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Physical Evidence for an Apolar Binding Site Near the Catalytic Center of Human α-Thrombin†

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ABSTRACT: Proflavin dye displacement studies, electron spin resonance active spin label studies and Tos-Arg-OMe esterase rate measurements on human α-thrombin have shown the presence of an apolar binding site for indole which may reside quite near the active center. Indole displaced proflavin from its binding locus at the thrombin active site with a dissociation constant K_{In} = 10.6 ± 1.3 mM (pH 6.5, 0.05 M sodium phosphate, 0.75 M NaCl, 25 °C) as measured by the decrease in the 468-nm difference spectral maximum for the human α-thrombin–proflavin complex. Only 3 isomorphous fluorosulfonylphenyl spin-labeled thrombins of a broad series were effected by indole binding whereas all 14 in the series were altered in their rotational mobility upon binding basic ligands such as benzamidine or *p*-chlorobenzylamine. This confirmed that indole binding was localized to a unique site in the active site region. Tos-Arg-OMe esterase activity of human α-thrombin was activated by indole while clotting activity was unaltered. The Tos-Arg-OMe activation was observed if the substrate concentration (1 mM) remained below those concentrations where (bovine) thrombin normally displayed substrate activation (e.g., 5 mM Tos-Arg-OMe). At 5 mM Tos-Arg-OMe indole did not activate the hydrolytic rate. These rate effects were examined in both no salt and in 0.3 M NaCl. The esterase activity was fourfold greater in the *absence* of NaCl. The apparent activation equilibrium dissociation constant for indole at pH 8.1 in *no* salt, 25 °C, was K_{In} = 4.3 ± 1.8 mM. The same constant in 0.3 M NaCl was not accurately measurable due to solubility problems but was approximately 10–50 mM. A model is proposed which places the indole binding site no farther than a proflavin molecular length from the basic substrate binding pocket.

Thrombin (EC 3.4.21.5) is a unique seryl protease in its (apparently) specific interactions at the physiological level with, e.g., its primary peptidyl substrate, fibrinogen, with platelets, and with several other coagulation proteins in serum (factors II, V, VIII, XIII, etc.). It is generated by proteolytic cleavages of its circulating zymogen (prothrombin or coagulation factor II) through a unique complex of several blood and tissue factors. Thus this enzyme is a serine protease of restricted "trypsin like" specificity yet functions *specifically* in several hemostatic events. Despite the importance of human thrombin, comparatively little is known about this latter species relative to the bovine enzyme, upon which most of the past structure–function work on thrombin has been focused. With the recent availability of isolation procedures for human thrombin of high purity and activity, this protein is now amenable to physicochemical investigations at the molecular level (Fenton et al., 1977a,b).

The importance of apolar or hydrophobic binding regions

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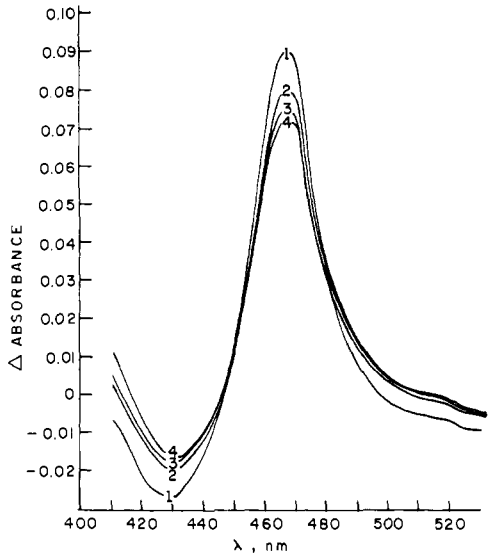

FIGURE 1: Difference absorbance spectrum of 12.5 μM proflavin–13.0 μM human α-thrombin, pH 6.5, 0.05 sodium phosphate, 0.75 M NaCl, 25 ± 0.5 °C, in the absence and presence of indole: (1) none, (2) 2.9 mM, (3) 5.0 mM, (4) 10.0 mM, respectively. Proflavin and enzyme stock solutions were made up fresh and kept at 4 °C until use.

