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# Conformational Differences between High Clotting Human α-Thrombin and Nonclotting γ-Thrombin†

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ABSTRACT: The conformations of human α-thrombin and γ-thrombin have been compared by circular dichroism, solvent perturbation difference spectroscopy, and chemical modification. Circular dichroism studies indicate that proteolytic conversion of α-thrombin to γ-thrombin is accompanied by considerable conformational changes which include a decrease in α-helical content from 5–7% to 0–1%. Solvent perturbation at pH 6.0 obtained with 20% ethylene glycol, 20% glycerol, and 20% dimethyl sulfoxide indicates an apparent exposure of 3.5 ± 0.2 tryptophan and 7.8 ± 0.1 tyrosine residues in α-thrombin and 4.6 ± 0.2 tryptophan and 9.2 ± 0.3 tyrosine residues in γ-thrombin. This increased exposure is substantiated by the greater reactivity of tryptophan residues in γ-thrombin toward dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide. It suggests that γ-thrombin is a less compact molecule than the parent α-thrombin. Solvent perturbation studies of α-thrombin and γ-thrombin inhibited by phenylmethanesulfonyl fluoride showed that 0.3 ± 0.4 tryptophan and 0.9 ± 0.3 tyrosine residues in α-thrombin and 0.6 ± 0.3 tryptophan and 1.3 ± 0.4 tyrosine residues in γ-thrombin were blocked by the inhibitor. These subtle differences in the extent of blocking of tyrosine and tryptophan suggest a tighter conformation in the catalytic site of γ-thrombin compared to that of α-thrombin.

It is known that multiple forms of thrombin with vastly differing specific activities toward protein and synthetic substrates can be isolated from activation mixtures of crude prothrombin (Mann et al., 1972; Seegers et al., 1968; Rosenberg & Waugh, 1970). Human α-thrombin, the form that is responsible for the clotting of blood, is a molecule composed of two polypeptide chains linked by a single disulfide bond (Thompson et al., 1977). This form of thrombin can be converted by autolysis or limited proteolysis to β-thrombin and subsequently to γ-thrombin. γ-Thrombin is characterized by a drastically reduced ability to clot fibrinogen, but its activity toward synthetic substrates and its ability to bind to antithrombin III are not significantly changed (Chang et al., 1979). In addition, γ-thrombin has retained its ability to activate factor XIII (Lorand & Credo, 1977) but has reduced ability to stimulate platelets (Charo et al., 1977).

Although the physiological significance of this form of thrombin is not known, γ-thrombin is an interesting model to study the active conformation of thrombin because proteolytic conversion of α-thrombin to γ-thrombin results in cleavage of the B chain at two places. On the basis of sequence homology with pancreatic proteases, this fragmentation separates the functional residues which are expected to participate in the charge relay system (His-57, Ser-195, and Asp-102). γ-Thrombin, however, retains these fragments through noncovalent association since complete sepration of the B-chain fragments could only be shown when subjected to dissociating conditions, i.e., reduction, urea, and $NaDodSO_4$[1] (Lundblad et al., 1979; Fenton et al., 1979).

The observations that the kinetic parameters (measured by proflavin displacement) of α-thrombin and γ-thrombin show no significant differences and have essentially identical behavior with ester substrate (Chang et al., 1979) have led to the suggestion that the active site and neighboring regions are unchanged by the α- to γ-thrombin conversion. Hence, it is believed that the loss of clotting activity of γ-thrombin during proteolysis must be related to as yet unexplained structural changes which are remote from the active site. Although reports have implicated conformational differences between these two forms because of different stabilities under denaturing conditions (Bauer et al., 1980), there is no direct experimental evidence, and the nature and extent of these differences are largely unknown.

In the present study, we have used solvent perturbation difference spectroscopy, circular dichroism, and chemical modification to investigate the conformational aspects of α-thrombin and γ-thrombin. The data presented here provide the first direct experimental proof of conformational differences between the two forms of thrombin in their native states.

## Materials and Methods

*Materials.* Highly purified α-thrombin and γ-thrombin were generous gifts of Dr. John Fenton II, New York State Department of Health, Albany, NY. The enzyme purity ($NaDodSO_4$–gel electrophoresis), specific activities (clotting and esterase), and active-site concentration (NPGB titration) were done in Albany and checked in our laboratory. Two α-thrombin preparations were used with the following enzymatic properties: 3153 and 3005 (NIH) clotting units/mg; 96.7% and 93.9% active by NPGB titration; 99.5% α, 0.5% β, 0.0% γ and 95.5% α, 0.6% β, 3.9% γ. Two γ-thrombin preparations were used with the following enzymatic properties: 0.74 and 0.31 (NIH) clotting units/mg; 65.1% and

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; PMSF, phenylmethanesulfonyl fluoride; PMS-α-thrombin, phenylmethanesulfonyl-α-thrombin; PMS-γ-thrombin, phenylmethanesulfonyl-γ-thrombin; tosyl-Lys-$CH_2Cl$, 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride; Tos-Arg-OMe, tosyl-L-arginine methyl ester; $NaDodSO_4$, sodium dodecyl sulfate.

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68.9% active by NPGB titration; 0.0% $\alpha$, 2.5% $\beta$, 97.5% $\gamma$ and 0.0% $\alpha$, 2.4% $\beta$, 97.6% $\gamma$.[2] Phenylmethanesulfonyl fluoride was purchased from Sigma Chemical Co., and dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide was from Calbiochem. *p*-Nitrophenyl *p'*-guanidinobenzoate hydrochloride (NPGB) was purchased from ICN Pharmaceuticals, and tosylarginine methyl ester (Tos-Arg-OMe) was from Schwarz/Mann Biochemical Co. Fibrinogen of bovine origin was purchased from Calbiochem and purified by the method of Blomback & Blomback (1956). All other reagents and solvents used were of the purest commercially available quality.

