Anion-Binding Exosite of Human α-Thrombin and Fibrin(ogen) Recognition†

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ABSTRACT: Activation of prothrombin to α-thrombin generates not only the catalytic site and associated regions but also an independent site (an exosite) which binds anionic substances, such as Amberlite CG-50 resin [cross-linked poly(methylacrylic acid)]. Like human α-thrombin with high fibrinogen clotting activity (peak elution at $I = 0.40 \pm 0.01$ M, pH 7.4, ~23 °C), catalytically inactivated forms (e.g., *i*-Pr_2P-α- and D-Phe-Pro-Arg-CH_2-α-thrombins) were eluted with only slightly lower salt concentrations ($I = 0.36$–0.39 M), while γ-thrombin with very low clotting activity was eluted with much lower concentrations ($I = 0.29$ M) and the hirudin complex of α-thrombin was not retained by the resin. In a similar manner, hirudin complexes of α-, *i*-Pr_2P-α-, and γ-thrombin were not retained by nonpolymerized fibrin–agarose resin. Moreover, the ionic strengths for the elution from the CG-50 resin of seven thrombin forms were directly correlated with those from the fibrin resin ($y = 0.15 + 0.96x$, $r = 0.95$). In other experiments, the 17 through 27 synthetic peptide of the human fibrinogen Aα chain was not an inhibitor of α-thrombin, while the NH_2-terminal disulfide knot (NDSK) fragment was a simple competitive inhibitor of α-thrombin with a $K_i \sim 3$ μM (0.15 M NaCl, pH 7.3, ~23 °C). These data suggest that α-thrombin recognizes fibrin(ogen) by a negatively charged surface, noncontiguous with the Aα cleavage site but found within the NDSK fragment. Such interaction involving an anion-binding exosite may explain the exceptional specificity of α-thrombin for the Aα cleavage in fibrinogen and α-thrombin incorporation into fibrin clots.

Procoagulant α-thrombin (EC 3.4.21.5) exhibits remarkable proteolytic specificity for cleaving fibrinopeptides A and B in the conversion of fibrinogen into clottable fibrin (Magnusson, 1971). However, neither the Aα nor the Bβ cleavages occur at predictably highly susceptible bonds to α-thrombin on the basis of those of various peptide and protein substrates (Chang, 1985). Rather, the third residue bond in the α-chain (Gly-Pro-Arg–Val) is predictably a highly susceptible bond, whereas that in the β chain (Gly-His-Arg–Pro) is not cleavable. While the third residue bond in the α-chain is only secondarily cleaved by thrombin (Blomback et al., 1967), it is cleaved exclusive of the Aα bond by trypsin (Lewis et al., 1987). Moreover, thrombin preferentially cleaves the two fibrinopeptide A's from fibrinogen to form fibrin I and subsequently the two fibrinopeptide B's to form fibrin II. The former is primarily involved in end-to-end polymerization, whereas the latter is required for later stages of fibril formation and fibrin cross-linking (Blomback et al., 1978).

Human γ-thrombin with very low clotting activity (Bing et al., 1977; Fenton et al., 1977b) releases fibrinopeptide A only slightly faster than fibrinopeptide B in marked contrast to α-thrombin. In comparing γ- to α-thrombin, specificity constants (k_{cat}/K_m)[1] are reduced ~2400- and ~800-fold for fibrinopeptides A and B, respectively, while the constant is reduced only ~5-fold for activating factor XIII (Lewis et al., 1987). This suggests that γ-thrombin has a disrupted fibrin(ogen) recognition exosite, independent of its catalytic site (Fenton, 1981). Consistent with three-dimensional models for thrombin, both the β- and γ-cleavages for forming γ-thrombin are removed from the catalytic site and occur within neighboring clusters of positively charged residues (Fenton, 1986; Fenton & Bing, 1986).

Like ^{125}I-labeled α-thrombin, labeled catalytically inactivated α-thrombin (i.e., ^{125}I-$PhCH_2SO_2$-α-thrombin) binds to fibrin clots (Liu et al., 1979). Furthermore, in the presence of α-thrombin sufficient to induce fibrin formation, either ^{125}I-$PhCH_2SO_2$- or ^{125}I-*i*-Pr_2P-α-thrombin incorporates into fibrin deposits on polystyrene beads to similar extents as ^{125}I-α-thrombin, whereas neither ^{125}I-γ-thrombin nor the hirudin complex of ^{125}I-α-thrombin incorporates (Wilner et al., 1981). These findings indicate that α-thrombin associates with fibrin during the clotting process and does so via an exosite, which is (i) independent of the catalytic site, (ii) lacking in γ-thrombin, and (iii) masked by hirudin. In order to examine the indicated association, we subsequently coupled fibrinogen to CNBr-activated agarose (Heene & Matthias, 1973), converted it with α-thrombin to the nonpolymerized fibrin resin, and found that various modified α-thrombins bound to the resin like the native enzyme, while γ-thrombin bound weakly

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[1] Abbreviations: Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; D-Phe-Pro-Arg-CH_2Cl, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; Fl(CHO), fluorescein carbohydrate conjugate; *i*-Pr_2P-F, diisopropyl phosphorofluoridate; K_a, association constant; k_{cat}, catalytic constant; K_d, dissociation constant; K_i, inhibition constant; K_m, Michaelis-Menten constant; *m*-CP(PBA)-F, *m*-[[*o*-[[2-chloro-5-(fluorosulfonyl)-phenyl]ureido]phenoxy]butoxy]benzamidine; $MeSO_2$-F, methanesulfonyl fluoride; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; $PhCH_2SO_2$-*F*, phenylmethanesulfonyl fluoride; SDM, standard deviation of the mean; Tos-Lys-CH_2Cl, *N*-tosyl-L-lysine chloromethyl ketone. Nomenclature used for thrombin forms follows that of Sonder and Fenton (1984).

