Binding Subsites in Human Thrombins†

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ABSTRACT: Human α- and γ-thrombins were studied in the presence of indole and nucleotide analogues which were structurally similar to platelet release products. One group of indole analogues (indole, tryptamine, D- and L-tryptophan, and tosylamide) were activators of thrombin-catalyzed tosyl-L-arginine methyl ester (Tos-Arg-OMe) hydrolysis. Serotonin (5-hydroxytryptamine), on the other hand, was an inhibitor of esterase activity. The indole analogues caused release of proflavin from thrombin–proflavin complexes and also caused mobility changes in apolar site sensitive spin-labeled irreversible inhibitors, as reported earlier with indole [Berliner, L. J., & Shen, Y. Y. L. (1977) *Biochemistry 16*, 4622–4626]. Furthermore, an activation of fibrinogen clotting activity was detected in the presence of several indole analogues. Several nucleotides (ATP, ADP, and UDP), as well as inorganic pyrophosphate, were potent inhibitors of fibrinogen clotting activity. A biphasic inhibition curve was observed which was consistent with (at least) two linked binding sites for the inhibitor. ATP was an inhibitor of Tos-Arg-OMe esterase activity, and it also induced proflavin release from thrombin–proflavin complexes, but it did not affect the mobility of apolar site sensitive spin-labels. ATP inhibition of clotting was reduced (but not completely eliminated) in the presence of the clotting activator indole. These effectors were classed into two general groups. One group of indole analogues binds to an apolar region, resulting in Tos-Arg-OMe activation, proflavin release, spin-label mobility changes, and clotting activation. The nucleotide analogues bind to at least two linked sites which result in inhibition of clotting, one or both of which cause proflavin release and Tos-Arg-OMe inhibition. The nucleotide site(s) and apolar (indole analogue) region appeared to be spatially distinct. The implications of these results to platelet interactions at the physiological level are discussed.

Platelets are small anucleate blood cells whose dense granules contain a concentrated mixture of the amine serotonin, calcium, ADP, and ATP. These metabolites both are released into the extracellular environment upon various platelet stimuli and are themselves capable of stimulating platelet aggregation [see, e.g., Phillips (1980) and references cited therein]. Thrombin (EC 3.4.21.5) also plays an important role in platelet aggregation and release reactions (Detwiler et al., 1975); the two distinct functions are believed to involve different structural aspects of the thrombin molecule (Tam et al., 1980). Since platelets also play several known roles in thrombin action (platelet factor 4, fibrinogen, factor V, etc.), we have investigated the role of the smaller platelet release products discussed above (and their analogues) as regulators of thrombin action.

Highly coagulant human α-thrombin may be converted to a three noncovalently associated chain *noncoagulant* γ form by controlled tryptic or autodigestive proteolysis (Fenton et al., 1977b). Although the latter form is structurally different, it retains estero/amidolytic activity toward small substrates while lacking only the ability to convert fibrinogen to fibrin. Our past work with α-thrombin had shown that the hydrophobic ligands indole and dioxane bound specifically to the enzyme, as demonstrated by proflavin dye displacement, interactions with active-site serine spin-labels, and activation of tosyl-L-arginine methyl ester (Tos-Arg-OMe)[1] esterase activity (Berliner & Shen, 1977a). In view of the above observations, we examined structurally similar indole analogues, serotonin, tryptamine, and other tryptophan metabolites as effectors for thrombin. We were also interested in comparing α- vs. γ-thrombin with some indole analogues as further confirmation of those structural similarities between the two forms. The work presented here comprises kinetic and physical studies of thrombin subsite binding by a series of effectors which are structurally related to platelet release products.

Experimental Procedures

Materials. Tos-Arg-OMe, Bz-Arg-OEt, NPGB (lot 9009), Trp-NH_2 hydrochloride (lots 95C-0257 and B7C-02461), serotonin oxalate (lot 77C-0081), indole-3-acetic acid (lot 57C-0341), ATP disodium salt (lot 110C-7410), ADP disodium salt, adenosine, UDP disodium salt (lot 94C-7270), indole (lot 84C-0233), and D- and L-Trp were from Sigma Chemical Co. Tosylamide was a gift of Dr. R. S. Bauer. Bovine fibrinogen (98% clottable) was from Miles Laboratories. Poly(ethylene glycol) 6000 was a gift from Dow Chemical Co. Skatole and 2.5-dimethylindole were gifts from Professor P. G. Gassman. Proflavin sulfate (lot W3716), from Schwarz/Mann, was recrystallized from methanol before use. Dioxane (lot PC-091387), from Aldrich Chemical Co., was passed through activated alumina before use to remove peroxides (Dasler & Bauer, 1946). Tosyl-ChromozymTH was a product of Boehringer Mannheim Biochemicals. Sulfonyl spin-labels were synthesized as described earlier (Wong et al., 1974).

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[1] Abbreviations: Tos-Arg-OMe, tosyl-L-arginine methyl ester; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Bz-Arg-OEt, benzoyl-L-arginine ethyl ester; Trp-NH_2, tryptamine; PEG, poly(ethylene glycol); tosyl-ChromozymTH, tosyl-Gly-Pro-Arg-*p*-nitroanilide; ESR, electron spin resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Table I

compound	λ (nm)	ε (M^{-1} cm^{-1})	ref
indole	216	3.56×10^4	this laboratory
tryptamine	279	5.00×10^3	Jackson & Smith (1965)
serotonin	275	5.80×10^3	Bretherick et al. (1961)
ATP, ADP, adenosine	259	1.54×10^4	Brewer et al. (1974)
Trp	280	5.60×10^3	Brewer et al. (1974)
proflavin	444	3.55×10^4	Koehler & Magnusson (1974)

bany, NY, as highly purified species (α-thrombin was ~3000 NIH units/mg, 95–98% NPGB active sites). Enzyme purity, specific activity, and analysis of polypeptide fragment structure were checked in Albany and in our laboratories by sodium dodecyl sulfate gel electrophoresis (nonreducing, reducing); clotting and esterase (Tos-Arg-OMe) activities and active-site concentration (NPGB) were checked by the procedures described in Fenton et al. (1977a).

