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Proflavin Binding within the Fibrinopeptide Groove Adjacent to the Catalytic Site of Human α-Thrombin†

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ABSTRACT: Human α-thrombin with high fibrinogen-clotting activity binds proflavin at a single specific site (n = 0.996 site/α-thrombin, K_d = 22.0 μM) with the same affinity as the bovine enzyme (K_d = 22 ± 3 vs. 24 ± 3 μM, respectively, at pH 7.4, ~23 °C). This human enzyme form further displayed no significant difference in its ability to bind the dye over a broad NaCl concentration range (0.15–3 μM), and its hydrolysis of Bz-Arg-OEt was inhibited by the dye in a simple competitive manner (K_i = 30 ± 3 μM). Conversion of the human α- to γ-thrombin by controlled tryptic digestion essentially destroyed clotting activity without appreciably altering synthetic substrate activities and caused only ~2-fold reduction in proflavin binding. Chemical modification of approximately four tryptophans or approximately four tyrosines per enzyme also caused analogous differential losses of clotting vs. synthetic substrate activities and reduced proflavin binding ~5- and ~10-fold, respectively. Inactivation of the enzyme by conjugation at the catalytic serine (Ser-195, chymotrypsin numbering) with $MeSO_2$-F, $PhMeSO_2$-F, or i-Pr_2P-F decreased binding ~4-, 26-, and 55-fold, respectively, following the increasing size and steric hindrance properties of the conjugated group. Conjugation of the catalytic histidine (His-57) with Tos-Lys-CH_2-Cl decreased binding only ~10-fold, suggesting partial displacement by the dye. Such partial displacement appeared to occur to a slightly greater extent with the conjugate of a large exo site affinity-labeling reagent, which covalently attaches to the enzyme within the fibrinopeptide groove distal to the catalytic site. On the other hand, D-Phe-Pro-Arg-CH_2-Cl, which specifically binds within the fibrinopeptide groove and covalently reacts at or adjacent to the catalytic site, reduced proflavin binding ~40-fold. These data strongly suggest that the proflavin binding site resides in an apolar active-site region, which is next to (or slightly overlaps) the catalytic site (His-57 and Ser-195) and extends into the fibrinopeptide groove. With the least sterically hindered inactivated form, $MeSO_2$-α-thrombin, hirudin displaced proflavin, while antithrombin III in the presence of heparin could not, indicating major differences in the active-site regions required for either of these protein inhibitors of α-thrombin.

Procoagulant α-thrombin has high fibrinogen clotting and all other thrombin-ascribed activities and is the central bioregulatory enzyme in hemostasis (Fenton, 1981). This serine proteinase more closely resembles the pancreatic enzyme chymotrypsin than trypsin, with respect to amino acid sequence and three-dimensional structure (Magnusson et al., 1975; Bing et al., 1981; Furie et al., 1982), although it preferentially cleaves protein substrates at arginine and secondarily at lysine bonds in a manner analogous to that of trypsin (Magnusson, 1971; Elmore, 1973). Like chymotrypsin and trypsin, α-thrombin also binds the acridine dye, proflavin, at or near its catalytic site (Koehler & Magnusson, 1974; Li et al., 1974; Berliner & Shen, 1977), while the closely related fibrinolytic enzyme, plasmin, does not bind the dye (Ryan et al., 1976). On the basis of displacement of proflavin by indole and related compounds, this dye appears to bind partially at an apolar binding site distinct from hydrophobic regions of the arginine side-chain bindng pocket (Berliner & Shen, 1977). Human γ-thrombin, which is the second autoproteolytic or tryptic derivative enzyme form, essentially lacks clotting activity yet retains most synthetic substrate activities (Fenton et al., 1977a,b). This form also binds proflavin in a manner similar to the parent α-thrombin form, suggesting that the proflavin binding site is removed from certain active-site regions required for fibrinogen recognition (Chang et al., 1979; Fenton, 1981).

The present studies were undertaken to further assess proflavin binding requirements and to localize the site in relation to the catalytic site, consisting of the His-57/Asp-102/Ser-195 triad (Figure 1). Dissociation constants (K_d)[1] were determined by spectrophotometric methods for specific proflavin binding to human and bovine α-thrombins, human γ-thrombin, chemically modified enzymatically active forms of α-thrombin with approximately four DNS-tryptophans or approximately