*Ultracentrifugal Measurements.* Sedimentation velocity experiments were performed at 20 °C with a Beckman Model E analytical ultracentrifuge. Double sector cells with a 12-mm optical path and quartz window were used. The speed of centrifugation was 47 660 rpm, and the progress of the sedimenting boundary was followed by using the schlieren optical system with exposure at 16-min intervals. PMS-thrombin was dissolved in 0.5M NaCl and 0.01M Mes, pH 6.0, at concentrations ranging from 3 to 5 mg/mL. Final corrections for the sedimentation coefficient were made in the customary manner (Schachman, 1957), to a solvent with density and viscosity of water at 20 °C.

The sedimentation equilibrium data were obtained by using the high-speed technique of Yphantis (1964). PMS-thrombin was dialyzed overnight against 0.5M NaCl and 0.01M Mes, pH 6.0, and centrifuged at a rotor speed of 23 150 rpm for 18 h. A partial specific volume $\bar{v}$ of 0.732 mL/g (Fenton et al., 1977) and a solvent density $\rho$ of 1.0197 g/mL were used.

The extinction coefficient of thrombin was determined by differential refractomery by using the analytical ultracentrifuge according to the procedure of Babul & Stellwagen (1969). The protein concentration ranged from 1 to 1.5 mg/mL, and a rotor speed of 10 000 rpm was used to form the solvent–protein boundary. The number of interference fringes observed for each protein solution was converted to milligrams per milliliter by using the average refractive increment of 4.1 fringes $mg^{-1}$ $mL^{-1}$ (Babul & Stellwagen, 1969).

*Solvent Perturbation Difference Spectroscopy.* Difference spectral measurement were made on a Cary 15 recording spectrophotometer equipped with a 10-fold scale expander. The automatic slit width was used, and the dynode voltage was set at 2 and slit control at 20. The difference spectral method and preparation of solutions were previously described (Herskovits & Sorensen, 1968a,b). Protein solutions containing 0% and 20% perturbants were prepared by 1:1 dilution of stock protein solutions in 0.5 M NaCl and 0.01 M Mes, pH 6.0, with the same buffer and with 40% perturbant. Buffer blanks containing 0% and 20% perturbant were prepared in the same manner. The protein absorbance at 280 nm of the final mixture ranged from 1.0 to 1.5. Each spectrum was recorded 4 times during a time period of approximately 30 min, and only those runs with identical raw traces were used. The reported spectra were averages of four to five determinations by using at least two different samples of each protein. The difference in $\Delta E$ values obtained for a given protein with a given perturbant were within the experimental uncertainties of difference spectral methods (±7%). The pH of each solution was measured after recording the spectra, and the values between solutions did not differ by more than 0.1 pH unit. There were no indications of conformational alteration in the proteins due to the presence of any of the three perturbants used (ethylene glycol, glycerol, and dimethyl sulfoxide) as shown by circular dichroism (see text).

*Circular Dichroism.* Circular dichroism spectra were obtained in a Cary 60 recording spectropolarimeter equipped with a Model 6002 circular dichroism attachment. The instrument was routinely calibrated with *d*-10-camphorsulfonic acid. All spectra were taken at 25 °C. Cylindrical cells with optical path length of 0.1 and 5.0 cm were used to measure the circular dichroism in the 200–250-nm region and 250–350-nm region, respectively. Protein concentrations ranged from 0.02% to 0.025%. The CD data was expressed as mean residue ellipticity, $[\theta]$, in degree centimeter squared per decimole on the basis of the mean residue weight of 112 for thrombin (Thompson et al., 1977).

*Reaction with Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Bromide.* The reaction of thrombin with this reagent was carried out essentially as described by Horton & Tucker (1970). Thrombin at a concentration of 1 mg/mL was dissolved in a buffer containing 0.5M NaCl and 0.01M Mes, pH 6.0. An aliquot of freshly prepared 2 mM solution of the reagent in water was added to the protein to give the desired molar ratio of the protein and reagent. The reaction mixture was incubated in the dark for 1 h at room temperature and the modified protein separated from excess reagent on a Sephadex G-25 (1.0 × 23 cm) column which was previously equilibrated with the same buffer. The extent of tryptophan modification was quantitated spectrophotometrically at pH 12 by using the extinction coefficient $\epsilon$ = 18 000 $M^{-1}$ $cm^{-1}$ at 410 nm (Barman & Koshland, 1967). A Beckman 120C amino acid analyzer was used to determine if modification of amino acids other than tryptophan residues has occurred. Hydrolysis was carried out under nitrogen atmosphere in a sealed tube at 110 °C for 24 h in 6 N HCl.

## Results

*Ultracentrifugal Measurements.* Before meaningful spectroscopic studies of the conformations of $\alpha$-thrombin and $\gamma$-thrombin can be made, it is necessary to demonstrate whether the noncovalently associated fragments of $\gamma$-thrombin remain intact under the condition of the experiments (0.5 M NaCl and 0.01 M Mes, pH 6.0). In addition, it is important to show that the absorption coefficients did not change during the proteolytic fragmentation of $\alpha$-thrombin to $\gamma$-thrombin.

