205–300-nm regions, respectively. Protein concentrations ranged from 0.010% to 0.015%. The mean residue ellipticity, $[\theta]$, was calculated using 113 as mean residue weight for antithrombin III and 112 for thrombin. The carbohydrate content of antithrombin III and thrombin are 9.0% (Kurachi et al., 1976; Danishefsky et al., 1977) and 7.6% (Thompson et al., 1977) by weight, respectively, and on a weight basis the 1:1 complex contains 61.4% antithrombin III and 38.6% thrombin. In a typical experiment, separate solutions of antithrombin III and thrombin were prepared such that they would be in a 2:1 molar ratio when mixed. After the spectrum of each solution was determined by itself, the composite spectrum of the two solutions in separate cells was measured. The two solutions were then mixed to allow for complex formation and the spectrum was redetermined. The optical rotatory dispersion data were reported as mean residue rotation, $[m]_\lambda$, without correction for the refractive index of the solvent.

able 1: Circular Dichroism and Optical Rotatory Dispersion Parameters of Thrombin, Antithrombin III, and Thrombin–Antithrombin III Complex[a]

	$-[\theta]_{222}$ (deg cm^2 dmol^{-1})	% α helix	$-[m]_{233}$ (deg cm^2 dmol^{-1})	% α helix
antithrombin III	5510 ± 500	7 ± 2	3770 ± 550	9 ± 2
thrombin	3200 ± 300	2 ± 1	2310 ± 400	3 ± 1
antithrombin III–thrombin complex (1:2) (exptl)	6500 ± 500	14 ± 2	4200 ± 550	13 ± 2
antithrombin III–thrombin complex (by additivity)	4550 ± 583	6 ± 2	3200 ± 680	6 ± 2
thrombin plus antithrombin III (combination spectra)[b]	4525 ± 500	6 ± 2	3350 ± 500	6 ± 2

[a] In 0.15 M NaCl, 0.01 M Tris, pH 7.5. [b] Obtained by measuring the composite CD or ORD spectra of thrombin and antithrombin III simultaneously in separate cells as described in Materials and Methods.

Results

Circular Dichroism and Optical Rotatory Dispersion. Figure 1 shows the CD and ORD spectra of antithrombin III, thrombin, and the thrombin–antithrombin III complex. The quantitative data are summarized in Table I. It is seen that the CD spectra vary in intensities but have essentially the same features with bands centered at 221–222 nm, 209–210 nm, and 190–192 nm. The mean residue ellipticities, $[\theta]_\lambda$, of thrombin at these wavelengths were −3200, −3750, and +4000 deg cm^2 dmol^{-1}, respectively. The values for antithrombin III

at these wavelengths were −5510, −6300, and +6300 deg cm^2 dmol^{-1}, respectively. By using the equation of Chen et al. (1972)

$$[\theta]_{222} = 30300f_h - 2340 \quad (2)$$

and by curve fitting, based on reference proteins (Chen et al., 1972), the thrombin was found to contain 2–3% α helix and almost no β structure, while antithrombin III has 7% α helix, 11% β structure, and the rest of the molecule in random conformation. We have shown previously that heparin produced far-UV circular dichroism changes in the antithrombin III spectrum (Villanueva & Danishefsky, 1977). These were characterized by a significant decrease in amplitude at 193 nm, while no detectable changes were observed at the 209 nm and 222 nm regions.[1] Such effects were not seen in thrombin.

The CD spectrum of the thrombin–antithrombin III complex in a 1:2 molar ratio has mean residue ellipticities of −6500, −7300, and +7500 deg cm^2 dmol^{-1} at the above wavelengths, respectively. The additivity of the mean residue ellipticity of the complex was investigated by calculating the theoretical curve for a thrombin–antithrombin III mixture in a 1:2 molar ratio. This curve (Figure 1A, dashed line) has values of −4550 and −5750 deg cm^2 dmol^{-1} at 222 and 210 nm, respectively. Thus, the experimental and calculated values differ by 1950 and 1550 deg cm^{-2} dmol^{-1} at 222 and 210 nm, respectively. The theoretical curve, based on additivity, corresponds to 5–7% α helix, 6–8% β structure, and 86–88% random coil. The experimental curve corresponds to 13–15% α helix, 0.1% β structure, and 85–87% random coil. The antithrombin III–thrombin complex formed in the presence of heparin has a consistently higher ellipticity at 222 nm than in the absence of heparin by −650 ± 50 deg cm^2 dmol^{-1}. This was not shown in the figure.