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[1] Abbreviations: Bz-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester; DNS-Br, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; D-Phe-Pro-Arg-CH_2-Cl, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; i-Pr_2P-F, diisopropyl phosphorofluoridate; K_d, dissociation constant; K_i, inhibition constant; m-CP(PBA)-F, m-[[o-[[2-chloro-5-(fluorosulfonyl)phenyl]ureido]phenoxy]butoxy]benzamidine; $MeSO_2$-F, methanesulfonyl fluoride; NO_2, nitro; NPGB, p-nitrophenyl p-guanidinobenzoate; $PhMeSO_2$-F, phenylmethanesulfonyl fluoride; SDM, standard deviation of the mean; Tos-Lys-CH_2-Cl, N^{α}-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Preparative Properties of Examined Thrombin Forms and Proflavin Binding Parameters[a]

preparation	evaluation properties: clotting activity (units/mg)	% NPGB active	% thrombin form: α	β	γ	proflavin binding parameter: λ_{max} (nm)	K_d (μM)
α-thrombin (lot no. 169B)	3025	90	99.2	0.4	0.4	465	21.7 ± 2.6
bovine α-thrombin (lot no. 10409)	2413	96	94.2	5.8	0.0[b]	465	23.7 ± 3.0
γ-thrombin (lot no. 61)	1.8	70	0.1	1.2	98.7	466	47.8 ± 8.6
DNS-α-thrombin (lot no. 202)	361	78	(100)	(0.0)	(0.0)	467	110 ± 30
NO_2-α-thrombin (lot no. 8)	0.8	58	(99.6)	(0.2)	(0.2)	465	216 ± 44
$MeSO_2$-α-thrombin (lot no. 6)	<0.3	<2	(97.9)	(1.8)	(0.3)	462	95.5 ± 9.7
$PhMeSO_2$-α-thrombin (lot no. 1)	1.0	9	(99.5)	(0.3)	(0.2)	464	560 ± 190
i-Pr_2P-α-thrombin (lot no. 10)	<0.4	<1	(96.6)	(3.4)	(0.0)	465	1220 ± 470
Tos-Lys-CH_2-α-thrombin (lot no. 4)	<0.3	<5	(99.5)	(0.3)	(0.2)	467	233 ± 30
D-Phe-Pro-Arg-CH_2-α-thrombin (lot no. 1)	<0.2	<2	(97.9)	(1.8)	(0.3)	461	830 ± 230
m-CP(PBA)-α-thrombin (lot no. 3)	<0.2	<2	(98.2)	(0.5)	(1.3)	461	148 ± 22

[a] Unless indicated, preparations are of human origin. Preparative methods are presented in the text; evaluation methods are described in Fenton et al. (1977a). Values of percent thrombin forms in parentheses are those of the α-thrombin preparation from which they were made and are assumed not to appreciably change during enzyme modification or inactivation. Proflavin binding was determined with a Varian Model 210 spectrophotometer, as described in the text, with 13 proflavin stock solutions ranging from 1.0 to 50 mM in 0.75 NaCl and 30 mM Tris at pH 7.4, ~23 °C. Spectral determinations of K_d values were based on five trials for human α-thrombin and four trials for all other enzyme forms. [b] Bovine thrombin does not have a form corresponding to human γ-thrombin (Fenton et al., 1977b, 1979).

four NO_2-tyrosines, and the catalytically inactivated forms *i*-Pr_2P-, $MeSO_2$-, $PhMeSO_2$-, *m*-CP(PBA)-, Tos-Lys-CH_2-, and D-Phe-Pro-Arg-CH_2-α-thrombins. In addition, the number of binding sites per α-thrombin and K_d were calculated by Scatchard analysis with the human enzyme. The K_i of proflavin was also determined with this enzyme form by potentiometric titration of Bz-Arg-OEt hydrolysis. Our data demonstrate that proflavin binds to a single specific site. This binding site occurs adjacent to the catalytic site and involves an apolar-site region, corresponding to the arginine (or lysine) side of a thrombin-susceptible peptide (e.g., the fibrinopeptide side of the Aα cleavage site in fibrinogen).

A preliminary report of some of the present findings was presented elsewhere (Sonder et al., 1979).

Materials and Methods

Material Sources. The following were gifts: *m*-CP(PBA)-F from Dr. David H. Bing (Cambridge Research Laboratory, Cambridge, MA); purified human antithrombin III from Dr. Richard D. Feinman (Downstate Medical Center, Brooklyn, NY); porcine heparin from Dr. William H. Holleman (Abbott Laboratories, North Chicago, IL); human fraction III paste and high-purity bovine α-thrombin from Dr. Bryan H. Landis (Armour Pharmaceutical Co., Kankakee, IL); highly purified hirudin from Dr. Fritz Markwardt (Medical Academy of Erfurt, Erfurt, GDR); DNS-Br modified human α-thrombin with approximately four derivatized tryptophans from Dr. German B. Villanueva (New York Medical College, Valhalla, NY). The following were purchased: Bz-Arg-OEt, *i*-Pr_2P-F, $MeSO_2$-F, tetranitromethane, and Tos-Lys-CH_2-Cl from Aldrich Chemical Co. (Milwaukee, WI); D-Phe-Pro-Arg-CH_2-Cl from Calbiochem-Behring (San Diego, CA); $PhMeSO_2$-F from Eastman Kodak Co. (Rochester, NY); proflavin sulfate from Sigma Chemical Co. (St. Louis, MO). All other materials were obtained from previously cited sources (Fenton et al., 1977a,b) or from other scientific vendors as reagent-grade materials.