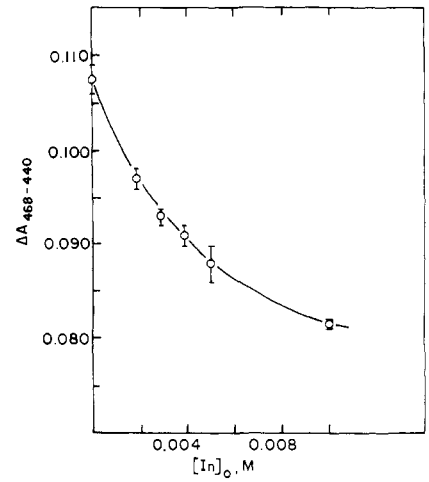

FIGURE 2: Decrease in absorbance with increasing indole concentration for the spectra in Figure 1. These data may be applied to eq 1 using the extinction coefficients noted in the text to calculate the dissociation constant, K_{In}, for the competitive binding of indole to the thrombin–proflavin complex. See Table I for results. Indole solubility at these salt concentrations was limited to ~10 mM.

in thrombin structure has been suggested from the affinity chromatography studies of Thompson and Davie (1971) and Thompson (1976). Human thrombin, when bound to *p*-chlorobenzylamido-ε-aminocaproylagarose, was removable only with very high concentrations of the relatively apolar ligand, dioxane, while 1 M benzamidine was practically ineffective in eluting the enzyme from the affinity gel.

While in the studies referred to above it was not possible to characterize such a site with respect to the catalytic center, we present here kinetic, spectroscopic, and magnetic resonance results which demonstrate the existence of an apolar binding site which lies near both the catalytic serine and the active site basic binding pocket.

Materials and Methods

The following chemicals were obtained from commercial sources: Proflavin sulfate (Lot No. W3716), Schwarz/Mann; benzamidine hydrochloride hydrate (Lot No. 022567) and *p*-chlorobenzylamine·HCl, Aldrich Chem. Co.; indole and tosylarginine methyl ester were from Sigma Chemical Co. and ICN-NBC, respectively. Electrophoretically pure human α-thrombin, 2000–2500 NIH units/mg, was a generous gift of Dr. John W. Fenton, II, New York State Department of Health, Albany. Thrombin (mol wt 36 500) concentration was estimated by its absorbance at 280 nm using a coefficient of 1.83 mL mg^{-1} cm^{-1} in 0.1 M NaOH (Fenton et al., 1977b). Fluorosulfonyl spin labels were synthesized by the procedures of Wong et al. (1974). Thrombin esterase activity was monitored either on a Radiometer pH stat system or a Unicam SP1800 spectrophotometer at 25 °C with Tos-Arg-OMe[1] (Hummel, 1959). Clotting activity was measured by the method of Fenton et al. (1977b) with a Fibrometer. Proflavin difference spectra and thrombin–proflavin equilibrium data were measured under conditions similar to those reported by Koehler and Magnusson (1974) for bovine thrombin. An extinction coefficient of 3.55×10^4 M^{-1} cm^{-1} for free proflavin was based on the work of Skalski et al. (1973). ESR spectra were measured at ambient temperature (26 ± 2 °C) on a Varian E-4 spectrometer.

[1] Abbreviations used: Tos-Arg-OMe, tosylarginine methyl ester; ESR, electron spin resonance; In, indole.