The sedimentation equilibrium data obtained in our laboratory showed that the molecular weight, $M_r$, of $\alpha$-thrombin and $\gamma$-thrombin used in this study were 36 900 ± 1700 and 36 000 ± 1600, respectively. Within the limit of experimental error, these values were fairly close and agreed substantially with the reported value of 36 000 (Fenton et al., 1977). The sedimentation velocity experiments showed that both $\alpha$-thrombin and $\gamma$-thrombin sediment as a single component with $s_{20,w}$ values of 3.25 ± 0.16 and 3.37 ± 0.16 ($I$ = 0.5 M), respectively. These values were slightly lower than the value of 3.55 for $s^0_{20,w}$ reported by Lanchantin et al. (1967).

The absorption coefficients at 280 nm determined by differential refractometry in the analytical ultracentrifuge were found to be $\epsilon_{1\%}$ = 18.5 ± 0.6 for $\alpha$-thrombin and $\epsilon_{1\%}$ = 18.2 ± 0.5 for $\gamma$-thrombin. These values were comparable to the

[2] The $\gamma$-thrombin used was derived from purified $\alpha$-thrombin by controlled proteolysis in immobilized trypsin (Fenton et al., 1979). In addition to the cleavage which yields $\beta$-thrombin, $\gamma$-thrombin has a cleavage site tentatively located at Lys-154. Since in bovine thrombin this residue is replaced by Glu-154, human $\gamma$-thrombin has no bovine counterpart. The [$^{14}$C]DFP-labeled Ser-205 fragments of trypsin-proteolyzed $\beta$ and $\gamma$ forms coelectrophorese in $NaDodSO_4$–polyacrylamide gel with the corresponding autolytic forms (Fenton et al., 1979), implying that the $B_2$ and $B_4$ fragments are practically the same. This suggests that although the proteolytic cleavage may be different from the autolytic cleavage site, trypsin proteolysis did not yield products of varying degrees of fragmentation.

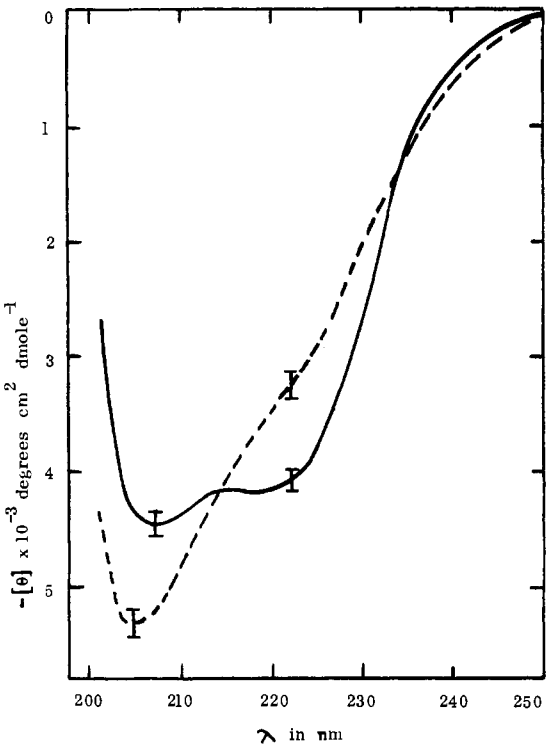


FIGURE 1: Circular dichroism spectra of α-thrombin and γ-thrombin in the far-ultraviolet region. α-Thrombin (solid line) and γ-thrombin (dashed line). Solvent: 0.5 M NaCl and 0.01 M Mes, pH 6.0. Protein concentration ranged from 0.02% to 0.025%.

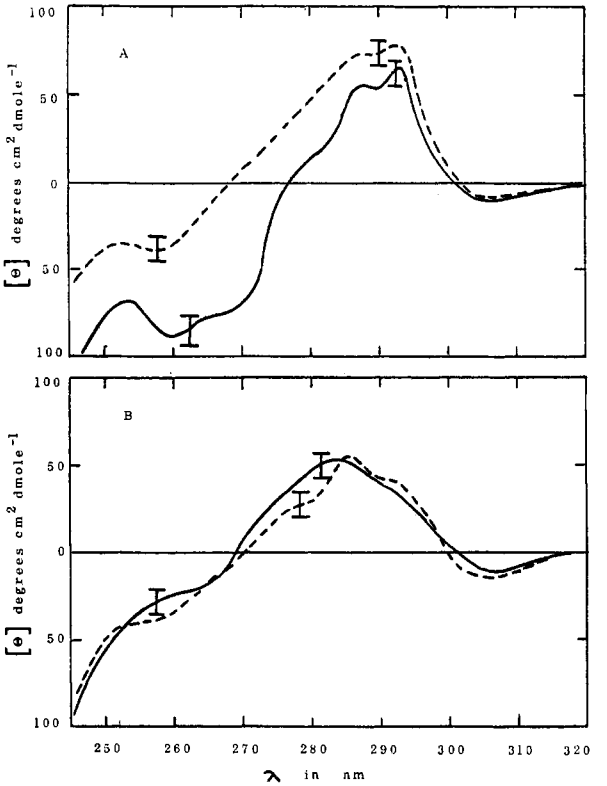


FIGURE 2: Effect of PMSF inhibition on the aromatic circular dichroism spectra of α-thrombin and γ-thrombin. (A) α-Thrombin (—); γ-thrombin (---). (B) PMS-α-thrombin (—); PMS-γ-thrombin (---). All conditions were the same as in Figure 1.

reported literature value of 18.3 (Fenton et al., 1977). On the basis of the above studies, an absorption coefficient $\epsilon_{1\%}$ of 18.3 and a molecular weight of 36 500 were used for both α-thrombin and γ-thrombin.