Thrombin Preparations and Modifications. Human α-thrombin (Table I) was prepared from fraction III paste by methods previously described and was stored frozen at −70 °C in 0.75 M NaCl at ~2–4 mg of protein/mL, pH ~6 (Fenton et al., 1977a,b). Nonclotting γ-thrombin was made from it by controlled passage through a column of agarose-immobilized trypsin, equilibrated in 0.75 M NaCl at pH 6.2,

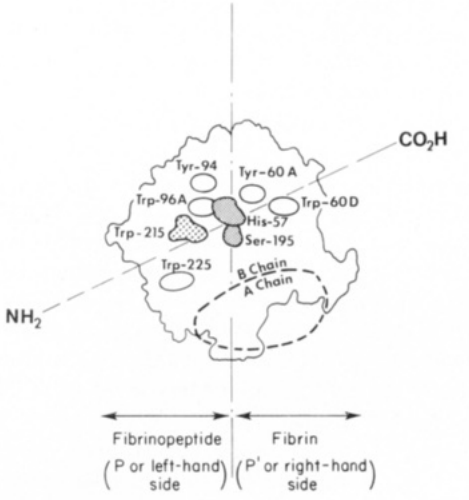

FIGURE 1: A freehand drawing of the bovine thrombin B-chain computer-generated three-dimensional model depicting the approximate locations of the catalytically functional residues His-57 and Ser-195 (chymotrypsin numbering), as well as accessible tyrosines and tryptophans near the catalytic site (Bing et al., 1981; Furie et al., 1982). In this drawing, the protein axis has been rotated 180° such that a peptide substrate would be oriented in the traditional manner (i.e., NH_2---CO_2H). The corresponding peptide groove is divided by the catalytic site (His-57 and Ser-195) into the fibrinopeptide (P or left-hand) side and the fibrin (P′ or right-hand) side. The catalytic site neighboring residue Trp-215 is shown by the crosshatch marks, since it is believed to be an important residue of the apolar binding site (Berliner & Shen, 1977; Bing et al., 1981). The thrombin A chain is drawn as a dashed ellipsoid near the catalytic site (Magnusson et al., 1975; Bing et al., 1977). The carbohydrate moiety and the cleavage site for forming β-thrombin are presumably on the back upper right-hand side and are therefore not shown. While the bovine α-thrombin has a 49-residue A and 259-residue B chain (Magnusson et al., 1975), the isolatable form of the human enzyme has a 13-residue peptide autolytically removed from the NH_2 terminus of its A chain. Amino acid replacements occur in either human chain and are clustered in sequences believed to be of minor structural importance (Fenton, 1981, and references cited therein).

~23 °C (Bing et al., 1977; Fenton et al., 1977b). Nonclotting NO_2-α-thrombin was prepared by using tetranitromethane (Sokolovsky et al., 1966) by diluting stock α-thrombin 1:1 with 100 mM Tris buffer at pH 8.0 and adding 100 μL of the reagent/mL of solution at ~23 °C. When the clotting activity had fallen to <2% within 1.5–2 h, the reaction was terminated by addition of a 0.5 volume of 1.0 M sodium phosphate at pH

6.5, and excess tetranitromethane was removed by centrifugation. The derivatized protein was then dialyzed against several changes of 0.75 M NaCl at 4 °C until the odor of the reagent was no longer present. As with the bovine enzyme (Lundblad & Harrison, 1971), the method produces approximately four NO_2-tyrosines per α-thrombin (Fenton et al., 1979).

Inactivated $MeSO_2$-α-thrombin was made by diluting stock α-thrombin 1:1 with 1 M Tris buffer at pH 8.0 and adding 10 μL of $MeSO_2$-F/mL of the reaction solution at ~23 °C. After 20 min and at 20-min intervals until the clotting activity declined to <2%, an additional 5 μL of reagent/mL of solution was added. Similarly, $PhMeSO_2$-α-thrombin was made by substituting 2.5 μL of 0.1 M $PhMeSO_2$-F (in acetone) per mL of the pH 8.0 buffered enzyme solution, and i-Pr_2P-α-thrombin was made with 10 μL of 0.2 M i-Pr_2P-F (in acetone) per mL of solution, except that clotting activities were monitored at 10-min intervals in the latter case.