The optical rotatory dispersion data for thrombin and antithrombin III were almost similar to the circular dichroism data. Thrombin was found to contain 2–3% α helix and no β structure. Antithrombin III has 9% α helix and 8% β structure. The apparent increase in the α-helix during complex formation was also observed in the ORD spectra. Figure 1B shows the ORD spectra of the experimental 1:2 molar ratio and the calculated curves based on additivity. The mean residue rotation, $[m]_\lambda$, of the experimental curve at 233 nm was found to be −4200 deg cm^2 dmol^{-1}, while the theoretical curve, based on additivity, was −3200 deg cm^2 dmol^{-1}. By using the parameters based on reference proteins (Chen et al., 1972), the difference in mean residue rotation of 1000 deg cm^2 dmol^{-1} between the experimental and the calculated curves corresponds to an increase in α-helix content from 5–7 to 12–14% and a decrease in β structure from 6–8 to 1–2%. The close correspondence of the values calculated, based on additivity and the experimental composite spectra determined by taking the spectrum of thrombin and antithrombin III simultaneously in separate cells, confirms the validity of the additivity approach (see Table I). Both ORD and CD data indicate an increase in α helix and a decrease in the β structure during complex formation. Since thrombin has almost no β structure, this loss must have occurred in the antithrombin III molecule.

The CD spectra of thrombin, antithrombin III, and the complex have been examined in connection with possible effects of 20% glycerol used in our difference spectral studies.

[1] It should be noted that, in a recent study, Nordenman & Bjork (1978) used a high concentration of Tris buffer which made spectral determination below 200 nm difficult. Consequently, they did not detect such changes. In addition, sodium fluoride was used in our study in order to maintain desired ionic strength at lower wavelength.

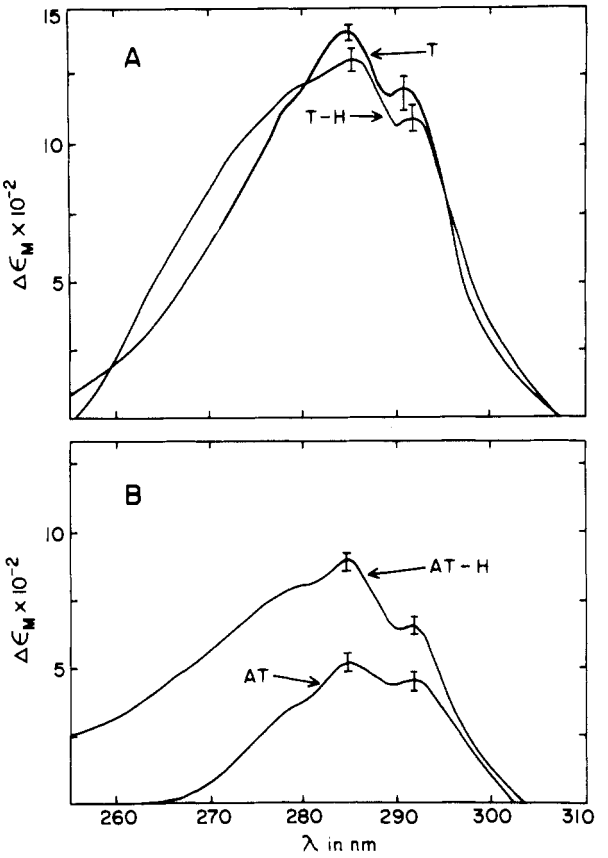

FIGURE 2: Solvent perturbation difference spectra of (A) thrombin and (B) antithrombin III in the presence and absence of heparin. Perturbant used: 20% glycerol. Solvent: 0.15 M NaCl, 0.01 M Tris, pH 7.5. Protein concentration ranged from 2 to 2.5 × 10^{-5} M for thrombin and 3.5 to 4.0 × 10^{-5} M for antithrombin III. Heparin concentration: 0.5–1.0 mg/mL.

Table II: Estimation of Exposed Tryptophan and Tyrosine Residues in Antithrombin III and Thrombin by Solvent Perturbation in 20% Glycerol[a]

	$\Delta\epsilon_{292}$	$\Delta\epsilon_{285}$	no. of exposed residues[b] Trp	Tyr
antithrombin III	475 ± 30	536 ± 30	1.4 ± 0.2	3.6 ± 0.2
antithrombin III in the presence of heparin	655 ± 40	890 ± 50	2.0 ± 0.2	6.0 ± 0.2
thrombin	1220 ± 50	1428 ± 65	3.7 ± 0.2	9.2 ± 0.2
thrombin in the presence of heparin	1100 ± 50	1310 ± 50	3.5 ± 0.2	8.5 ± 0.2

[a] 0.15 M NaCl, 0.01 M Tris, pH 7.5. [b] Based on curve fitting, according to the method of Herskovits & Sorensen (1968a).

No significant changes in the 222- and 210-nm bands were observed indicating that 20% glycerol has no effect on the solution conformation of these proteins.