*Circular Dichroism Studies.* The circular dichroism spectra of α-thrombin and γ-thrombin in the region of the amide bond transition are shown in Figure 1. These spectra are averages of four to five determinations by using different stock solutions. Due to reduced signal to noise ratio below 200 nm, it was not possible to extend the measurements far into the ultraviolet region. It is seen, however, that within the narrow spectral range in which accurate determination can be made, the two spectra vary both in shape and intensity. In α-thrombin, there is a pronounced band at 222 nm, whereas in γ-thrombin, it is reduced to a less resolved shoulder which is accompanied by intensification of the band at the 205–208-nm region. The mean residue ellipticities, $[\theta]$, of α-thrombin at 222 and 205–207 nm are 4400 ± 200 and 4500 ± 200 deg cm$^2$ dmol$^{-1}$, respectively. The corresponding values of γ-thrombin at these wavelengths are 3500 ± 150 and 5500 ± 200 deg cm$^2$ dmol$^{-1}$, respectively. When α-thrombin is curved fitted on the basis of the reference proteins (Chen et al., 1972), it is found to contain 5–6% α helix, 14–15% β structure, and 79–80% unordered structure. γ-Thrombin is found to contain 0–1% α helix, 15–16% β structure, and the rest in an unordered structure.

The ellipticity reported here for human α-thrombin is slightly higher than that reported elsewhere for the bovine enzyme (Villanueva et al., 1975). This is probably due to the difference in ionic strength (0.5 vs. 0.125 M) which has been demonstrated to have significant effect on the structure and activity of thrombin (Orthner & Kosow, 1980; Landis et al., 1981).

Figure 2A presents the circular dichroism spectra of α-thrombin and γ-thrombin in the region of the aromatic absorption. The spectrum of α-thrombin is characterized by two major positive bands at 294 and 288 nm and a less resolved positive shoulder at 278 nm. These bands have mean residue ellipticities, $[\theta]$, of 60 ± 5, 50 ± 5, and 15 ± 5 deg cm$^2$ dmol$^{-1}$, respectively. It has a crossover at 276 nm and two well-defined negative bands at 260 and 268 nm corresponding to 90 ± 10 and 80 ± 10 deg cm$^2$ dmol$^{-1}$, respectively. γ-Thrombin exhibits slightly higher positive bands at 294 and 288 nm corresponding to 80 ± 5 and 70 ± 5 deg cm$^2$ dmol$^{-1}$. It has a crossover at 268 nm and a weak and less-resolved negative band at 258 nm corresponding to 40 ± 5 deg cm$^2$ dmol$^{-1}$. In addition, both forms of thrombin consistently show a negative trough at 305 nm corresponding to 15 ± 5 deg cm$^2$ dmol$^{-1}$. Repeated measurements have shown these bands and crossovers to be quite reproducible. These indicate differences in the relative degrees of assymmetry in the environments of tryptophan, tyrosine, and phenylalanine residues.

The CD spectra of PMS-α-thrombin and PMS-γ-thrombin were also investigated. The spectra of the inhibited enzymes in the far-ultraviolet peptide region were almost indistinguishable from those of the native enzymes. However, the spectra of the inhibited enzymes in the aromatic absorption region were considerably different from those of the native enzymes. This is shown in Figure 2B. They are characterized by a positive band centered at 285 nm and two less resolved shoulders at 293 and 275 nm. The ellipticities at these wavelengths correspond to 50 ± 5, 40 ± 5, and 45 ± 5 deg cm$^2$ dmol$^{-1}$, respectively. They have the same crossover at 269–271 nm. It is interesting to note that the apparently different circular dichroism of native α-thrombin and γ-thrombin (see Figure 2A) appears to have been normalized to almost identical spectra in the inhibited enzymes.

Since the enzyme conformation may be altered by the perturbants used in this study, the CD spectra of the proteins were examined as a function of perturbant concentration. The data on Figure 3 indicates that neither the ellipticity in the

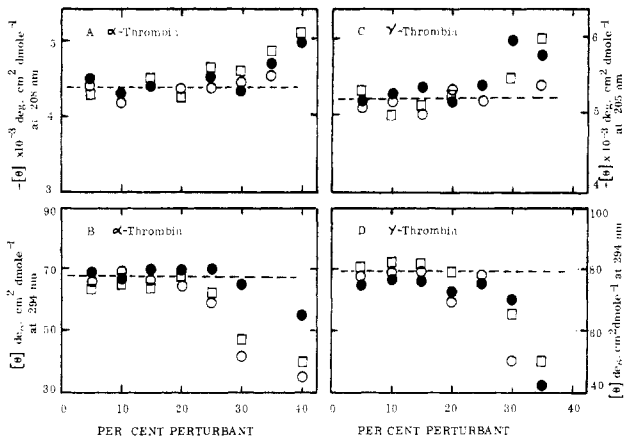


FIGURE 3: Effects of perturbants on the CD parameters of $\alpha$-thrombin and $\gamma$-thrombin. (A) Ellipticities of $\alpha$-thrombin at 208 nm. (B) Ellipticities of $\alpha$-thrombin at 294 nm. (C) Ellipticities of $\gamma$-thrombin at 205 nm. (D) Ellipticities of $\gamma$-thrombin at 294 nm. (O) Ethylene glycol; (●) glycerol; (□) dimethyl sulfoxide.

peptide region nor the ellipticity in the aromatic region is adversely affected at the levels of 20% ethylene glycol, 20% dimethyl sulfoxide, and 20% glycerol used in the perturbation experiments.