Results

Proflavin Dye Displacement. Figure 1 shows difference spectra for human α-thrombin–proflavin complex (curve 1). The peak at 465 to 470 nm represents the complex while the trough near 440 nm accounts for free proflavin. The dissociation constant for this complex at pH 6.5, $K_{EF} = 8.7$ μM, was measured from the absorbance difference $\Delta A_{468-440nm}$, where $\Delta\epsilon_{468-440nm} = 2.0 \times 10^4$ M^{-1} cm^{-1} which was comparable to the value of 21.2 μM for the bovine enzyme at pH 7.2 (Koehler and Magnusson, 1974). As found with the bovine enzyme, proflavin was displaced from human α-thrombin upon addition of the basic competitive inhibitors benzamidine or *p*-chlorobenzylamine. However, a similar effect was observed in the presence of the *apolar* ligands indole or dioxane, as demonstrated in Figure 1 by the decrease in the proflavin–thrombin complex difference spectrum with increasing indole concentrations (curves 2, 3, 4). Figure 2 shows a plot of this spectral decrease with increasing indole concentration. When these data were analyzed by the treatment of Brandt et al. (1967) assuming a competitive dissociation model

$$K_{In} = \frac{(EF)}{(PF)_0 - (EF)} K_{EF}(In)_0 \times \left[E_0 - (EF)\left(1 + \frac{K_{EF}}{(PF)_0 - (EF)}\right)\right]^{-1} \quad (1)$$

[where (EF) = concentration of thrombin–proflavin complex, $(PF)_0$ = total proflavin concentration, $(E)_0$ = total active thrombin concentration, $(In)_0$ = total indole concentration, and K_{EF} = thrombin–proflavin complex dissociation constant], a dissociation constant, K_{In}, of 10.6 ± 1.3 mM was obtained. Similar spectral changes were observed with dioxane; however, the deviation from hyperbolicity of these data suggested that both displacement and aspecific effects were elicited on the proflavin–thrombin complex, precluding any accurate estimate of equilibrium parameters.

ESR Results. In the course of our extensive comparison of active site conformations of related serine proteases (Berliner and Shen, 1977; Shen, 1977) with a series of spin-labeled sulfonyl fluorides (Berliner and Wong, 1974), we found a

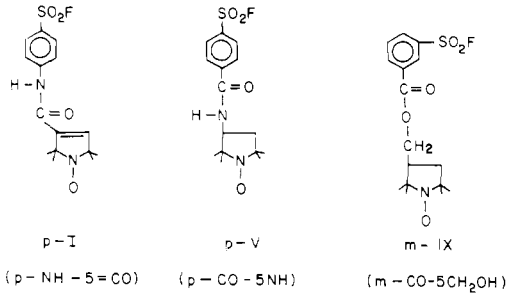

FIGURE 3: Spin-labeled sulfonyl fluorides which were affected by indole binding.

general ESR spectral effect on spin-labeled thrombins when exposed to basic ligands such as benzamidine, *p*-chlorobenzylamine, or valerylamidine. This was not totally unexpected since the basic substrate side chain binding pocket in thrombin should be adjacent to the catalytic serine if analogies to trypsin, α-chymotrypsin, or elastase tertiary structure are relevant (Stroud, 1974). However, the exposure of these spin-labeled thrombins to indole was without effect *except* for the three labels shown in Figure 3; one example, p-V (p-CO-5NH), is shown in Figure 4. Thus upon exposure to a saturated (~10 mM) indole solution at pH 6.5, 0.05 M sodium phosphate, 0.75 M NaCl, the nitroxide moiety in the upper spectrum becomes more immobilized (center spectrum) as evidenced particularly by the further downfield shift of the broad line feature on the left side of the spectral line shape (arrow). For comparison the lower spectrum shows the effects of exposure to either *p*-chlorobenzylamine or benzamidine.