Affinity-labeled Tos-Lys-CH_2-α-thrombin was prepared by adding a 100-fold molar excess of solid Tos-Lys-CH_2-Cl to the enzyme in 0.75 M NaCl buffered with 100 mM Tris at pH 8.0 and allowing the reaction to proceed at ~23 °C for ~2 h, at which time essentially no further loss of clotting activity occurred. Similarly, D-Phe-Pro-Arg-CH_2-α-thrombin was made by reaction with a 6-fold molar excess of reagent for 30 min. Exo site affinity-labeled m-CP(PBA)-α-thrombin was prepared essentially by the method of Bing et al. (1977).

All modified thrombin preparations were dialyzed overnight against 0.75 M NaCl at 4 °C prior to frozen storage at −70 °C. The purity and activities of thrombin preparations (Table I) were examined by methods previously described (Fenton et al., 1977a). Protein concentrations were calculated from the absorbance at 280 nm assuming an extinction coefficient of 1.83 mL mg^{-1} cm^{-1} for all thrombin in 0.1 M NaOH. An M_r of 36 500 was used for human thrombins, while 38 000 was assumed for the bovine enzyme.

Proflavin Binding Spectral Measurements. The majority of spectral data presently reported were collected at ~23 °C with a Varian Model 210 dual-beam recording spectrophotometer, equipped with automatic base-line correction and sample measurement timing systems. The data obtained with this more sensitive instrument reconfirmed our initial data obtained with an Aminco-Chance dual-beam spectrophotometer (Sonder et al., 1979).

Proflavin stock solutions were made at concentrations ranging from 1.0 to 50 mM in 0.75 M NaCl (or in appropriate salt solution from 0.15 to 3.0 M NaCl) buffered with 30 mM Tris at pH 7.4 and were stored at ~23 °C, shielded from light with aluminum foil. For proflavin in 0.75 M NaCl under these conditions, the absorbency at 444 nm was 27.9 ± 6.2 mM^{-1} cm^{-1}.

Proflavin difference spectra for various thrombin forms were determined in the following manner. To 1.00 mL of 50 mM proflavin stock solution was added 100 (or 200) μL of ~50–100 μM thrombin solution to the sample cuvette, while 100 (or 200) μL of the corresponding salt solution was added to the same volume of the proflavin stock solution in the reference cuvette. Following mixture by inverting the capped cuvettes, the difference spectrum was scanned from 500 to 400 nm to determine the wavelength of maximum absorbance (λ_{max}) for the bound dye with each thrombin form examined. Absorbance measurements were made at the appropriate λ_{max} with 13 individual proflavin stock solutions in the same manner as described above. In order to correct for signal fluctuations at low absorbance measurements (e.g., i-Pr_2P-α-thrombin), 2-s measurements were recorded every 60 s over a 15-min period, and an average absorbance ($\bar{A}$) from these measurements was used for calculating dye binding.

For each thrombin form, absorbance corrections were made by linear regression of $\bar{A}$ vs. proflavin concentration, [P], with a Texas Instruments TI-55-II calculator ($r > 0.92$, $n > 5$). The absorbance at [P] = 0 was then subtracted from $\bar{A}$ to give the corrected absorbance ($\bar{\bar{A}}$). The infinite dye concentration absorbance ($\bar{\bar{A}}_\infty$) was obtained by linear regression of $\bar{\bar{A}}^{-1}$ vs. $[P]^{-1}$ and solving for the reciprocal of $\bar{\bar{A}}^{-1}$ at $[P]^{-1} = 0$ ($r > 0.93$, $n > 5$). From this $\bar{\bar{A}}_\infty$ value and the known enzyme concentration, [E], the difference extinction coefficient ($\Delta\epsilon$) was calculated for the individual enzyme–proflavin complexes. The complex concentration, [EP], was then calculated for each dye concentration trial from the observed absorbance (A_{obsd}), where $[EP] = A_{obsd}/\Delta\epsilon$. The free enzyme, [E], and proflavin, [P], concentrations were obtained by subtracting the complex concentration from the initial enzyme, $[E_0]$, and proflavin, $[P_0]$, concentrations ($[E] = [E_0] - [EP]$; $[P] = [P_0] - [EP]$) and used to obtain K_d values ($[K_d] = [E][P]/[EP]$). Values of ±1 SDM were calculated for independently determined K_d values. The number of binding sites per α-thrombin and K_d were calculated for the human enzyme by the method of Scatchard (1949). These were obtained by linear regression ($r = 0.997$, $n = 8$) of [EP] $([E_0][P])^{-1}$ vs. $[EP]/[E_0]$, where the slope is $-K_d^{-1}$ and the intercept is nK_d^{-1}.