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and hirudin complexes did not bind (Fenton et al., 1981). Similar findings were also reported by others (Kaminski & McDonagh, 1983, 1987). We further found that various anionic substances (e.g., ATP and pyrophosphate) eluted α-thrombin from nonpolymerized fibrin resin (Berliner et al., 1985) and that various thrombin forms bound to the negatively charged resin, heparin–agarose (Miller-Andersson et al., 1974), in a manner resembling that of the fibrin resin (Olson et al., 1986). In the present studies, we demonstrate that thrombin binding to Amberlite CG-50 resin [cross-linked poly(methacrylic acid)] directly correlates ($r = 0.95$) with that to nonpolymerized fibrin resin and that the binding is blocked by hirudin. We also found that the first 11-residue homologue of the fibrin α-chain is not an inhibitor, while the fibrinogen NDSK fragment (Blomback et al., 1972) is a competitive inhibitor of α-thrombin. These findings indicate that a multifunctional anion-binding exosite participates in fibrin(ogen) recognition and orients fibrinogen for preferential Aα cleavage.

Experimental Procedures

Materials. The following were gifts: human plasma fraction III paste from Dr. James E. Cavanaugh (Armour Pharmaceutical Co., Kankakee, IL); hirudin from Dr. Fritz Markwardt (Medical Academy Erfurt, Erfurt, DDR); Ancrod from Grant H. Barlow (Michael Reese Blood Center, Chicago, IL); *m*-CP(PBA)-F from Dr. David H. Bing (CBR Laboratories, Boston, MA); D-Phe-Pro-Arg-CH_2-Cl from Dr. Elliott Shaw (Brookhaven National Laboratory, Upton, NY). Hirudin was also purchased from American Diagnostica (Greenwich, CT) and D-Phe-Pro-Arg-CH_2-Cl also from Calbiochem-Behring (San Diego, CA). Bovine fibrinogen (>90% clottable) was purchased from Miles Laboratories (Elkhart, IN), human fibrinogen (grade L) was from AB Kabi (Stockholm, Sweden), bovine α-chymotrypsin and β-trypsin were from Worthington Biochemicals (Freehold, NJ), Amberlite CG-50 resin (200–400 mesh) was from Mallinckrodt Chemical Work (St. Louis, MO), Sepharose 4B and Sepharose G-100 were from Pharmacia Fine Chemicals (Piscataway, NJ), and Bio-Gel P-2 was from Bio-Rad Laboratories (Richmond, CA). All other materials were obtained from sources indicated in publications cited below.

Thrombin Preparations. Human α-thrombin was prepared from fraction III paste (Fenton et al., 1977a,b). From these preparations, modified forms were made: γ-thrombin (Bing et al., 1977; Fenton et al., 1977b); Fl(CHO)-α-thrombin (Atha et al., 1984); NO_2-α-, $MeSO_2$-α-, $PhCH_2SO_2$-α-, *i*-Pr_2P-α-, and Tos-Lys-CH_2-α-thrombins (Fenton et al., 1979; Sonder & Fenton, 1984); D-Phe-Pro-Arg-CH_2-α-thrombin (Kettner & Shaw, 1979; Sonder & Fenton, 1984); *m*-CP(PBA)-α-thrombin (Bing et al., 1977; Sonder & Fenton, 1984). Hirudin complexes were formed by adding a 10% molar excess of the inhibitor to various thrombin forms. Throughout, an absorption coefficient of 1.83 mL mg^{-1} cm^{-1} at 280 nm was used for thrombin forms in 0.10 M NaOH, and an M_r of 36 500 was assumed for all forms. Thrombin preparations were evaluated (Table I) and stored frozen at −70 °C in 0.75 M NaCl until used (Fenton et al., 1977a,b, 1979).

Amberlite CG-50 Resin Chromatography. Previously recycled CG-50 resin used for thrombin preparation was washed with 0.5 M HCl, 0.5 M NaOH, and water (Fenton et al., 1977a,b, 1979). A column of ~20 mL of resin was packed in a 30-mL plastic syringe barrel fitted with a glass wool pad (resin dimensions of ~7.5 cm × 2 cm i.d.). The packed resin was washed with 100 mL of 3 M NaCl, 200 mL of water, and 150 mL of 0.15 M NaCl buffered with 50 mM Tris-HCl at pH 7.4, ~23 °C. Thrombin samples were dialyzed overnight against the equilibration buffer at 4 °C, and ~1–2 mg of protein was applied to the resin at ~23 °C. After the resin was rinsed with 20 mL of the equilibration buffer, a linear gradient was generated from 100 mL of the buffer (first chamber) and 100 mL of 0.50 M NaCl buffered with 50 mM Tris-HCl at pH 7.4 (second chamber). Eluates were withdrawn at ~0.5–1.0 mL/min with an LKB 12000 peristaltic pump and passed through an ISCO UA-5 UV monitor, recording the absorbancy at 280 nm. Individual 3-mL fractions were collected, and their conductivity was measured with a Radiometer CDM2e conductivity meter. Conductivity measurements were subsequently converted into corresponding NaCl concentrations from calibration plots. The CG-50 resin column was regenerated by the above 3 M NaCl washing procedure.