Esterase and Clotting Activity. Tos-Arg-OMe esterase activity was measured at pH 8.1, 25 °C, under four sets of conditions. Since bovine thrombin catalyzed Tos-Arg-OMe hydrolysis had been reported previously to be subject to both ionic strength and substrate activation effects, we examined these kinetics as a function of added indole in the presence and absence of NaCl at Tos-Arg-OMe concentrations below (1 mM) and well above (5 mM) the substrate activation region (Curragh and Elmore, 1964). The latter reference reported a K_m of 0.032 mM at pH 8.4, 25 °C, while substrate activation appeared over the 1 mM to 5 mM range.[2] Figure 5 shows relative apparent rates for Tos-Arg-OMe hydrolysis vs. indole concentration in the absence of NaCl (upper curve, squares) and in 0.3 M NaCl (lower curve, circles). The data points at 0 mM indole show that for human α-thrombin the rate in no salt (□) was about four times greater than in 0.3 M NaCl (○). Also, under any ionic strength, substrate activation was apparent as evidenced by the rates at 1 mM and 5 mM Tos-Arg-OMe respectively in no salt (□, ■) and 0.3 M NaCl (○, ●), respectively. In no salt (□) indole appeared to "activate" 1 mM Tos-Arg-OMe to a maximal rate which was close to that attained at maximal substrate activation; 5 mM Tos-Arg-OMe produced the same rate effect whether indole was present or not (■). The apparent dissociation constant, K_{In}, for this indole mediated activation was 4.3 ± 1.8 mM as calculated by a nonlinear regression analysis program based on CURFIT from Bevington (1969). This compares well with the proflavin result earlier (Figure 2) of 10.6 ± 1.3 mM when considering the differences in pH and ionic strength conditions.

In 0.3 M NaCl, the lower curve (○) also showed that indole

[2] Since there are no published kinetic analyses of human α-thrombin catalyzed Tos-Arg-OMe hydrolysis, we have assumed that the significance of the substrate concentration range chosen here was similar to that expected for the bovine enzyme.

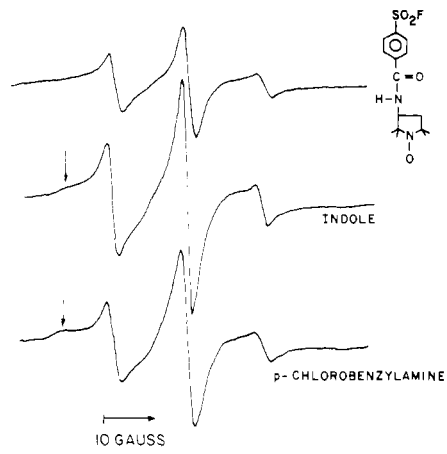

FIGURE 4: X-Band ESR spectra of human α-thrombin spin labeled with the sulfonyl fluoride p-V (p-CO-5NH), pH 6.5, 0.05 M sodium phosphate, 0.75 M NaCl, 26 ± 2 °C. (Upper spectrum) "Native" enzyme; (center spectrum) in the presence of saturated indole (~10 mM); (lower spectrum) in 100 mM *p*-chlorobenzylamine. The arrows denote the principal shift in these spectra relative to the native (upper) spectrum.

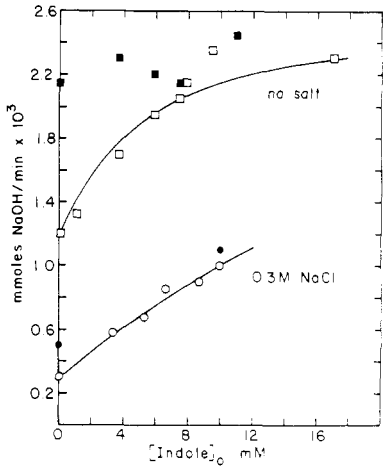

FIGURE 5: Relative rate constants for human α-thrombin catalyzed Tos-Arg-OMe hydrolysis by the pH stat method vs. increasing indole concentration at pH 8.1, 25 °C. Salt and substrate concentrations were (upper curve) no salt with 1 mM or 5 mM Tos-Arg-OMe (□, ■) and (lower curve) 0.3 M NaCl with 1 mM or 5 mM Tos-Arg-OMe (○, ●). The dissociation constant associated with the apparent activation of Tos-Arg-OMe esterase activity was K_{In} = 4.3 ± 1.8 mM for no salt and $K_{In} \approx$ 10–50 mM for 0.3 M NaCl.

activated Tos-Arg-OMe hydrolysis and that the maximal rate attained was the same whether Tos-Arg-OMe was saturating (1 mM, ○) or not (5 mM, ●). While we were limited by solubility to 10 mM indole in the 0.3 M NaCl study, it is interesting to note that the maximal rate in 0.3 M NaCl appeared to approach that for the enzyme in no salt at nonsubstrate activating conditions (□). An accurate measurement of K_{In} was not possible; however, it was approximately 10–50 mM. Table I summarizes physical parameters for the experiments described in this report.