*Solvent Perturbation Studies.* Figure 4 shows a comparison of the original tracings of the difference spectra of $\alpha$-thrombin and $\gamma$-thrombin obtained in 20% ethylene glycol and 20% dimethyl sulfoxide at identical protein concentrations. The difference spectra of $\alpha$-thrombin are characterized by three well-resolved bands at 291, 285, and 280 nm. The first band is characteristic of tryptophan perturbation whereas the other two are the first and second peaks due to tyrosine perturbation. The difference spectral maxima of $\gamma$-thrombin at 291 and 285 nm in 20% dimethyl sulfoxide are about 20% greater than those of $\alpha$-thrombin. Similarly, these spectral maxima in 20% ethylene glycol are about 25% greater in $\gamma$-thrombin. In addition, the second tyrosine peak at 280 nm is a well-defined shoulder in $\alpha$-thrombin whereas in $\gamma$-thrombin it is virtually lost. This is consistent for all the perturbants used including 20% glycerol. It has been reported that this second tyrosine peak is very sensitive to instrumental setting and experimental conditions, i.e., dynode voltage, slit width, and absorbance (Herskovits & Sorensen, 1968a). Since the two protein solutions were run at identical instrumental settings and at absorbance not exceeding 1.5, it is reasonable to suggest that this is a reflection of the difference in the microenvironment of the tyrosine residues in the two proteins.

The data was analyzed by using the procedure of Herskovits & Sorensen (1968a,b) to obtain quantitative information. Estimates of the apparent number of exposed tryptophan and tyrosine residues were obtained by the best fit to the protein curves by

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

where $a$ represents the number of exposed tryptophan residues and $b$ the number of exposed tyrosine residues. The $\Delta\epsilon_\lambda$(Trp) and $\Delta\epsilon_\lambda$(Tyr), respectively, are the molar absorptivitiy differences of model compounds of tryptophan and tyrosine as a function of wavelength. The problems that pertain to curve fitting and interpretation of protein difference spectra have been fully described (Herskovits & Sorensen, 1968 a,b; Villanueva & Herskovits, 1971). The loss and obliteration of difference spectral detail commonly observed with protein curves (i.e., shallow minima at 288–289 nm) have been attributed to the heterogeneity of the environment of surface residues in proteins as compared to the homogeneous environment of model the compound in solution. The poor fit of the protein curves below 280 nm is due to phenylalanine perturbation in the protein spectra which is not taken into account in the model spectra. Figure 5 shows the perturbation difference spectra of native and urea-denatured $\alpha$-thrombin in 20% ethylene glycol. In native $\alpha$-thrombin, the best fit curve (dotted line) corresponds to 3.7 tryptophan and 7.8 tyrosine residues exposed. In 8 M urea, the apparent number of exposed tryptophan and tryosine residues are found to be 6.5 and 9.5, respectively. Further reduction in the presence of 2-mercaptoethanol results in exposure of one additional tyrosine, but the extent of tryptophan exposure remains unchanged. Since human thrombin is known to contain 9 tryptophan and 10 tyrosine residues (Thompson et al., 1977), it is evident that even in 8 M urea about 2–3 tryptophan residues are still inaccessible to the solvent. This suggests that residual secondary structure is probably still present even under this condition and may partly explain the reported ability of thrombin to refold after exposure to the denaturing condition with complete recovery of catalytic activities (Chang et al., 1980). Table I presents a summary of the perturbation data of $\alpha$-thrombin and $\gamma$-thrombin. The last two columns in this table are estimates of the apparent number of exposed tryp-

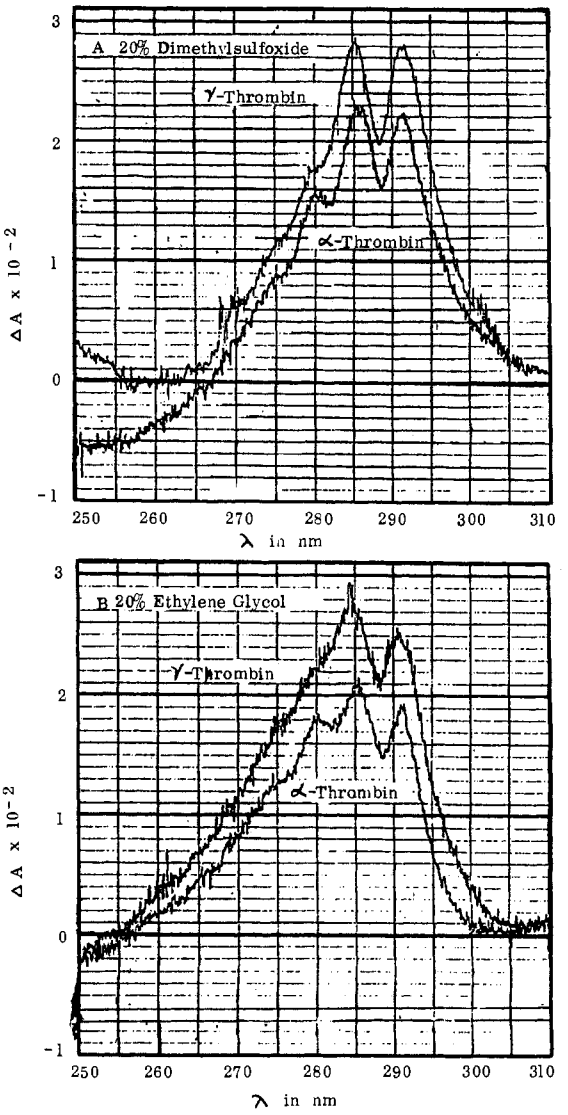


FIGURE 4: Original tracings of the solvent perturbation difference spectra of $\alpha$-thrombin and $\gamma$-thrombin in 20% ethylene glycol and 20% dimethyl sulfoxide. The proteins have identical concentrations which are $1.1 \times 10^{-5}$ M in (A) and $1.53 \times 10^{-5}$ M in (B). Solvent used: 0.5 M NaCl and 0.01 M Mes, pH 6.0.