Nonpolymerized Fibrin(ogen) Resin Chromatography. Bovine fibrinogen was immobilized on CNBr-activated agarose (Heene & Matthias, 1973) as previously described (Berliner et al., 1985). This resin was used directly as the fibrinogen resin or converted with ~0.1 unit of α-thrombin/mL of moist resin to the fibrin resin, which was washed with 500 mL of 0.15 M NaCl buffered with 50 mM Tris-HCl at pH 7.6. Resins were used immediately after preparation or storage at 4 °C for brief periods.

As described for CG-50 resin, ~20 mL of fibrin(ogen) resin was packed in a 30-mL syringe. The resin was equilibrated by passage of ~100 mL of 50 mM NaCl buffered with 50 mM Tris-HCl at pH 7.6, ~23 °C. Thrombin samples were dialyzed overnight against the equilibrating buffer at 4 °C, and ~0.2–1 mg of protein in 0.5 mL of the equilibration buffer was applied to the resin. The resin was then rinsed with 20 mL of this buffer prior to beginning a linear gradient generated from 100 mL of the buffer (first chamber) and 100 mL of 0.30 M NaCl and 50 mM Tris-HCl at pH 7.6 (second chamber). With the exception of collecting 2-mL fractions, eluates were withdrawn and monitored, as described for the CG-50 resin.

Fibrin(ogen) Peptide and Fragment Inhibitor Experiments. The 11-residue peptide homologue (H-Gly-Pro-Arg-Val-Val-Glu-Arg-His-Gln-Ser-Ala-OH) of positions 17–27 of the human fibrinogen Aα chain (Blomback et al., 1967) was synthesized by modification of the Merrifield solid-phase method (Wilner et al., 1976). Following deprotection and cleavage from the supporting resin with anhydrous HF at 0 °C, the peptide was dissolved in 50% (v/v) acetic acid, freezed-dried, and passed through a column (~70 cm × cm i.d.) of Bio-Gel P-2 in 50 mM NH_4HCO_3. The peptide was verified by its TLC and TLE mobilities and gave the following composition upon amino acid analysis: 1.0 Gly, 1.0 Pro, 1.9 Arg, 2.0 Val, 1.8 Glx, 0.96 His, 0.87 Ser, and 0.87 Ala. The NDSK fragment of human fibrinogen was prepared and gel filtered through Sephadex G-100 as described (Blomback et al., 1972). The preparation was fibrin(ogen) free, and an M_r of 11 000 was assumed for it.

Potentiometric titration of α-thrombin hydrolysis of Bz-Arg-OEt was carried out with a Radiometer Model TTT 60 titrator, equipped with a PHM 62 pH meter, an ABU 12 autoburette, and an REA 160 recorder. Titrations were performed at pH 7.25 and ~23 °C by addition of 2 mM NaOH under N_2 to 100 nM α-thrombin and 25–200 μM Bz-Arg-OEt. The concentration of the synthetic peptide ranged up to 10 mM without any apparent inhibition being shown. Those of the NDSK fragment varied from 0.10 to 100 μM. For this fragment, reciprocal initial velocities (v_0^{-1}) were plotted against fragment concentrations to determine the K_i