Measurement of Proflavin Inhibition of Esterolytic Activity. Hydrolysis of Bz-Arg-OEt by human α-thrombin was monitored potentiometrically with a Radiometer Model TTT 60 titrator equipped with a PHM 62 pH meter, an ABU 12 autoburet, and an REA 160 recorder. Stock solutions of 18 mM Bz-Arg-OEt and 100 μM proflavin were prepared in 0.75 M NaCl. These and 0.75 M NaCl diluent solution were degassed under vacuum, titrated to pH 7.4, and stored under N_2. Concentrations of 50–500 μM Bz-Arg-OEt and 0.5–50 μM proflavin were made by bringing the reactant mixture to 9.0 mL with 0.75 M NaCl diluent. To this was added 5 μL of ~100 μM α-thrombin in 0.75 M NaCl, and hydrolysis of the substrate was followed by titration to pH 7.4 with 8 mM NaOH under N_2 at ~23 °C. Initial velocities (v_0) were obtained for four trials each with four substrate and ten proflavin ($[P_0]$) concentrations. Plots of v_0^{-1} vs. $[P_0]$ were constructed to determine the type of enzyme inhibition and to estimate K_i. The K_i was also obtained by linear regression ($r > 0.97$, $n = 6$), calculated for the four independently determined K_i values.

Results

Well-characterized thrombin preparations were used in all experiments (e.g., Table I). With the highly sensitive Varian Model 210 spectrophotometer, the K_d values determined for proflavin binding to human α-thrombin did not significantly differ in 0.15–3.0 M NaCl buffered with 30 mM Tris at pH 7.4, ~23 °C (eight NaCl concentrations, three trials each with seven proflavin concentrations; data not shown). This validated our previous (Sonder et al., 1979) and present use of 0.75 M NaCl to avoid solubility problems with certain derivative thrombin forms. Despite the use of a highly sensitive spectrophotometer with an automatic base-line correction and the collection of large numbers of data points (e.g., four or five trials with 13 proflavin concentrations), the coefficient of variability was large, ranging from 12% for human α-thrombin to 38% for i-Pr_2P-α-thrombin (Table I; Figure 2). In fact, ±2 SDM for the 95% confidence level with the latter form (±940 μM) is sufficiently large to include the other low-binding forms (i.e., $PhMeSO_2$-α- and D-Phe-Pro-Arg-CH_2-

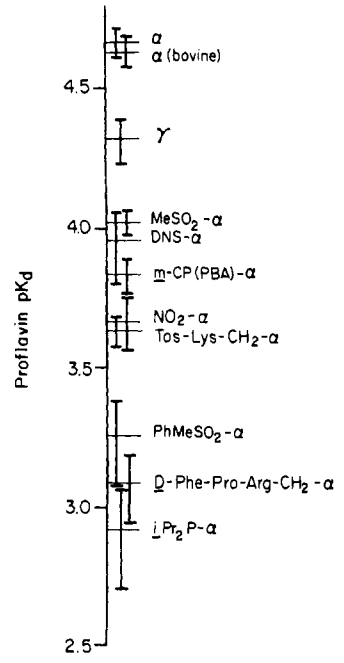

FIGURE 2: Proflavin binding scale for various thrombin forms. Unless indicated, values are for the human enzyme. Data were collected for enzyme forms at ~23 °C in 0.75 M NaCl buffered with 30 mM Tris at pH 7.4, as described in Table I, and are expressed as pK_d values ($-\log K_d$). Error bars give ±1 SDM.

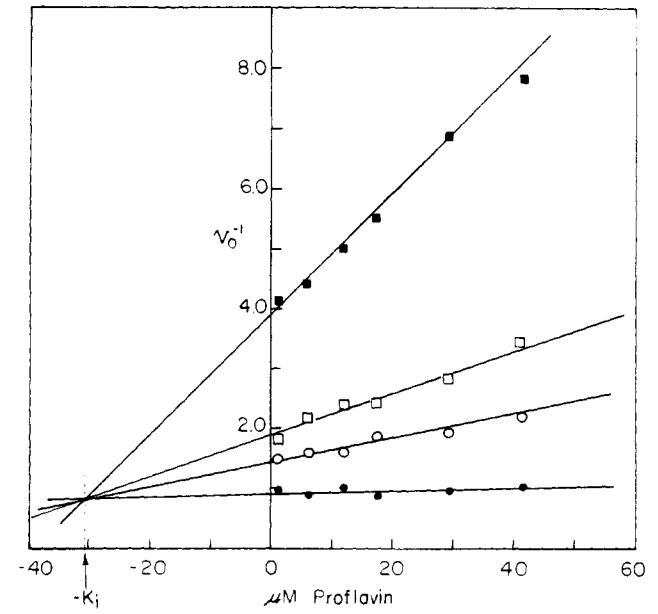

FIGURE 3: Proflavin inhibition of human α-thrombin catalyzed hydrolysis of Bz-Arg-OEt. Concentrations of proflavin ranged from 0.5 to 50 μM, while those of Bz-Arg-OEt were 50 (■), 75 (□), 100 (○), and 500 μM (●). Reactions were started by addition of 5 μL of 100 mM α-thrombin. Initial velocities were obtained by titration with 8 mM NaOH (see Materials and Methods). As shown, the extrapolated value for $-K_i$ is −31 μM.