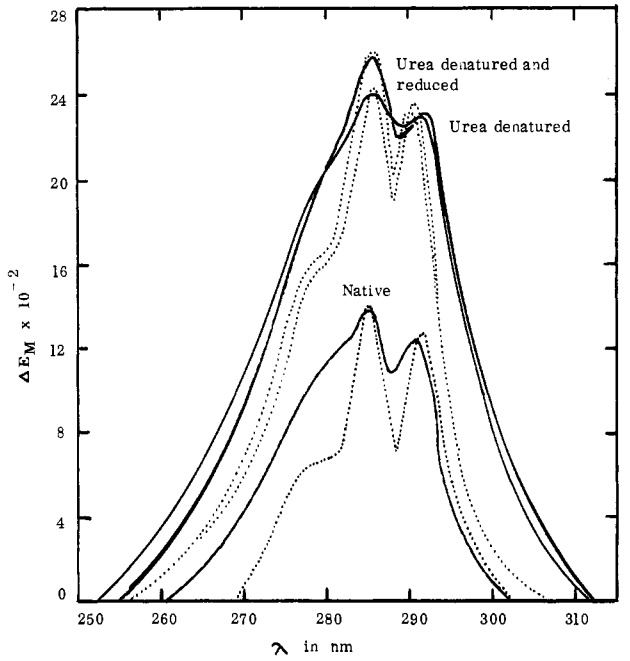


FIGURE 5: Effects of urea denaturation and reduction on the solvent perturbation difference spectra of α-thrombin obtained with 20% ethylene glycol as perturbant. The solid lines represent the spectra obtained with native α-thrombin (0.5 M NaCl and 0.01 M Mes, pH 6.0), denatured α-thrombin (8 M urea), and denatured disulfide-cleaved α-thrombin (8 M urea and 0.02 M mercaptoethanol). The dotted lines represent the calculated curves giving the best fit of the data. They correspond to 3.7 Trp and 7.8 Tyr for native α-thrombin, 6.5 Trp and 9.5 Tyr for the denatured α-thrombin, and 6.5 Trp and 10.5 Tyr for the denatured and disulfide-cleaved α-thrombin.

Table I: Difference Spectral Parameters of α-Thrombin and γ-Thrombin[a]

| perturbants (20%) | molar absorptivity difference[b] $\Delta\epsilon_M$, 291–292 nm | $\Delta\epsilon_M$, 286–285 nm | app no. of exposed residues[c] Trp | Tyr |
|---|---|---|---|---|
| | α-Thrombin | | | |
| dimethyl sulfoxide | 2100 | 2272 | 3.6 | 7.8 |
| ethylene glycol | 1258 | 1375 | 3.7 | 7.8 |
| glycerol | 1011 | 1222 | 3.3 | 7.7 |
| | | | 3.5 ± 0.2[d] | 7.8 ± 0.1[d] |
| | γ-Thrombin | | | |
| dimethyl sulfoxide | 2642 | 2600 | 4.7 | 9.0 |
| ethylene glycol | 1606 | 1770 | 4.8 | 9.3 |
| glycerol | 1433 | 1575 | 4.4 | 9.0 |
| | | | 4.6 ± 0.2[d] | 9.2 ± 0.3[d] |

[a] Solvent: 0.5 M NaCl and 0.01 M Mes, pH 6.0. [b] Average of four to five determinations. [c] Calculated from the best fit of the theoretical curve using eq 1. [d] Average exposure.

tophan and tyrosine residues based on the best fit of the data. The data obtained with α-thrombin using three perturbants gave an average exposure of 3.5 ± 0.2 tryptophan and 7.8 ± 0.1 tyrosine residues exposed. The average exposure obtained with γ-thrombin were 4.6 ± 0.2 tryptophan and 9.2 ± 0.3 tyrosine residues. Subtraction of these values gives 1.1 tryptophan and 1.4 tyrosine residues or their equivalents. These represent the average increase in exposure of the aromatic chromophores when α-thrombin is converted to γ-thrombin. It should be emphasized that although solvent perturbation is an acceptable measure of solvent accessibilites of aromatic residues in proteins, they are not without pitfalls, and the data obtained, especially the absolute numerical values of solvent exposure, should be interpreted with caution. These values

Table II: Effects of Tryptophan Modification on α-Thrombin and γ-Thrombin by Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Bromide[a]

| molar excess of reagent | no. of tryptophan modified | clotting act. (%) | esterase act. (%) |
|---|---|---|---|
| | α-Thrombin | | |
| 15 | 0.6 | 60 | 92 |
| 25 | 1.0 | 54 | 86 |
| 50 | 1.7 | 31 | 57 |
| 75 | 3.2 | 27 | 45 |
| 100 | 5.8 | 20 | 51 |
| | γ-Thrombin | | |
| 15 | 1.4 | | 95 |
| 25 | 2.1 | | 87 |
| 50 | 2.7 | | 66 |
| 75 | 5.7 | | 54 |
| 100 | 6.9 | | 43 |

[a] Solvent: 0.5 M NaCl and 0.01 M Mes, pH 6.0.

generally overestimate solvent accessibilities due to spectral contributions from inaccessible surface residues (Kosen et al., 1980). The absolute numerical values of solvent exposure are not that significant in the present study. It is, rather, the comparative differences of the relative degrees of solvent accessibilities of these chromophores that is of prime importance.