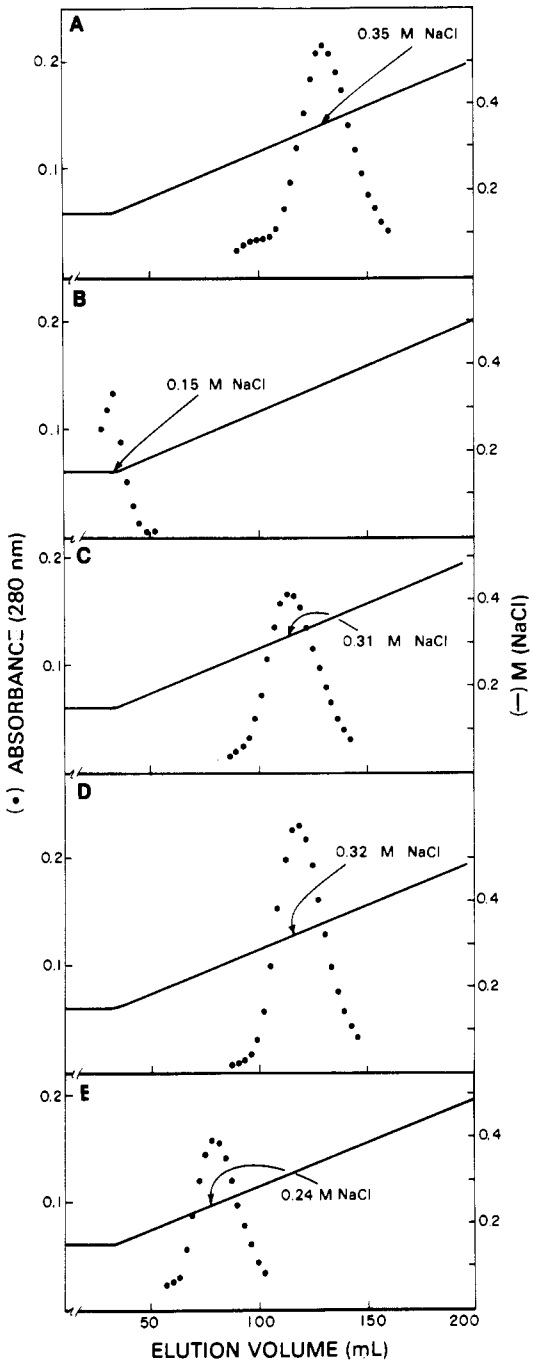

FIGURE 1: Elution profiles of representative human thrombin forms from Amberlite CG-50 resin [cross-linked poly(methylacrylic acid)]. Experiments were performed at pH 7.4 and ~23 °C, where absorbances at 280 nm are those directly recorded with a 5-mm path-length flow cell and are amplified 10-fold. Other details are given in text. The panels shown are (A) α-thrombin, (B) α-thrombin premixed with a 10% molar excess of hirudin, (C) i-Pr_2P-α-thrombin, (D) D-Phe-Pro-Arg-CH_2-α-thrombin, and (E) γ-thrombin. The NaCl concentrations for midpeak elutions are indicated.

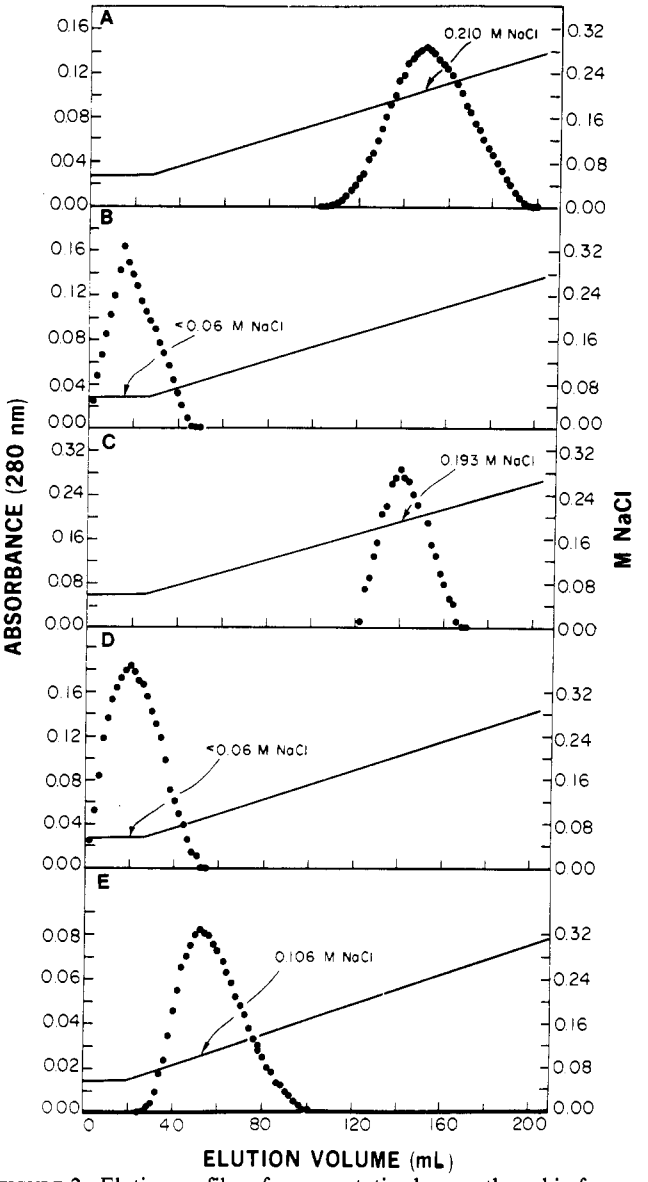

FIGURE 2: Elution profiles of representative human thrombin forms from nonpolymerized fibrin immobilized on Sepharose 4B. Experiments were carried out at pH 7.6 and ~23 °C, as in Figure 1. The panels shown are (A) α-thrombin, (B) α-thrombin premixed with a 10% molar excess of hirudin, (C) i-Pr_2P-α-thrombin, (D) i-Pr_2P-α-thrombin premixed with a 10% molar excess of hirudin, and (E) γ-thrombin. The NaCl concentrations for midpeak elutions are indicated.

and inhibition pattern (Mahler & Cordes, 1971). The K_i was also determined by linear regression with a Texas Instruments TI-55-II calculator.

RESULTS

Upon development with a shallow salt gradient, human α-thrombin was eluted from CG-50 resin as a sharp peak with 0.35 ± 0.01 M NaCl in 50 mM Tris-HCl at pH 7.4 (Table I; Figure 1A). In contrast, its hirudin complex was not retained (eluted in ~1 column volume; Figure 1B). Both i-Pr_2P-α- and D-Phe-Pro-Arg-CH_2-α-thrombins were eluted with slightly lower salt concentrations than α-thrombin (Figure 1C,D), while γ-thrombin was eluted with a significantly lower salt concentration (Figure 1E). In addition, other catalytically inactivated thrombins [$MeSO_2$-α-, $PhCH_2SO_2$-α-, Tos-Lys-CH_2-α-, and m-CP(PBA)-α-thrombins] behaved like i-Pr_2P-α- and D-Phe-Pro-Arg-CH_2-α-thrombin (Table I).