Solvent Perturbation. The method of solvent perturbation was used to investigate the accessibility of the aromatic amino acid residues and to determine if the exposure of these residues are altered as a consequence of the conformational changes indicated by ORD and CD studies. Figures 2A and 2B show the solvent perturbation difference spectra of thrombin and antithrombin III both in the absence and presence of heparin. The molar absorptivity differences of antithrombin III increases in the presence of heparin. In thrombin these parameters did not change significantly, although a small decrease in intensity could be observed in the presence of heparin. These results indicate that, in the presence of heparin, there is an increased accessibility of the tyrosine and tryptophan

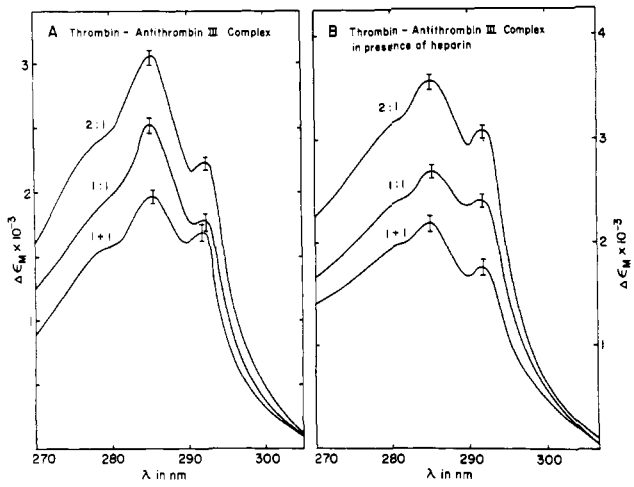

FIGURE 3: (A) Solvent perturbation difference spectra of thrombin–antithrombin III complex without heparin. (B) Solvent perturbation difference spectra of thrombin–antithrombin III complex in the presence of heparin. Perturbant used: 20% glycerol. The upper curves correspond to the experimental 1:2 molar ratio. The middle curves are the calculated 1:1 molar ratio obtained by subtracting $\Delta\epsilon_M$ values equivalent to 1 mol of antithrombin III from the upper curves. The lower curves are the sum of the $\Delta\epsilon_M$ of 1 mol of antithrombin III and 1 mol of thrombin. Protein concentrations used were 5–6 × 10^{-6} M for thrombin and 1–1.5 × 10^{-5} M for antithrombin III. The protein concentration of the complex was determined by additivity.

chromophores in antithrombin III, while in thrombin the effect is that of small burial of these residues. The calculated number of exposed chromophores corresponding to these observed difference spectra are shown in Table II. The data show that, in thrombin, an average of 3.7 out of 9 tryptophan and 9.2 out of 10 tyrosine residues were exposed. In the presence of heparin, the average exposure decreased slightly to 3.5 tryptophan and 8.5 tyrosine residues. The data on antithrombin III showed an average exposure of 1.4 out of 6 tryptophan and 3.6 out of 10 tyrosine residues which in the presence of heparin was increased to 2.0 tryptophan and 6.0 tyrosine residues. It should be noted that, in our previous studies using 20% propylene glycol, antithrombin III in the presence of heparin showed similar effects on the exposure of tryptophan and tyrosine residues (Villanueva & Danishefsky, 1977) by about the same extent. These difference spectral data clearly demonstrate that the heparin adduct interacts and unfolds antithrombin III, whereas the effects on thrombin are minor and in the expected direction of burial on interaction.

In the studies on the solvent perturbation difference spectra of the thrombin–antithrombin III complex, it was assumed that the absorbances of the chromophores are additive. This has previously been shown to be true for proteins and model compounds (Herskovits & Sorensen, 1968a). Figure 3A shows the solvent perturbation difference spectra of the thrombin–antithrombin III complex in the absence of heparin. Table III summarizes the quantitative data. As mentioned earlier, complex formation has to be carried out in a molar excess of the inhibitor in order to minimize the degradation of the primary complex. In order to derive the values for a 1:1 molar ratio, $\Delta\epsilon_M$ values equivalent to 1 mol of antithrombin III were subtracted from the experimental difference spectrum obtained in a 1:2 molar ratio. The upper curve shows the experimental spectrum of the complex in a 1:2 molar ratio. The best fit to this curve corresponds to an average exposure of 6.4 tryptophan and 23.5 tyrosine residues (Table III). The middle curve is the resulting spectrum corresponding to a 1:1 molar ratio after an equivalent of 1 mol, of antithrombin III was subtracted from the upper curve. This represents an exposure of 5.0

Table III: Estimation of Exposed Tyrosine and Tryptophan Residues in the Thrombin–Antithrombin III Complex by Solvent Perturbation in 20% Glycerol[a]