α-thrombin). By this criterion, the intermediate binding forms differ from the parent human enzyme form, and human α- and γ-thrombins are marginally different, whereas human and bovine α-thrombins are clearly not significantly different (i.e., ≤±1 SDM of each other).

The present values for human and bovine α-thrombins compared favorably with that of 21 μM for the bovine enzyme at neutral pH as reported by Koehler & Magnusson (1974) and the range of values for either enzyme reported by Li et al. (1974). When absorbance corrections were not made, our K_d values were ~33% lower, more closely in agreement with those of the lower range of reported values (Berliner & Shen, 1977; Chang et al., 1979). On the other hand, our corrected values (Table I) are similar to those of 25, 170, and 730 μM for α-, Tos-Phe-CH_2-α-, and *i*-Pr_2P-α-chymotrypsin, as reported by Weiner (1966). Since the active-site-containing B chain of α-thrombin structurally resembles α-chymotrypsin (Magnusson et al., 1975; Bing et al., 1981, Furie et al., 1982), such close agreement between K_d values is not unexpected. In addition, Scatchard analysis of the corrected spectral data for human α-thrombin gave a K_d of 22.0 μM, in agreement with 21.7 ± 2.6 μM (Table I) calculated by assuming 1:1 stoichiometry. Scatchard analysis further gave a value of 0.996 binding site/α-thrombin, confirming a single specific site.

Neither clotting nor synthetic substrate activities (i.e., percent NPGB activities in Table I) were obligatory for proflavin binding (Table I; Figure 2). The $MeSO_2$-α-thrombin form, for example, was enzymically inactive (<0.3 clotting unit/mg; <2% NPGB active), yet it bound the dye with only an ~4-fold increased K_d relative to that of the native enzyme. Thus, neither the unmodified Ser-195 (Figure 1) nor the intact catalytic apparatus is necessary for proflavin binding, as they are for enzymic activity. However, as the Ser-195 derivatizing group was increased in size, the K_d increased ~26- and 55-fold for $PhMeSO_2$-α- and *i*-Pr_2P-α-thrombins, respectively (Table I), suggesting that the dye nevertheless bound near the catalytic site. The relative K_d for Tos-Lys-CH_2-α- and *m*-CP-(PBA)-α-thrombins did not follow such steric hindrance effects, implying that the dye could partially displace these relatively large ligands with differing sites of attachment (see Discussion). On the other hand, the K_d for D-Phe-Pro-Arg-CH_2-α-thrombin approached that of *i*-Pr_2P-α-thrombin, and clearly this tripeptide ligand must be covalently attached within the upper fibrinopeptide groove adjacent to or at the catalytic site (Figure 1).

To further demonstrate that proflavin bound at or near the catalytic site, the dye was examined as an inhibitor of Bz-Arg-OEt hydrolysis by human α-thrombin. The v_0^{-1} vs. $[P_0]$ plot gave a common intercept, which was above the abscissa for a simple competitive inhibitor (Mahler & Cordes, 1971) and extrapolated to −31 μM for $-K_i$ (Figure 3). The K_i calculated directly for the data was 30.5 ± 2.8 μM and did not differ significantly from the corresponding K_d of 21.7 ± 2.6 μM (Table I), suggesting that both methods measured binding at the same site.

In other experiments, since $MeSO_2$-α-thrombin bound proflavin moderately well, we attempted to displace the dye with protein inhibitors of the enzyme (Table II). The results confirmed that either antithrombin III in the presence of heparin (Chang et al., 1979) or hirudin (Landis et al., 1978) can displace proflavin from γ-thrombin, as well as from α-thrombin, and therefore does not require certain active-site regions necessary for fibrinogen clotting activity. On the other hand, only hirudin could displace the dye from catalytically inactivated $MeSO_2$-α-thrombin, while neither protein inhibitor could displace proflavin from *i*-Pr_2P-α-thrombin.

Discussion

The location of the catalytic site within the cleft or groove for protein substrates defines active-site regions on either side (Figure 1). A variety of evidence has indicated that a major noncovalent protein binding or the fibrin(ogen) recognition site region resides on the right-hand side of the catalytic site (the corresponding fibrin side), while other evidence has indicated the existence of an important apolar binding site on the left-hand side, adjacent to the catalytic site (Fenton, 1981).