Although elution was with lower salt concentrations, the same general pattern was obtained for the elution for various thrombin forms from nonpolymerized fibrin resin. Again the hirudin complex of α-thrombin was not retained (Figure 2, panel A vs panel B) nor was the complex of i-Pr_2P-α-thrombin (Figure 2, panel C vs panel D) or that of γ-thrombin (data not shown). Furthermore, γ-thrombin was eluted with a significantly lower salt concentration than α-thrombin (Table I; Figure 2, panel A vs panel E). For seven thrombin forms, where comparable data were obtained, a direct correlation (r = 0.95) was obtained for the ionic strength for peak elution from either resin (Figure 3).

Table I: Properties of Thrombin Preparations and Their Binding to CG-50 Resin or Fibrin Monomer Immobilizing Sepharose 4B at 23 °C[a]

preparation	characterization data: clotting activity (units/mg)	% original clotting activity	% NPGB activity	% thrombin form: α	β	γ	concn of NaCl in 50 mM Tris-HCl, pH 7.4, for elution from CG-50 resin (M)	concn of NaCl in 50 mM Tris-HCl, pH 7.6, for elution from fibrin resin (M)
α-thrombin	>3000		100	96.6	2.7	0.7	0.35 ± 0.01 (n = 4)	
	2920		92.5	98.1	0.6	1.3		0.209 ± 0.007 (n = 4)
Fl(CHO)-α-thrombin							not determined	
	2278	100	92.6	97.6	0.7	1.7		0.195 (n = 1)
γ-thrombin	46.7	1.50	80.0	0.0	2.0	98.0	0.24 (n = 2)	
	0.8	0.04	63.8	0.0	2.7	97.3		0.107 (n = 2)
NO_2-α-thrombin	0.5	0.03	66.5	(97.9)	(1.8)	(0.3)	not retained (n = 3)	
	86.4	2.9	51.9	(88.2)	(3.1)	(8.7)		(biomodal) 0.12–0.15 (n = 2)
$MeSO_2$-α-thrombin	0.3	<0.02	2.1	97.9	1.8	0.3	0.31 (n = 1)	
	<0.5	<0.01	1.6	(96.5)	(2.8)	(0.7)		0.162 (n = 1)
$PhCH_2SO_2$-α-thrombin	<0.4	<0.01	<1.0	(100)	(0.0)	(0.0)	0.32 (n = 1)	
	<0.3	<0.01	<1.0	(95.4)	(0.8)	(3.8)		0.175 (n = 1)
i-Pr_2P-α-thrombin	<0.4	<0.01	<0.01	(98.2)	(1.1)	(0.7)	0.31 (n = 2)	
	0.7	<0.01	<0.1	(88.2)	(3.1)	(8.7)		0.191 (n = 2)
Tos-Lys-CH_2-α-thrombin	<0.3	<0.02	5.0	(99.5)	(0.3)	(0.2)	0.34 (n = 1)	
	0.6	<0.01	1.9	(96.5)	(2.8)	(0.7)		0.298 (n = 2)
m-CP(PBA)-α-thrombin	0.9	0.1	1.4	(98.2)	0.5	(1.3)	0.33 (n = 1)	
	0.9	0.1	1.4	(98.2)	(0.5)	(1.3)		0.205 (n = 1)
D-Phe-Pro-Arg-CH_2-α-thrombin	<0.2	<0.01	1.3	(100)	(0.0)	(0.0)	0.325 (n = 2)	
	<0.1	<0.01	<0.1	(95.7)	(2.8)	1.5		0.185 (n = 2)

[a] Methods are described in the text. The percent original clotting activity is based on the starting α-thrombin preparations. Values of percent thrombin forms in parentheses are those of the starting preparations which are assumed not to change during enzyme modification. Average values for NaCl concentrations for midpeak elution of thrombin forms are shown with the number of determinations in parentheses. That of α-thrombin is given ±1 SDM.

Omitted from this correlation was the carbohydrate-derivatized form Fl(CHO)-α-thrombin, which behaved like α-thrombin on the fibrin resin but was not presently run on CG-50 resin. Nevertheless, this form is known to bind to CG-50 resin or heparin–agarose similar to α-thrombin (Atha et al., 1984), in agreement with other evidence that the carbohydrate side chain is removed from active-site regions involved in complexing with hirudin (Olson et al., 1986).

In addition, NO_2-α-thrombin was excluded because it was not retained by CG-50 resin (like the hirudin–α-thrombin complex) and because it was eluted as a bimodal peak from the fibrin resin (Table I). The latter indicates that this chemically derivatized form is a mixed product. Like the bovine product (Lundblad & Harrison, 1971), our human preparations contain ca. five nitrated tyrosines (Fenton et al., 1979). However, the preparations also appear to be partially cross-linked, since they do not enter sodium dodecyl sulfate containing, 7.5% cross-linked polyacrylamide gels like other thrombin forms (data not shown). Both heterogeneity and cross-linking raise serious questions as to the interpretation of data obtained with this form. Nevertheless, NO_2-α-thrombin did elute from the fibrin resin more like γ-thrombin than other forms, consistent with its substantially reduced clotting activity and moderate retension of NPGB activity (Table I).