	$\Delta\epsilon_{292}$	$\Delta\epsilon_{285}$	no. of exposed residues[b] Trp	Tyr
Thrombin–Antithrombin III Complex Alone				
thrombin–antithrombin III complex (1:2 molar ratio)	2245 ± 80	3060 ± 120	6.4 ± 0.5	23.5 ± 1
thrombin–antithrombin III complex (1:1 molar ratio)[c]	1770 ± 75	2524 ± 100	5.0 ± 0.4	19.9 ± 1
sum of antithrombin III and thrombin[d]	1695 ± 58	1964 ± 74	5.1 ± 0.3	12.8 ± 0.3
net change in exposure due to complex formation			0.1 ± 0.4	7.1 ± 1
Thrombin–Antithrombin III Complex in the Presence of Heparin				
thrombin–antithrombin III complex (1:2 molar ratio)	3080 ± 110	3590 ± 125	9.3 ± 0.5	21.5 ± 1
thrombin–antithrombin III complex (1:1 molar ratio)[c]	2425 ± 100	2700 ± 110	7.3 ± 0.4	15.5 ± 1
sum of antithrombin III and thrombin (1 + 1)[d]	1755 ± 64	2200 ± 70	5.5 ± 0.3	14.5 ± 0.3
net change in exposure due to complex formation			1.8 ± 0.4	1.0 ± 1

[a] 0.15 M NaCl, 0.01 M Tris, pH 7.5. [b] Based on curve fitting according to the method of Herskovits & Sorensen (1968a). [c] Calculated by subtracting $\Delta\epsilon_{285}$ and $\Delta\epsilon_{292}$ values equivalent to 1 mol of antithrombin III from those in the 1:2 molar ratio. [d] Sum of $\Delta\epsilon$ values from Table II.

tryptophan and 19.9 tyrosine residues. The lower curve represents the sum of the $\Delta\epsilon_M$ values of 1 mol of thrombin and 1 mol of antithrombin III which is equivalent to an exposure of 5.1 tryptophan and 12.8 tyrosine residues. Comparison of the experimental difference spectrum (middle curve, 1:1) and the calculated spectrum (lower curve, 1 + 1) showed that, although there is a pronounced effect at the 285-nm peak, no significant difference is observed at the 292-nm peak. This would indicate that formation of the 1:1 complex leaves the tryptophan exposure essentially unaltered in the absence of heparin.

The $\Delta\epsilon_M$ value of free tryptophan (*N*-acetyltryptophan methyl ester) at 292 nm obtained with 20% glycerol is 304 M^{-1} cm^{-1} (Herskovits & Sorensen, 1968a). Thus, the observed difference in $\Delta\epsilon_M$ values between the 1:1 molar ratio (derived from the experimentally determined 1:2 molar ratio) and the calculated 1:1 molar ratio (sum of antithrombin III and thrombin values) of 75 M^{-1} cm^{-1} represents an increased exposure of a small fraction of a tryptophan residue. The $\Delta\epsilon_M$ value of free tyrosine (*N*-acetyltyrosine methyl ester) at 285 nm obtained with 20% glycerol is 80 M^{-1} cm^{-1}. The observed difference at 285 nm between the experimental and calculated values was 560 M^{-1} cm^{-1}. This represents an increased exposure of seven tyrosine residues. With both tyrosine and tryptophan contributions taken into account by using eq 1, the

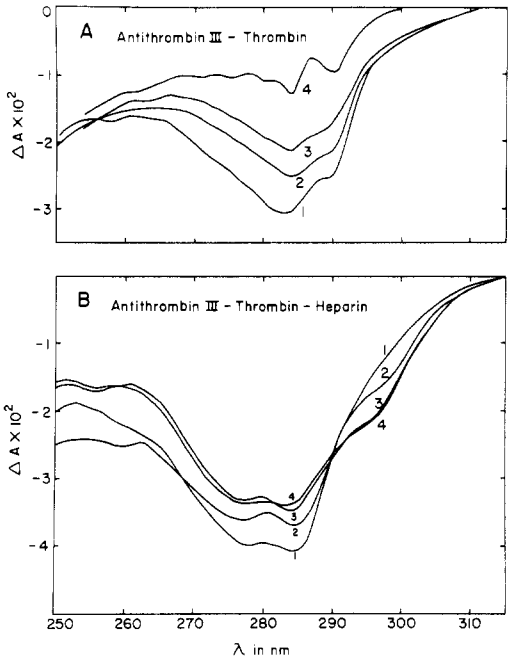

FIGURE 4: Ultraviolet difference spectra of the thrombin–antithrombin III complex without heparin (A) and in the presence of heparin (B). The numbers indicate incubation time as follows: (1) 3 min; (2) 30 min; (3) 1 h; and (4) 24 h. Protein concentrations used were 5.8×10^{-6} M for thrombin and 1.3×10^{-5} M for antithrombin III.

average estimate, based on curve fitting, indicates an average due to complex formation of 7.1 tyrosine residues and essentially no change in the exposure of the tryptophan residues.

The solvent perturbation difference spectrum in the presence of heparin (Figure 3B and Table III) shows an increase in both $\Delta\epsilon_M$ values at 292 and 285 nm, indicating that both tyrosine and tryptophan residues are perturbed. Analysis of the data reveals that the net increase in $\Delta\epsilon_M$ of the experimental values, over that calculated for a 1:1 molar ratio, corresponds to an increased exposure of 1.8 tryptophan and 1.0 tyrosine residues.