Table II: Displacement of Proflavin from Various Human Thrombin Preparations by Hirudin and Antithrombin III in the Presence of Heparin[a]

preparations	% proflavin displaced: antithrombin III plus heparin	% proflavin displaced: hirudin
α-thrombin	>90	>90
γ-thrombin	>90	>90
$MeSO_2$-α-thrombin	<1	>90
i-Pr_2P-α-thrombin	<1	<2

[a] Preparations used in these experiments are similar to those shown in Table I. Experiments were performed with an Aminco Chace spectrophotometer at λ_{max} 465 nm and ~23 °C. Proflavin and thrombin concentrations were ~20 and 3–6 μM, respectively, in 0.75 M NaCl buffered with 25 mM Tris at pH 7.4. Hirudin and antithrombin III concentrations were made equal molar to that of α-thrombin. Heparin was used at final concentration of ~4 μM.

Such an apolar binding site has been implicated in proflavin binding, where this dye binds between the site for benzamidine (the arginine side-chain pocket) and an apolar site for indole [Figure 6 in Berliner & Shen (1977)]. Although the proflavin binding site has been thought to reside on the left-hand side, by the relatively conserved nature of the site in γ-thrombin (Chang et al., 1977; Figure 2) and by the location of Trp-215 in the B-chain model (Bing et al., 1981; Figure 1), the location of the site has lacked experimental verification.

No significant difference was found between the proflavin K_d for either human or bovine α-thrombin (Figure 2). This clarifies discrepancies in the literature, which are most likely due to experimental differences (e.g., pH) rather than those attributable to the species of origin [cf. Koehler & Magnusson (1974) and Li et al. (1974)]. Thus, the 13-residue peptide, which is autolytically removed from the NH_2 terminus of the A chain of human α-thrombin and is not required for clotting activity (Fenton et al., 1977a), is also not required for proflavin interactions. The competitive inhibition of Bz-Arg-OEt hydrolysis with human α-thrombin by proflavin agrees with the predicted proximity of the dye binding to the catalytic site (Figure 3). The graphically determined K_i of ~31 μM and the calculated value of 30 ± 3 μM agree closely with the K_d of 21.7 ± 2.6 μM (Table I). A K_d of 22.0 μM was further calculated from spectral data by Scatchard analysis, where 0.996 binding site/α-thrombin was obtained. The absence of major salt effects of increasing NaCl concentrations on the proflavin K_d values is consistent with the small effects of this salt relative to other group Ia chlorides on synthetic substrate binding parameters (Landis et al., 1981).

Of the various modified human enzyme forms, *i*-Pr_2P-α-thrombin bound proflavin with the lowest affinity (Figure 2). The very sterically hindering *i*-Pr_2P– group attached to Ser-195, therefore, blocked regions that neighbor the catalytic site and contribute to proflavin binding. Similarly, the $PhMeSO_2$– group, which also conjugates to Ser-195, interfered with proflavin binding to about the same extent. On the other hand, the much smaller $MeSO_2$– group, which attaches to Ser-195, had only a modest effect on decreasing proflavin binding. In fact, hirudin (Markwardt, 1970) displaced proflavin from $MeSO_2$-α-thrombin (Table II), confirming that the catalytic serine is not necessary for hirudin complexing (Landis et al., 1978; Fenton et al., 1979). In contrast, antithrombin III in the presence of heparin was incapable of displacing the dye (Table II). Since the K_d is ~4 μM for the α-thrombin–antithrombin III complex in the presence of heparin (Olson & Shore, 1982), the dye should have been partially displaced, if the initial interaction is a simple equilibrium at binding sites other than the catalytic site per se. As with α-thrombin, antithrombin III readily displaced proflavin from γ-thrombin (Table II), as previously reported (Chang et al., 1977).

The exo site affinity-labeling reagent *m*-CP(PBA)-F caused an intermediate reduction in proflavin binding (Figure 2). This relatively large reagent presumably is bound via its benzamidine and subsequent apolar moieties [Figure 2 in Bing et al. (1977)]. Its fluorine-hydrolyzed derivative has a K_i of ~4 μM, and the reagent interacts with active-site regions common among α- and γ-thrombins. Since the reactive sulfonyl fluoride group of the reagent is distal to the arginine side-chain pocket-directed benzamidine group, the reagent conjugates with residues removed from the catalytic site (e.g., ~80% on the B and ~20% on the A chains; Bing et al., 1977). The conjugated ligand, thus, could be rotated away from regions adjacent to the catalytic site by active-site-directed reagents. This ligand, therefore, appears to have been partially displaced by proflavin, which is also a relatively large apolar compound [Figure 6 in Berliner & Shen (1977)]. The corresponding apolar binding sites for either of these compounds appear to be on the left-hand side of the catalytic site (Figure 1), since both *m*-CP(PBA)-F and proflavin have essentially no difference in their interactions with either α- or γ-thrombin (Bing et al., 1977; Chang et al., 1979).