Although Tos-Lys-CH_2-α-thrombin behaves like other catalytically inactivated forms on CG-50 resin, it bound substantially more firmly to the fibrin resin than α-thrombin (Table I). Since the Tos-Lys-CH_2- ligand is attached to the catalytic histidine (Glover & Shaw, 1971) and its chloromethyl ketone is a relatively poor reagent with a low affinity for thrombin (Kettner & Shaw, 1979), the conjugate ligand is most likely solvated. Furthermore, the fibrinogen used for preparing the fibrin resin was only partially purified (see Experimental Procedures) and may well have contained plasminogen among other components (Mosesson & Sherry, 1966). Therefore, immobilized plasminogen could potentially bind the lysine side chain of Tos-Lys-CH_2-α-thrombin, but

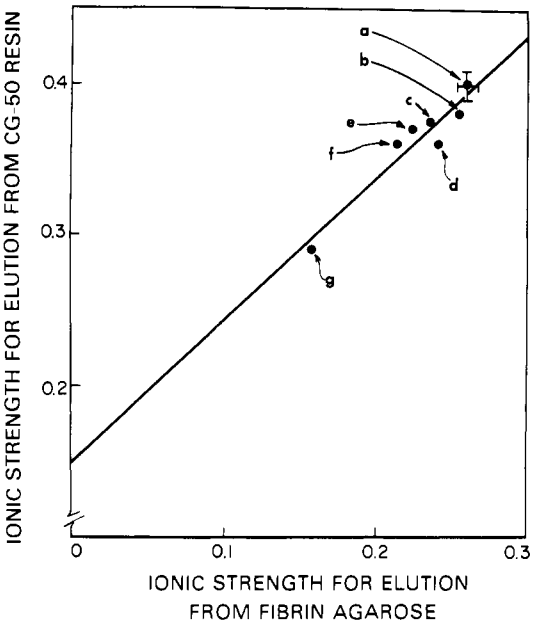

FIGURE 3: Correlation of ionic strengths for the elution of various human thrombin forms from CG-50 resin and fibrin agarose at ~23 °C. Ionic strength values are those for the midpeak NaCl concentrations plus 50 mM for the Tris-HCl contribution (Table I). Points are (a) α-thrombin, (b) m-CP(PBA)-α-thrombin, (c) D-Phe-Pro-Arg-CH_2-α-thrombin, (d) i-Pr_2P-α-thrombin, (e) $PhCH_2SO_2$-α-thrombin, (f) $MeSO_2$-α-thrombin, and (g) γ-thrombin. The relationship of $y = 0.15 + 0.96x$ was determined by linear regression analysis, where $r = 0.95$.

such complexes are resistant to high salt concentrations yet dissociate with ε-aminocapronic acid (Deutsch & Mertz, 1970). Since Tos-Lys-CH_2-α-thrombin eluted with an ionic strength of 0.35 M, some other explanation seems likely.

In other experiments, i-Pr_2P-α-thrombin was found to bind to immobilized fibrinogen (prior to exposure to α-thrombin) in essentially the same manner as to the fibrin resin, as recently reported for $PhCH_2SO_2$-α-thrombin (Kaminski & McDonagh,

1987). The fibrinogen clotting enzyme from snake venom, Ancrod, bound weakly to fibrin resin, resembling γ-thrombin. Neither inactivated *i*-Pr_2P-α-chymotrypsin nor *i*-Pr_2P-β-trypsin bound to fibrin resin (i.e., eluted in ~1 column volume), despite the fact that the thrombin B chain closely resembles the structure of chymotrypsin and that thrombin exhibits a preferential specificity for cleavage at arginine bonds like that of trypsin (Magnusson, 1971; Fenton & Bing, 1986).

In order to assess where α-thrombin might predominantly interact with fibrin(ogen), the peptide of residues 17–27 of the Aα chain and the NDSK fragment of human fibrinogen were chosen as likely candidates. The synthetic substrate Bz-Arg-OEt was selected for potentiometric titration because of its relatively simple kinetic behavior and moderately low affinity for thrombin [e.g., k_{cat}(limited) and K_m of ~6 μM for human α-thrombin in 0.15 M NaCl], which should permit detection of moderately weak inhibitors (Landis et al., 1981). When an 11-residue synthetic peptide was examined, no inhibition was obtained even up to 10 mM concentrations. This suggests that the first 11 residues following the Aα cleavage contribute little to fibrin(ogen) binding of α-thrombin. Since these residues correspond to the first product of proteolytic cleavage at the Aα site, such low-affinity binding would favor release of the α-chain NH_2 terminus from α-thrombin. In this regard, this NH_2 terminus must be accessible for it to bind to the D domain (Kudryk et al., 1974; Landano & Doolittle, 1978; Olexa & Budzynski, 1981).

The NDSK fragment of human fibrinogen consists of residues 1–51 of the Aα chain, 1–115 of the Bβ chain and 1–78 of the γ-chain and thus has its fibrinopeptide cleavage sites intact, while its thrombin-generated product is incapable of polymerizing (Blomback et al., 1972; Kudryk et al., 1974). Upon potentiometric titration, this fragment behaved as a simple competitive inhibitor (Mahler & Cordes, 1971) of Bz-Arg-OEt hydrolysis by human α-thrombin, where a plot of reciprocal velocity versus NDSK concentration at three substrate concentrations had a common intercept (above the abscissa) corresponding to a K_i of 3.2 μM (data not shown). This results suggests that the dominant site(s) recognized in fibrin(ogen) by α-thrombin is (are) contained within the NDSK fragment.