Ultraviolet Difference Spectroscopy. The ultraviolet difference spectra of thrombin–antithrombin III complex obtained in a 1:2 molar ratio were also investigated. The spectrum obtained without heparin (Figure 4A) was characterized by negative bands centering at 284 and 289 nm. These bands decreased in intensity with increasing reaction time. Based on several studies with proteins and model compounds (Donovan, 1973; Bigelow, 1961), these bands, although at slightly different wavelengths, represent the characteristic 279- and 287-nm bands, which are indicative of tyrosine perturbation. They are due to polarization blue shifts which occur when tyrosine residues have entered more polar environments or regions of decreased refractive index. This generally represents an increased exposure of tyrosine residues to the solvent (Donovan, 1973). It can be assumed that the band at 287 nm observed by these authors corresponds to the 289-nm band observed in this study. When the ΔA_{289} at 3-min incubations is converted to $\Delta\epsilon_M$ based on additivity, this value corresponds to -4475 M^{-1} cm^{-1}. As a close approximation and assuming no contribution from tryptophan residues, this value corresponds to an exposure of 6 tyrosine residues, based on $\Delta\epsilon = 750$ per mol of tyrosine (Klee, 1977). The close agreement between this value and the value of 7 obtained from the solvent perturbation data (Table III) is additional proof that there is essentially very little or no tryptophan pertubation accompanying the complex formation in the absence of heparin.

Figure 4B shows the difference spectra of the complex in the presence of heparin. The major features of these spectra were two negative bands at 278 and 284 nm. They decreased in intensities with increasing reaction time up to 1 h. A band at 297 nm appeared with increasing reaction time. It was already present in 3-min incubation, since the ΔA_{298} is 1.8 times greater in the presence of heparin than in its absence. This band which is apparently not present when there is no heparin can be ascribed to tryptophan perturbation. The difference spectrum below 290 nm that appears to be larger than one would expect based on the solvent perturbation data is probably due to increased contribution of tryptophan transition at 284 nm which Andrews & Forster (1972) have observed to occur when the tryptophan perturbation is due to solvent and charge effects.

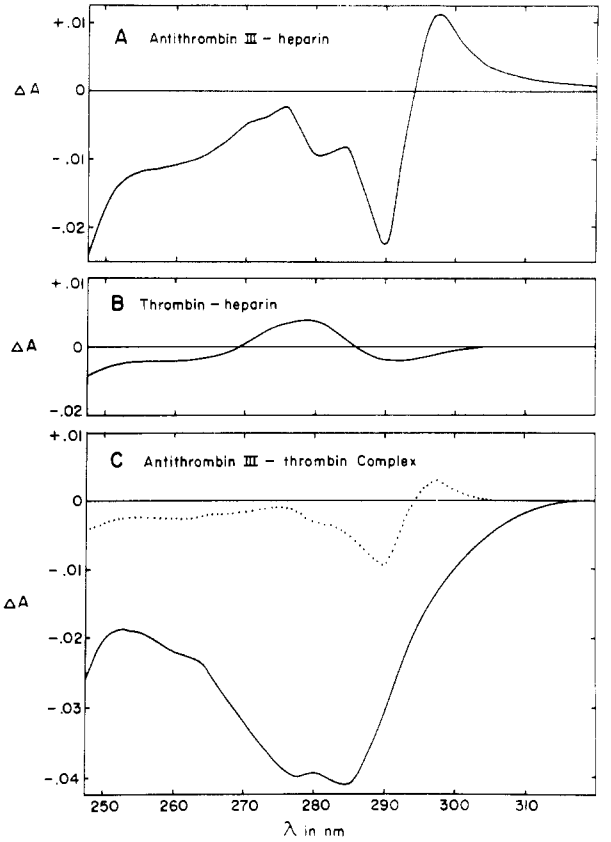

FIGURE 5: Ultraviolet difference spectra of antithrombin III (A), thrombin (B), and the thrombin–antithrombin III complex (C) in the presence of heparin. The dotted line is the sum of the difference spectra of thrombin and antithrombin III normalized to the concentrations that are present in the complex. In A the concentration of antithrombin III was 3.57×10^{-5} M, and in B the concentration of thrombin was 2.5×10^{-5} M. In C, the concentrations of thrombin and antithrombin III were 6.2×10^{-6} and 1.29×10^{-5} M, respectively.