*Chemical Modification Studies.* The increased exposure of tryptophan and tyrosine residues in γ-thrombin relative to α-thrombin, as detected by solvent perturbation, could be due to differential effect of the perturbants used. This is possible because the presence of internal cleavages in γ-thrombin could impart a relatively loose structure which could unfold easily when exposed to the perturbing influence of the solvent. So that this possibility could be checked, the chemical reactivity of tryptophan residues in both α-thrombin and γ-thrombin were investigated by using dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide. Table II shows the effect of increasing the molar excess of the reagent. It is obvious that even under relatively mild condition (15 molar excess), where the esterase activity is still intact, differences in reactivity between the two forms of thrombin already exist which are greatly enhanced at higher molar excess. At less than 15 molar excess, when greater than 95% of the esterase activity is still remaining, one tryptophan residue in γ-thrombin is already modified. In α-thrombin, it requires about 25 molar excess of the reagent to modify one tryptophan residue, and this is accompanied by a drop in esterase activity to 86% and the clotting activity to 54% of the control. No changes in the recovery of the other amino acid residues are detected with up to 75 molar excess of the reagent. However, modification above 100 molar excess results in destruction of methionine and tyrosine residues. This greater reactivity of tryptophan in γ-thrombin indicates that the increased solvent accessibility of this chromophore as detected by solvent perturbation is due to its more exposed state rather than to any nonspecific effect of the perturbants used.

The lower molar excess of the reagent required to modify a tryptophan residue in the present study, compared to similar studies on bovine α-thrombin (Uhteg & Lundblad, 1977), is probably not due to species differences but rather because of the different pH and reagent used. In their study, 2-hydroxy-5-nitrobenzyl bromide was used at pH 4. Preliminary studies by this author have demonstrated a pH-dependent conformational change of thrombin even within a narrow range of pH 6 to 7.5. This is the subject of a succeeding paper (G. B. Villanueva and J. Fenton II, unpublished results).

Table III: Difference Spectral Parameters of PMS-α-thrombin and PMS-γ-thrombin[a]

| perturbants (20%) | molar absorptivity difference $\Delta\epsilon_M$, 291–292 nm | $\Delta\epsilon_M$, 284–285 nm | app no. of exposed residues Trp | Tyr | changes in molar absorptivity due to PMSF inhibition[b] $\Delta\epsilon_M$, 291–292 nm | $\Delta\epsilon_M$, 284–285 nm | app no. of groups buried due to PMSF inhibition[b] Trp | Tyr |
|---|---|---|---|---|---|---|---|---|
| | | | PMS-α-thrombin | | | | | |
| dimethyl sulfoxide | 2038 | 2046 | 3.5 | 6.8 | −62 | −226 | 0.1 | 1.0 |
| ethylene glycol | 1190 | 1320 | 3.2 | 7.1 | −68 | −55 | 0.5 | 0.6 |
| glycerol | 995 | 1145 | 3.0 | 7.0 | −16 | −77 | 0.3 | 0.7 |
| | | | 3.2 ± 0.3[d] | 6.9 ± 0.2[d] | | | 0.3 ± 0.4[d] | 0.9 ± 0.3[d] |
| | | | PMS-γ-thrombin | | | | | |
| dimethyl sulfoxide | 2386 | 2252 | 4.1 | 7.5 | −256 | −348 | 0.6 | 1.5 |
| ethylene glycol | 1395 | 1485 | 4.1 | 7.8 | −2.11 | −275 | 0.7 | 1.5 |
| glycerol | 1300 | 1385 | 3.8 | 8.1 | −133 | −190 | 0.6 | 0.9 |
| | | | 4.0 ± 0.3[d] | 7.8 ± 0.3[d] | | | 0.6 ± 0.3 | 1.3 ± 0.4 |

[a] Solvent: 0.5 M NaCl and 0.01 M Mes, pH 6.0. [b] Differences in $\Delta\epsilon_M$ values between thrombin (see Table I, columns 2 and 3) and PMS-thrombin. [c] Differences in exposure between thrombin (see Table I, columns 4 and 5) and PMS-thrombin. [d] Average.

*Inhibition Studies.* Solvent perturbation studies were extended to the proteins inhibited by phenylmethanesulfonyl fluoride in order to gain insights into the structural features of the active center of thrombin. Table III summarizes the solvent perturbation difference spectral data of PMS-α-thrombin and PMS-γ-thrombin at the two spectral maxima. The data indicate that PMS-α-thrombin has an average of 3.2 tryptophan and 6.9 tyrosine residues exposed whereas PMS-γ-thrombin shows an average of 4.0 tryptophan and 7.8 tyrosine residues exposed (Table III, columns 4 and 5). Manual subtraction of the $\Delta\epsilon_M$ values of the inhibited enzymes (Table III, columns 2 and 3) from those of the native unmodified forms (Table I) provides a measure of the burial of these chromophores due to the presence of the phenylmethanesulfonyl group in the active site of the enzyme. These values are shown in columns 6 and 7 of Table III.

The $\Delta\epsilon_M$ value of free tryptophan at 291–293-nm tryptophan maximum obtained with the three perturbants used in this study (ethylene glycol, glycerol, and dimethyl sulfoxide) ranges from 304 to 489 (Herskovitis & Sorensen, 1968a,b). Thus, the observed decrease in $\Delta\epsilon_M$ of PMS-α-thrombin relative to α-thrombin ranging from −16 to −68 represents a burial of about 0.1 to 0.3 tryptophan and that of PMS-γ-thrombin relative to γ-thrombin ranging from −133 to −256 represents a burial of 0.6 to 0.7 tryptophan. This assumes that the contribution of exposed tyrosines can be neglected at this wavelength. When both tryptophan and tyrosine contributions are taken into account, about the same estimates of tryptophan burial are obtained. By following a similar method of analysis at the 284–285-nm tyrosine maximum and after correction for tryptophan contribution at this wavelength, it is found that the average tyrosine burial due to phenylmethanesulfonyl inhibition ranges from 0.6 to 1.0 tyrosine in α-thrombin and from 0.9 to 1.5 tyrosine residues in γ-thrombin. Thus, on the average, the apparent number of buried tryptophan and tyrosine residues due to phenylmethanesulfonyl fluoride inhibition is 0.2 ± 0.4 tryptophan and 0.9 ± 0.3 tyrosine in α-thrombin and 0.6 ± 0.3 tryptophan and 1.3 ± 0.4 tyrosine in γ-thrombin.