In contrast to the Tos-Arg-OMe esterase results above, the clotting activity of human α-thrombin at pH 7.4, 0.15 M NaCl was totally unaffected by indole concentrations as high as 10 mM nor did indole binding affect benzamidine inhibition of clotting (B. H. Landis, unpublished results).

Discussion

The results presented here clearly show the existence of an indole binding site on human α-thrombin. The location of this

TABLE I: Summary of Physical Parameters.

α-Thrombin species	Human[a]	Bovine[b]
Proflavin dye binding		
λ_{max} (nm)	468	468
K_{EF} (μM)	8.7	21.2
$\Delta\epsilon_{468-440nm}$ (M^{-1} cm^{-1})	2.0×10^4	2.69×10^4
Indole binding, K_{In}		
Proflavin dye displacement[a]	10.6 ± 1.3 mM	
Tos-Arg-OMe esterase activation (pH 8.1, 25 °C)	4.3 ● 1.8 mM	
Tos-Arg-OMe esterase activation (pH 8.1, 0.3 M NaCl, 25 °C)	10–50 mM	

[a] This work, pH 6.5, 0.05 M sodium phosphate, 0.75 M NaCl, 25 °C. [b] Koehler and Magnusson (1974), pH 7.2, 0.1 M phosphate, 25 °C.

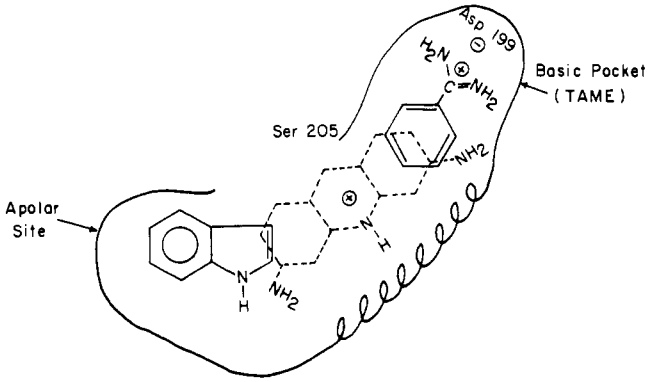

FIGURE 6: Model of active site binding regions in human α-thrombin. The displacement of proflavin (broken lines) by both benzamidine and indole suggests that the proflavin binding locus must overlap both mutually exclusive binding sites. The benzamidine or Tos-Arg-OMe binding site is assumed to be homologous to that for trypsin with the acidic residue Asp-199 at the bottom of the basic binding pocket. The catalytic serine-205 lies near the base of this pocket.

apolar site with respect to the essential catalytic groups and substrate basic side chain binding pocket was inferred from the following considerations:

The spectroscopic data of Figures 1 and 2 suggest that proflavin is displaced from the thrombin active site region in a manner similar to that for basic ligands such as benzamidine or *p*-chlorobenzylamine. Since both proflavin and benzamidine are also competitive inhibitors for thrombin catalyzed Tos-Arg-OMe esterase or clotting activity, it has been concluded that proflavin must overlap with the basic binding pocket (Koehler and Magnusson, 1974). Thus bound indole must overlap with the proflavin binding locus (but not that for benzamidine, which is a substrate inhibition site). We consider it less likely but still possible that the indole could impart a structural change on the enzyme which "reduces" the α-thrombin–proflavin binding interaction. Thus a steric competition or conformational change model for indole binding are considered as two alternatives.