Our previous studies on the difference spectrum of the antithrombin III–heparin complex (Villanueva & Danishefsky, 1977) were examined in greater detail in order to determine if correlation exists between these spectral changes and the one that occurred in the thrombin–antithrombin III complex. Figures 5A and 5B show the ultraviolet difference spectra of thrombin and antithrombin III in the presence of heparin. The difference spectrum of antithrombin III in the presence of heparin showed a positive band at 298 nm, indicating tryptophan perturbations, and negative bands at 290 and 282 nm which could be assigned to tyrosine perturbations. Thrombin showed no significant difference spectrum in the presence of heparin which suggests very little perturbation, if any, of the aromatic amino acid residues. Figure 5C shows the difference spectrum of the thrombin–antithrombin III complex in the

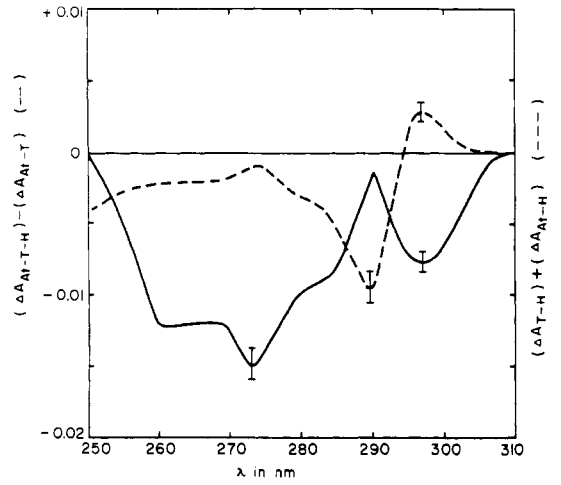

FIGURE 6: Changes in the ultraviolet difference spectra of the antithrombin III–thrombin complex due to the presence of heparin. The solid line is a double difference spectrum obtained by subtracting the difference spectra of the antithrombin III–thrombin complex from the spectra of the complex in the presence of heparin. The dashed line is the sum of the separate difference spectra of antithrombin III and thrombin in the presence of heparin (see Figure 5C).

presence of heparin. The dotted lines correspond to the sum of the difference spectra of thrombin and antithrombin III normalized to the concentrations in which they were present in the complex. It is evident that the difference spectra due to the formation of the complex is about five times greater than the sum of the separate spectra. The differences between the experimental and the calculated spectra are, therefore, reflections of the perturbation that have occurred on complex formation. In addition to increased intensity, the profile of the spectrum of the complex was different from the additive spectrum. The characteristic positive tryptophan transition that was present in the antithrombin III difference spectrum was evident in the spectrum of the complex as a negative shoulder at 298 nm. When the difference spectra of the complex in the presence and absence of heparin were compared, it appeared that the time dependent changes that occur after complex formation were different. Interestingly, it appeared also that tyrosine and tryptophan residues were perturbed in the presence of heparin, while only tyrosine residues were perturbed in the absence of heparin which is in accord with the solvent perturbation data. Manual subtraction of the difference spectrum of the antithrombin III–thrombin complex formed without heparin from that formed in the presence of heparin results in a spectrum shown in Figure 6. The solid line is a reflection of a different perturbation that has occurred during complex formation in the presence of heparin. The difference between the solid line and the dashed line is, therefore, a reflection of the changes induced by heparin during complex formation in addition to the changes induced on antithrombin III before complex formation.

In order to determine whether the spectral changes produced by heparin are due to nonspecific effects of the charged mucopolysaccharide, studies were conducted on the effect of chondroitin 6-sulfate in the binding of thrombin to antithrombin III. Like heparin, this is a sulfated mucopolysaccharide; however, it does not have anticoagulant activity. The results of experiments on the ultraviolet spectra, as well as the solvent perturbation difference spectra (Figures 7 and 8), demonstrate that chondroitin 6-sulfate does not have the same effect as heparin; i.e., the spectra are indistinguishable from those exhibited by thrombin–antithrombin reaction in the absence of heparin.

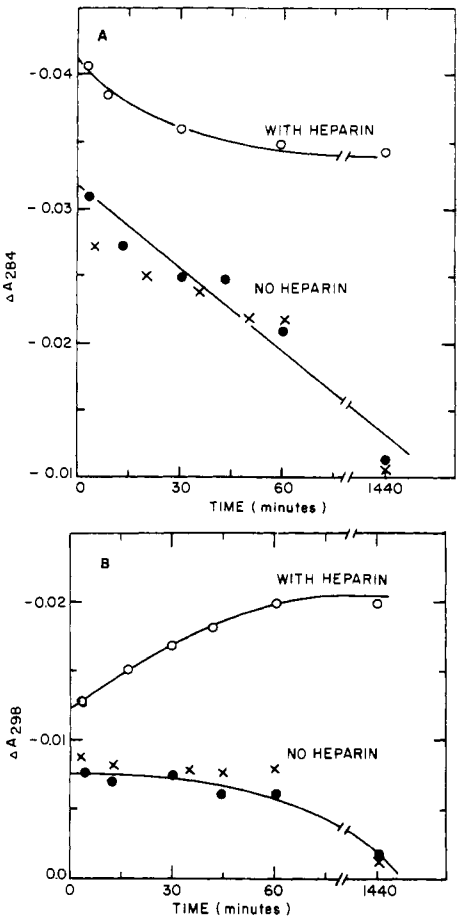

FIGURE 7: The time-dependent ultraviolet difference spectral changes in the antithrombin III–thrombin complex in the presence and absence of heparin. (A) Difference spectral maxima at 284 nm. (B) Difference spectral maxima at 289 nm. (O) With heparin; (●) without heparin; and (X) with chondroitin 6-sulfate.