Discussion

Thrombin has been known for some time to adhere to glass (Seegers et al., 1952) and cation-exchange resins (Rasmussen, 1955). Since prothrombin does not bind to Amberlite CG-50 resin, this cation-exchange resin can be used to remove any thrombin-like binding substances from prothrombin complexes and subsequently to recover from such activated prothrombin complexes high-purity α-thrombin (Fenton et al., 1977a,b). Isolated in this manner, human α-thrombin labeled with [^{14}C]-*i*-Pr_2P-F electrophoresed in 0.1% SDS–polyacrylamide gels with 99.8% of the radioactivity as a homogeneous component but exhibited three major labeled isoelectric forms with p*I* values of 7.0, 7.3, and 7.6 (Fenton et al., 1977a). Therefore, under the present conditions of pH 7.4 for CG-50 resin chromatography, α-thrombin is predominantly a noncharged protein and should not bind to this resin if such binding were attributable to "general ion exchange effects" for evenly distributed charges on the protein. That the retention by the resin might be due to nonionic adsorption and solvation with increasing salt concentrations seems unlikely since human α-thrombin has a solubility of up to 0.3 g/L in deionized water (Landis et al., 1981), and by use the neutral pH absorption coefficient for the protein (Fenton et al., 1977a), concentrations of roughly one-third this can be calculated from the present peak absorbances (Figures 1 and 2). Thus, the binding of α-thrombin to CG-50 resin is by uneven charge distribution, which is supported by the clustering of arginines and lysines in thrombin three-dimensional models (Bing et al., 1981; Fenton, 1986; Fenton & Bing, 1986). In addition to forming the required components of the catalytic site for enzymic activity, prothrombin activation must also expose such cation clusters accounting for binding to CG-50 resin.

The elution of β- and γ-thrombins in front of α-thrombin from CG-50 resin developed with a salt gradient has suggested a relationship between this resin affinity and fibrinogen clotting activity (Fenton et al., 1979). In the present studies, γ-thrombin exhibited very low clotting activity and eluted (with the exception of thrombin–hirudin complexes) from either CG-50 or nonpolymerized fibrin resin with lower salt concentrations than other thrombin forms (Table I; Figures 1 and 2). Moreover, the direct correlation ($r = 0.95$) of elution ionic strength for seven thrombin forms from either resin implies binding by a common site (Figure 3). Since CG-50 resin is cross-linked poly(methylacrylic acid) and catalytically inactivated α-thrombins elute similar to α-thrombin, this site binds negatively charged substances independent of the catalytic site of the enzyme and is therefore an anion-binding exosite.

The present findings agree with those of others that the lysine-derivatizing reagents acetic anhydride (Landaburu & Seegers, 1959) and pyridoxal 5′-phosphate (Griffin, 1979) inhibit clotting without destroying synthetic substrate activity. Phosphopyridoxylated α-thrombin does not bind to nonpolymerized fibrin resin (Kaminski & McDonagh, 1987), as predicted if the fibrin(ogen) binding involves lysines. In thrombin three-dimensional models, such a clustering of lysines (as well as arginines) exists removed from and to the side of the catalytic site and further contains the cleavage site(s) for forming β-thrombin (Fenton, 1986; Fenton & Bing, 1986). In the human system, α-thrombin is autoproteolytically or tryptically converted to β-thrombin with loss of clotting activity and subsequently to γ-thrombin (Fenton et al., 1977a,b), whereas in the bovine system there is no γ-thrombin counterpart and clotting activity is lost with the formation of β-thrombin (Sonder & Fenton, 1986). Moreover, human β-thrombin has recently been isolated from partial tryptic digests consisting of primarily α- and β-thrombins with little γ-thrombin. The undigested α-thrombin was retained by a cation-exchange resin (similar to CG-50 resin), where predominantly β-thrombin eluted with lower ionic strength. This product possessed very low clotting activity and exhibited little or no affinity for nonpolymerized fibrin resin (Bezeaud & Guillin, 1987), as presently found for γ-thrombin (Table I). Thus, the anion-binding exosite appears to be partially destroyed or obstructed upon forming β-thrombin.

In the present studies, hirudin was found to block the binding of α-thrombin to CG-50 resin (Figure 1), as well a α-, *i*-Pr_2P-α-, and γ-thrombins to nonpolymerized fibrin (Figure 2). This confirms our results with the latter resin (Fenton et al., 1981) and those of others (Kaminski & McDonagh, 1983, 1987). It also agrees with our findings that the α-thrombin–hirudin complex is not retained by heparin–agarose (Olson et al., 1986), indicating that hirudin blocks a common site involved in the binding to all three resins.