A piece of evidence which may further support the steric competition model is the ESR spin label indole effect (Figure 4). While the effect of basic ligand binding to spin labeled human α-thrombins was not sensitive to the nitroxide geometry on the phenyl ring (Berliner and Shen, 1977; Shen, 1977), this latter effect was probably imparted on the phenylsulfonyl group. On the other hand, indole affected just the three of many possible structurally varied spin-labeled sulfonyl fluorides. When Corey–Pauling–Koltun space filling models of the three labels in Figure 3 were compared, both the phenyl groups and nitroxide moieties overlapped significantly. Furthermore they swept out isomorphous volumes in space when rotated. Thus one specific locus peripheral to the phenylsulfonyl ring was sensitive to indole binding. While the possibility of an indole elicited conformational change at the protein region sensed by these nitroxide moieties cannot be ruled out, the simpler steric competition model is more consistent with the proflavin data above which suggested the close proximity of the indole site to the catalytic center.

Since the x-ray crystallographic structures of either bovine or human thrombins are not yet completed (McKay et al., 1977; Tsernoglou and Petsko, 1977), we can only refer to the high resolution x-ray results on α-chymotrypsin, trypsin, or elastase structures for comparison (Stroud, 1974). With relation to our steric competition model it was interesting to note that the α-chymotrypsin–indole complex was shown to be structurally unchanged from the native enzyme (Steitz et al., 1968). An example of a steric competition effect with fluorosulfonyl spin-labeled proteases was with α-chymotrypsin where the ESR spectral indole effect was consistent with a steric displacement[3] of the spin label from its binding orientation, assuming no protein structural changes occurred as with the native enzyme (Berliner and Wong, 1974). While the ligand binding sites in thrombin are different than for α-chymotrypsin, the example cited above serves at least as a precedent for such a model.

The Tos-Arg-OMe esterase results were another indicator of the effects of indole binding on the active site. While indole mimicked the substrate activation properties of Tos-Arg-OMe, whether it binds at a site separate from or identical with the Tos-Arg-OMe activation site was not assessible from these experiments. It was interesting to note also that some earlier reports had appeared of Tos-Arg-OMe activation by cholate and other detergents (Curragh and Elmore, 1964; Engel and Alexander, 1972; Cole, 1974) as well as by prothrombin fragment II (Myrmel et al., 1976). It is also interesting to note that with trypsin Howard and Mehl (1965) found an 8% activation of Tos-Arg-OMe hydrolysis by tosylamide. Consequently it would not be unexpected if Tos-Arg-OMe and indole were found to bind at the same (apolar) activation site. Since indole did not alter clotting activity, as was the case with prothrombin fragment II (Myrmel et al., 1976), it is likely that fibrinogen binding was not hindered by indole and that the apolar site was distinct from those implicated in fibrinopeptide B binding (Blömback, 1963).

Dioxane, which was required to displace human or bovine α-thrombin from *p*-chlorobenzylamino-ε-aminocaproylagarose (Thompson, 1976), was also shown to activate Tos-Arg-OMe esterase activity of bovine thrombin while inhibiting clotting (Villaneuva et al., 1974). Unfortunately at the dioxane concentrations where these various effects above manifested themselves (~2 M), it becomes difficult to rule out other aspecific dielectric and water structuring effects.

From the combined results of the measurements described above we favor the steric competition model depicted in Figure 6. Thus the maximum separation between the indole and basic binding pocket must be spanned by the acridine ring system of the proflavin molecule (dotted structure). This model is most

[3] This effect has been termed an *orientation shift* as opposed to a protein *conformation change* (Berliner, L. J. (1977), *Methods Enzymol. 49* (in press)).

consistent with the proflavin and ESR results while the Tos-Arg-OMe esterase activation effects are more difficult to assign to one model or another. If, however, an indole induced conformational change were responsible for all of the phenomena in the spectrophotometric, ESR and kinetic results, the proximity of this site to the catalytic center would be less certain.

The existence of apolar secondary sites in thrombin structure had been suggested indirectly by earlier work (Thompson, 1976). This work has presented direct physical and kinetic evidence for the existence of one (or more) apolar sites for indole on human α-thrombin and also in preliminary experiments with the lower clotting activity molecular form, γ-thrombin. The significance of these binding interactions at the physiological level poses a challenging problem for further study.

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