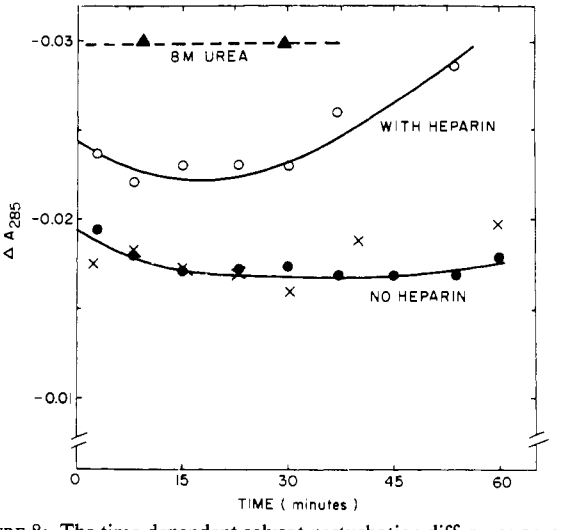

FIGURE 8: The time-dependent solvent perturbation difference spectral changes in the antithrombin III–thrombin complex in the presence and absence of heparin; (Δ) solution in 8 M urea. The other legends are similar to Figure 7.

Since heparin accelerates the otherwise slow reaction of thrombin and antithrombin III, the spectral differences observed could be due to the different extent of complex formation and/or differences in the relative level of degradation in the presence and absence of heparin. To investigate this possibility, the spectral changes were plotted as a function of time. If the role of heparin is to accelerate the same con-

formational change that occurs slowly in its absence, then the spectral data should be similar after a certain period of time, i.e., when the concentration of complex and the relatively small amounts of degradation products are the same. Moreover, if only a rate difference was involved, the spectrum for the heparin-absent reaction at a later time (e.g., 30 min) should be similar to that for the heparin-present reaction at an early time (e.g., 5–10 min). Figures 7A and 7B show the ultraviolet difference spectral changes as a function of time. It is evident that the spectra do not approach each other and are qualitatively different throughout the whole range of incubation time. In the presence of heparin, the difference spectral maxima become constant after 60 min, while, in the absence of heparin, the spectra continue to diminish up to 24 h. Figure 8 shows that the solvent perturbation difference spectra also do not approach each other and that they are qualitatively different. In the presence of heparin, the spectra approach that of the fully unfolded protein in 8 M urea but in the absence of heparin after 20 min, the spectra remain constant corresponding to only 50% unfolding. From these data, it is reasonable to conclude that there is a different conformation in the presence or absence of heparin.

Discussion

The results of the spectroscopic studies demonstrate that, when thrombin binds with antithrombin III, the complex undergoes considerable conformational changes. The data from the CD and ORD studies indicate an increase in α-helix content from 5–7 to 12–15% and a corresponding loss in β structure. This change is somewhat higher when the interaction occurs in the presence of heparin. It should be noted that this estimate of the change in secondary structure is a minimum value since large fractions of the molecules are in random conformation and any change from one random structure to another is not detected by the methods employed. An additional consideration is the fact that complex formation is relatively slow in the absence of heparin. The changes observed after a 3 min incubation of thrombin with antithrombin III are for only their partial transformation to the complex. Thus, in the presence of heparin, which accelerates the reaction, a relatively higher ellipticity is observed.

The solvent perturbation results indicate that, in the absence of heparin, the conformational change is accompanied by exposure of approximately 7 tyrosine residues. When heparin is present, there is an exposure of an average of 1 tyrosine and 2 tryptophan units. The significant "blue shift" perturbations in the ultraviolet difference spectra for the complexes in both cases are consistent with increased exposures of aromatic amino acid residues. Moreover, the difference spectra for complex formation in the absence of heparin indicate bands which are only characteristic for tyrosine perturbation, whereas, in the presence of heparin, the difference spectra implicate both tyrosine and tryptophan. Thus, the results of the ultraviolet difference spectral studies support the conclusions based on perturbation studies. The results obtained in the absence of heparin would indicate that the exposed tyrosine residues must be distant from the tryptophan residues, since the latter did not experience any perturbation. However, since both tyrosine and tryptophan were perturbated in the presence of heparin, these chromophores must be neighboring groups or multiple changes occurred during complex formation in this case. At any rate, it is evident that the complex formed in the presence of heparin differs in conformation from that produced in its absence. In both cases, however, the molecules undergo considerable unfolding as shown by increased exposure of the aromatic chromophores. It is conceivable that the susceptibility of the complex to degradation is due to this unfolding process which exposes more thrombin-susceptible bonds to the surface of the complex.