Likewise, affinity labeling α-thrombin with Tos-Lys-CH_2-Cl caused an intermediate decrease in proflavin binding (Figure 2), even though this reagent reacts in the opposite direction at His-57 in the catalytic site (Glover & Shaw, 1971). The Tos-Lys-CH_2– ligand clearly should reside to the left of the catalytic site (Figure 1) but with a relatively low affinity, since the reagent reacts slowly in comparison to with D-Phe-Pro-Arg-CH_2-Cl (see Materials and Methods). Thus, proflavin appears to also be able to partially displace the Tos-Lys-CH_2– ligand, although to a lesser extent than the *m*-CP(PBA)– ligand.

On the other hand, the thrombin-selective reagent D-Phe-Pro-Arg-CH_2-Cl (Kettner & Shaw, 1977) decreased proflavin binding almost to the same extent as the sterically obstructing *i*-Pr_2P– group (Figure 3). The tripeptide ligand of this reagent should occupy the arginine side-chain pocket and associate with neighboring apolar binding sites through interactions with the D-Phe-Pro– groups (Figure 4). Moreover, the tripeptide ligand corresponds to the fibrinopeptide side of Aα cleavage in fibrinogen, confirming that the proflavin binding site is located on the left-hand side and adjacent to the catalytic site, as suggested in the model of Berliner & Shen (1977). Their model further depicted the proflavin binding site overlapping that for benzamidine within the arginine side-chain pocket and that for indole or similar apolar compounds. Our data thus support their model, as also does the nature of the thrombin cleavage site on antithrombin III (Björk et al., 1981).

Human γ-thrombin has its B chain proteolytically cleaved into three similar size, noncovalently associated fragments, where each fragment contributes components of the charge relay system [Figure 1 in Bing et al. (1977)]. This form, unlike α-thrombin, essentially lacks clotting activity but like the parent enzyme form retains reactivity with proflavin and antithrombin III (Chang et al., 1979), as confirmed by the present data (Tables I and II). Our data also confirm that γ-thrombin binds the dye with slightly less affinity than the native enzyme (Figure 2). This finding suggests that the apolar region adjacent to the catalytic site might be slightly constrained in γ-thrombin, as suggested by spin-label reagents conjugated at Ser-195 (Berliner et al., 1981).

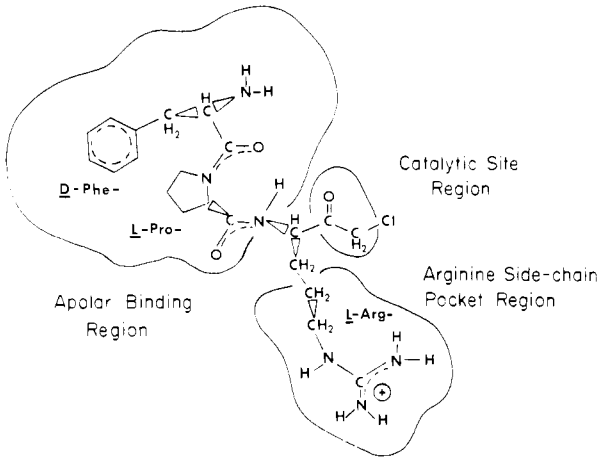

FIGURE 4: A schematic drawing of the thrombin affinity-labeling reagent, D-Phe-Pro-Arg-CH_2-Cl, of Kettner & Shaw (1979) and probable corresponding regions within the active site of thrombin. The orientation shown is such that the inhibitor would align with active-site components shown in Figure 1. For example, the arginine side-chain pocket would project downward and slightly to the right of the catalytic site (His-57 and Ser-195), while the apolar binding region would reside to the left of the catalytic site (e.g., being partially formed by Trp-215).

Differential loss of clotting vs. synthetic substrate activities also can be achieved by selective chemical modification. Nitration of approximately four tyrosines in α-thrombin (Table I) caused an intermediate reduction in proflavin binding (Figure 2). This suggests that the modification of a nearby tyrosine (e.g., Tyr-60A or -94 in Figure 1) might induce microenvironmental conformational changes constraining the dye binding site. On the other hand, modification of approximately four tryptophans (Table I) caused a lesser effect than nitration (Figure 2). Therefore, if Trp-215 participates in the formation of the apolar binding site adjacent to the catalytic site (Bing et al., 1981), this residue must be less accessible to chemical modification than other residues involved in fibrinogen recognition (e.g., Trp-60D in Figure 1).

In summary, our data strongly suggest that the proflavin binding occurs at a single site within the fibrinopeptide groove proximal to the catalytic site. This dye binding site appears to correspond to a prominent apolar binding site region, which is important for interactions with synthetic substrates and inhibitors, as well as with antithrombin III. This site might also be important in various other nonclotting thrombin functions.

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Registry No. Proflavin, 92-62-6; thrombin, 9002-04-4.

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