## Discussion

The results of spectroscopic studies demonstrate that proteolytic fragmentation of α-thrombin and γ-thrombin is accompanied by considerable conformational changes. These changes are characterized by a drop in α-helical content of α-thrombin from 5–7% to 0–1% and suggest that a small helical region in the protein may be essential to the clotting activity since γ-thrombin has virtually no clotting activity. It should, however, be noted that the changes observed here are a minimum value since a large fraction of the molecule appears to be in random conformation and any change from one random conformation to another is not detected by the method employed. Thus, it is also possible that the loss of a helical region is secondary to a major configurational change that directly affects the clotting mechanism.

Investigation of the states of tryptophan and tyrosine residues by solvent perturbation indicates that approximately one tryptophan and one tyrosine residues become exposed as a consequence of the proteolytic conversion of α-thrombin to γ-thrombin. This is in accord with the observed increase in reactivity of tryptophan residues toward dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide. It should be emphasized, however, that this is merely a qualitative agreement. Attempts to equate increased reactivity with increased exposure in quantitative terms should be interpreted with caution because there is a need to take into account the effect of local environment on the spectra of modified residues in proteins (Myers & Glazer, 1971). The quantitative agreement of these results is probably coincidental. Since the sedimentation coefficient, $s_{20,w}$, appears to have increased slightly from 3.25 ± 0.16 to 3.37 ± 0.16, these results suggest that γ-thrombin is a less compact molecule than the parent α-thrombin.

The results of solvent perturbation studies on PMS-α-thrombin and PMS-γ-thrombin provide an interesting observation. The data in Table III indicate that PMSF inhibition in both enzyme forms results in the burial of a tyrosine and a fraction of tryptophan residue. Since changes in CD spectra during inhibition were observed only in the aromatic side-chain region, this burial could be due solely to a combination of both direct shielding or blocking of those groups from the solvent by the inhibitor and subtle changes involving side-chain reorientation without any effect in the polypeptide backbone. The apparent normalization of the aromatic CD spectra of α-thrombin and γ-thrombin when they are inhibited by PMSF is consistent with the presence of these chromophores in the immediate environment of the active site. This shielding effect is in accord with the observations that pretreatment of thrombin with either tosyl-Lys-$CH_2Cl$ or phenylmethanesulfonyl fluoride resulted in a decreased extent of tryptophan modification by 2-hydroxy-5-nitrobenzyl bromide (Uhteg & Lundblad, 1977) and a decrease in the number of $H_2O_2$-oxidizable tryptophan in thrombin when the reaction is carried out in the presence of benzamidine or diisopropyl fluoro-

phosphate (Villanueva et al., 1975). Similarly, the present results are in agreement with the reduced degree of tyrosine acetylation by *N*-acetylimidazole in DIP-thrombin relative to the native enzyme (Villanueva et al., 1975) and the differential effect of tetranitromethane on the clotting and esterase activities of thrombin (Lundblad & Harrison, 1971). Thus, the present data justifies the conclusion from these previous observations that tyrosine and tryptophan residues are located in the vicinity of the active site.

The extent of burial of tryptophan and tyrosine residues due to inhibition of γ-thrombin by phenylmethanesulfonyl fluoride (0.6–0.7 Trp; 0.9–1.5 Tyr) is within the limits of experimental error, slightly greater than that in α-thrombin (0.1–0.5 Trp; 0.6–1.0 Tyr; Table III). The subtle difference suggests that proteolysis of α-thrombin has a small but significant effect in the immediate vicinity of the active center and indicates a tighter conformation of γ-thrombin compared to α-thrombin in this region. This is consistent with the greater immobilization of certain Ser-205 conjugated spin-labeling reagents in γ-thrombin compared to α-thrombin (Berliner et al., 1981). In that study, they concluded that a portion of the active site of γ-thrombin might be more constrained than those of α-thrombin. The present data are also in accord with the observation that γ-thrombin is a poorer enzyme toward certain synthetic tripeptide chromgenic substrates (Mattler & Bang, 1977). On the other hand, the observation that proflavin displacement and esterase activity do not show significant differences might suggest that the structural changes involving tryptophan and tyrosine residues are not sufficiently close to the area of contact of proflavin and Tos-Arg-OMe. The same argument could explain the similar behavior of bovine α-thrombin and β-thrombin toward dansylarginine (3-ethyl-1,5-pentanediyl)amide (Nesheim et al., 1979).

These subtle changes in the active center, however, may not be sufficient to account for the drastic loss of clotting activity. Our circular dichroism studies indicate that large gross conformational changes which involve the polypeptide backbone accompany the conversion of α-thrombin to γ-thrombin. These suggest that greater structural alterations occur outside the reach of the phenylmethanesulfonyl group, and it may be that the subtle changes observed in the active site are merely the tail of larger conformational changes remote from the active site but drastically affecting fibrinogen binding. Since both forms of the enzyme have identical behavior toward antithrombin III (Chang et al., 1979), the present data suggest that the changes involving the environment around tryptophan and tyrosine residues do not involve the antithrombin III binding region and support the contention for separate binding sites for antithrombin III and fibrinogen.

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