The effect of heparin on the conformation of the complex may be due to its binding to antithrombin III, to thrombin, or to both components. Since the binding of heparin to antithrombin III is accompanied by spectral perturbations of tryptophan residues (Villanueva & Danishefsky, 1977), whereas addition of heparin to thrombin does not have this effect, it is probable that the action of heparin on complex formation is due to its binding to antithrombin III. The binding of heparin to antithrombin III has been postulated as the pivotal step in a mechanism by which heparin accelerates the inhibition of thrombin (Rosenberg & Damus, 1973). Support for this mechanism was also presented by Li et al. (1976), by Nordenman et al. (1978), and by a previous report from this laboratory (Villanueva & Danishefsky, 1977). Other reports have given evidence that the primary reaction is the binding of heparin to thrombin (Machovich et al., 1975, 1978; Sturzebecker & Markwardt, 1977; Smith & Craft, 1975) or that heparin binds to both components of the complex (Danishefsky et al., 1977; Pomerantz & Owen, 1978). The present findings give additional support that heparin binds to antithrombin III; however, it does not exclude the possiblity for another interaction with thrombin.

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Molecular Weight of (Na^+,K^+)ATPase from Shark Rectal Gland†

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ABSTRACT: The (Na^+,K^+)ATPase from the rectal gland of *Carcharhinus obscurus* has been solubilized in Lubrol WX as an active complex containing 379 900 g of protein and 61 mol of phospholipid. This detergent–lipid–protein complex contains two catalytic subunits of molecular weight 106 400 and four glycopeptide subunits of *protein* molecular weight 36 600. The latter subunit has a total molecular weight of 51 700 when the carbohydrate is included. Attempts to dissociate this active enzyme complex to smaller size by increasing the detergent concentration led to inactivation. Thus, the smallest active particle in the presence of Lubrol WX contains the two polypeptide subunits in a mole ratio of 2:4 under conditions where the micellar form of the detergent is present at a 70:1 molar ratio. This large excess of Lubrol WX eliminates any possibility of artificial togetherness as the result of statistical considerations.

The active translocation of Na^+ and K^+ in animal membranes is mediated by an enzyme which hydrolyzes ATP[1] and is stimulated by Na^+ and K^+, (Na^+,K^+)ATPase. A number of investigators have reported purification (primarily in membrane bound form) of the enzyme from a variety of sources (e.g., Kyte, 1971; Lane et al., 1973; Jorgensen, 1974; Uesugi et al., 1973; Dixon & Hokin, 1974; Hokin et al., 1973), but to date the molecular weights of the active complex and of its subunits have not been determined. This fundamental information can be obtained only when the protein complex and its polypeptide components are soluble in homogeneous form so that the standard techniques of physical solution chemistry can be applied. Neither electrophoretic nor gel filtration techniques provide a correct measurement of molecular weight (Maddy, 1976; Nielsen & Reynolds, 1978). Equilibrium analytical ultracentrifugation is a rigorous thermodynamic method which is most easily used for the determination of protein mass in detergent solubilized complexes.

The purified enzyme has been subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and has been observed by most investigators to contain two classes of polypeptides. The mobilities of these species correspond to apparent molecular weights of 95 000–105 000 and 45 000–55 000 when compared with water-soluble proteins. We shall use the convention of referring to these two polypeptides as α and β, respectively.

This paper reports the solubilization and purification of an active (Na^+,K^+)ATPase from the rectal gland of *Carcharhinus obscurus*, the molecular weight of this complex particle, and the stoichiometry and molecular weights of the polypeptide components.

Materials and Methods

All reagents except those specified were standard reagent grade. Sodium dodecyl sulfate was obtained from Gallard-Schlessinger as "specially pure" grade. Sodium dodecyl [^{35}S]sulfate was supplied by Amersham/Searle. D_2O from Bio-Rad Laboratories was used with the precaution of periodic density measurements to guard against possible contamination by atmospheric H_2O.

Protein concentrations were determined using a modified Lowry procedure of Bensdoun & Weinstein (1976). Organic phosphate was measured according to Bartlett (1959). ATPase and pNPPase activities were determined as previously described by Ottolenghi (1975) using the procedure of Baginski et al. (1967) for the measurement of inorganic phosphate.

Polyacrylamide gel electrophoresis was carried out according to Weber & Osborne (1969) using Bio-Rad precast 7.5% gels and a modified buffer system containing 0.1% sodium dodecyl sulfate, 50 mM phosphate, pH 7.2. Staining and destaining conditions and times were identical for the purified polypeptides and those obtained from the native enzyme. Gels were scanned at 560 nm and peak areas integrated by planimetry.

Solubilization of an active enzyme was accomplished by titration of the enzyme containing vesicles at a concentration of 0.2 mg/mL of protein with increasing concentrations of Lubrol WX in a buffer containing 3 M glycerol, 0.5 mM EDTA, 20 mM recrystallized imidazole, pH 7.4. The solution was allowed to stand for 30 min and activity measured on the total mixture. The solution was then centrifuged at 100000*g* for 1 h and the activity of the supernatant determined as a

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[1] Abbreviations used: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; pNPPase, *p*-nitrophenylphosphatase.

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