Hirudin is a small 65-residue protein secreted from the salivary glands of European medicinal leeches and forms a highly specific, high-affinity noncovalent thrombin complex (Walsmann & Markwardt, 1981). The first 40 some residues constitute a growth factor like, apolar entity linked by three disulfide bridges and followed by an unusually negatively

charged tail, where the overall net charge is −10 (Dodt et al., 1984). Consequently, the α-thrombin–hirudin complex should have a decreased net charge of −10 relative to that of α-thrombin, which would facilitate the solubility of the complex and might explain why it is not retained by either CG-50 or the fibrin resin. However, the hirudin tail is necessary for high antithrombin activity (Chang, 1984), and synthetic peptide homologues of it are moderately potent inhibitors of clotting but not of synthetic substrate activities (Krstenansky & Mao, 1987), suggesting that the tail combines with and masks a positively charged region on α-thrombin. When α- is compared to γ-thrombin, there is a shift from noncompetitive to competitive inhibition and a drop of about 2 orders of magnitude in affinity (Landis et al., 1978; Fenton et al., 1979; Stone et al., 1987). In addition, a more pronounced conformational shift occurs with α- versus γ-thrombin indicating more than a single interactive site and the partial loss or obstruction of a site in α-thrombin (Villanueva et al., 1987). These and the present findings are in accord with the hypothesis that hirudin is bound by its negatively charged tail to the anion-binding exosite (which is intact in α-thrombin) and by its apolar entity to the catalytic site and adjacent regions of thrombin (Fenton, 1986; Fenton & Bing, 1986).

Within the fibrinopeptide groove, adjacent to the catalytic site is a prominent apolar binding site (Sonder & Fenton, 1984) which appears to be of major importance in determining thrombin specificity with tripeptide *p*-nitroanilide (Sonder & Fenton, 1986) and protein substrates (Chang, 1985). Although certain structural aspects of fibrinopeptides appear to contribute to thrombin specificity (Marsh et al., 1983), the overall structure of fibrinogen (and particularly that on the fibrin side of fibrinopeptide cleavage) contributes to recognition by α-thrombin (Witting et al., 1987). In the present study, the first 11-residue peptide following the Aα cleavage in fibrinogen was not an inhibitor of α-thrombin, and the corresponding peptide in fibrin(ogen) appears to contribute little to interactions with thrombin. In contrast, the NDSK fragment of fibrinogen was a simple competitive inhibitor with a K_i of 3.2 μM (see Results). This value approximates the K_m for Aα cleavage by α-thrombin (Higgins et al., 1983) and the reciprocal K_a determined for α-thrombin binding to nonpolymerized fibrin resin (Kaminski & McDonagh, 1983). Since the NDSK fragment is contained within the E domain, our data support the correction that α-thrombin binds to this domain [Kaminski and McDonagh (1983) vs Kaminski and McDonagh (1987)]. The E domain, moreover, appears to contain the necessary structural components for α-thrombin recognition in the cleavage of fibrinopeptide A (Hanna et al., 1984).

From the above considerations, we suggest that α-thrombin recognizes fibrin(ogen) via a prominent negatively charged surface, which is spatially near (but noncontiguous) to the Aα cleavage site. Since α- relative to γ-thrombin has an 800-fold greater specificity for the Bβ cleavage (Lewis et al., 1987), the intact anion-binding exosite in α-thrombin might also facilitate this cleavage. Furthermore, the exosite might serve to shuttle α-thrombin into fibrin clots. Since this incorporation occurs only during clotting (Wilner et al., 1981) and the incorporated enzyme behaves as if it were solvated, not protein associated (Carney et al., 1979), fibrin-bound thrombin most likely is displaced upon fibrin polymerization. Such a displacement would constitute an irreversible event and cause the process to proceed even if the thrombin–fibrin interaction is a weak ionic association. Such an active entrapment, partitioning thrombin within the clot and removing it from blood, would readily explain the antithrombin properties of fibrin and may be an important process in hemostasis (Seegers, 1962).

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Analysis of the Structure of Synthetic and Natural Melanins by Solid-Phase NMR†

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ABSTRACT: The structures of one synthetic and two natural melanins are examined by solid-state NMR using cross polarization, magic angle sample spinning, and high-power proton decoupling. The structural features of synthetic dopa melanin are compared to those of melanin from malignant melanoma cells grown in culture and sepia melanin from squid ink. Natural abundance ^{13}C and ^{15}N spectra show resonances consistent with known pyrrolic and indolic structures within the heterogeneous biopolymer; ^{13}C spectra indicate the presence of aliphatic residues in all three materials. These solid-phase experiments illustrate the promise of solid-phase NMR for elucidating structural information from insoluble biomaterials.

Melanins are a class of pigments widespread in the animal and plant kingdoms (Nicolaus, 1968). A subcategory known as eumelanins are unique solid biopolymers derived from tyrosine and characterized by their insolubility, intense dark color, resistance to hydrolysis, lack of molecular regularity, and paramagnetism. After years of study, no generally accepted molecular structures have been defined for these materials. The single periodic structural feature observed in melanins is a 0.34-nm spacing believed to correspond to the adventitious parallel stacking of aromatic units in randomly oriented local domains (Nicolaus, 1968; Blois, 1978; Swan, 1974).

As materials, melanins have been described as both insulators and semiconductors (Swan, 1974; Pullman & Pullman, 1961), ion-exchange resins (Lindquist, 1986), redox polymers (Froncisz et al., 1980; Sarna et al., 1980) and free-electron scavengers (Packer et al., 1981). Their biological functions range from an obvious role as pigmentation agents in skin, hair, and feathers to protective agents against light-induced damage (an empirically evident action that still needs further mechanistic clarification) (Pathak et al., 1976), as possible participants in one- and two-electron reduction systems (Felix et

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