The enzyme was stored in 0.75 M NaCl at −72 °C and thawed only immediately before use. Standard thrombin (lot B-3), 21.7 NIH units/mg, was supplied by Dr. D. Aronson, Bureau of Biologics, FDA, Bethesda, MD.

Methods. All spectrophotometric measurements were made on a Unicam SP1800 spectrophotometer. The extinction coefficients used for the compounds examined in this study are shown in Table I. Thrombin concentration was estimated spectrophotometrically at 280 nm, $\epsilon_{280nm} = 1.83$ mL mg^{-1} cm^{-1} in 0.1 M NaOH (Fenton et al., 1977b), or by NPGB active-site titration. A molecular weight of 36 600 was used throughout. Spin-labeled thrombins were prepared as previously described (Berliner & Shen, 1977a).

Esterase activity with Tos-Arg-OMe or Bz-Arg-OEt was measured with a Radiometer pH stat. Clotting activity was measured on a BBL fibrometer based on the assay of Fenton et al. (1977a). ESR measurements were performed on a Varian E-4 spectrometer at X-band frequency. Proflavin displacement was measured spectrophotometrically after the procedure of Koehler & Magnusson (1974). Kinetic and equilibrium constants were derived by computer best fit nonlinear or linear regression using a Hewlett-Packard 9835 or 97S computer.

Results

Tos-Arg-OMe Esterase Activity: Activation. We first examined the activation of Tos-Arg-OMe hydrolysis by both α- and γ-thrombin forms in the presence of indole. Figure 1 shows the rate of Tos-Arg-OMe hydrolysis in the absence (upper curve) and presence (lower curve) of 0.3 M NaCl for identical concentrations of α-thrombin (open symbols) and γ-thrombin (closed symbols) respectively. The rate data were fit by nonlinear regression analysis as before (Berliner and Shen, 1977a) to a simple nonessential activation model in which the activator binds with equal affinity to the free enzyme or enzyme–substrate complex. Both the extent of activation and the apparent activation rate constants were almost identical for both species under each set of conditions.

Several indole analogues were also found to activate α-thrombin-catalyzed Tos-Arg-OMe hydrolysis. The results are summarized in Table II. As compared to the ca. 2-fold activation by indole (see Figure 1), tryptamine activated the rate of Tos-Arg-OMe hydrolysis by 3-fold under the same

Table II: Summary of Binding of Platelet-Release Products and Tryptophan Metabolites to Human α- and γ-Thrombins[a]

	Tos-Arg-OMe[b]		proflavin[c]	clotting[d]	
compound	K_{actn}(app)[e] (mM), no salt (0.3 M NaCl)	K_i(app) (mM)	K_d (mM)	K_i(app) (mM)	K_d (mM)
indole analogues					
indole					
α-thrombin	11.0[f] (11.0)		10.6[g]		
γ-thrombin	8.0[f] (12.2)				
tryptamine					
α-thrombin	6.8 (7.1)		6.9	3–7	>10
γ-thrombin	5.4 (5.4)				
serotonin					
α-thrombin		≤3.0	2.2	3–7	>10
γ-thrombin		≤3.0			
D-tryptophan[h]	4.1				
L-tryptophan[h]	4.8				
indoleacetic acid[h]		4.3			
tosylamide[h]	~3.0				
nucleotide analogues[h]					
ATP		2.6	2.8	biphasic	
ADP				biphasic	
UDP				biphasic	
adenosine				2.1[i]	
PP_i			no effect	17–20	
P_i				~50	

[a] Where only apparent constants were determinable, their comparison with direct physically measured constants should be considered qualitative confirmation of a particular binding phenomenon. Furthermore, it is prudent to point out that since equilibrium dialysis determinations of binding stoichiometry were impossible due to the relatively large dissociation equilibrium constants and the solubility limits of thrombin (Fenton et al., 1977a), the operational definition of a subsite must remain qualitative. [b] Tos-Arg-OMe experiments were performed in 0.4 mM Tris-HCl, pH 8.1, 27 °C, in a pH stat. [c] Proflavin dye binding was conducted in 0.05 M sodium phosphate and 0.75 M NaCl, pH 6.5, 27 °C. [d] Clotting experiments were performed in 0.024 M sodium phosphate, 0.1 M NaCl, pH 7.0, and 0.66% (w/v) PEG 6000, 37.2 °C. [e] The apparent equilibrium constant for a simple nonessential activation model where the activator binds with equal affinity (K_{actn}) to the free enzyme or enzyme–substrate complex. The results are obtained from a hyperbolic nonlinear regression analysis fit to the increase in activity vs. activator concentration. [f] Although these two constants are just within experimental error of one another (±2 mM), the more pronounced difference when shifting to 0.3 M NaCl may be attributable to several differences in their relative stabilities (Bauer et al., 1981; Landis et al., 1981) which have been characterized structurally by both solution studies (Berliner et al., 1981) and molecular modeling (Furie et al., 1982). [g] Berliner & Shen (1977a). [h] All data are for α-thrombin only. [i] Fit to a simple noncompetitive inhibition model.

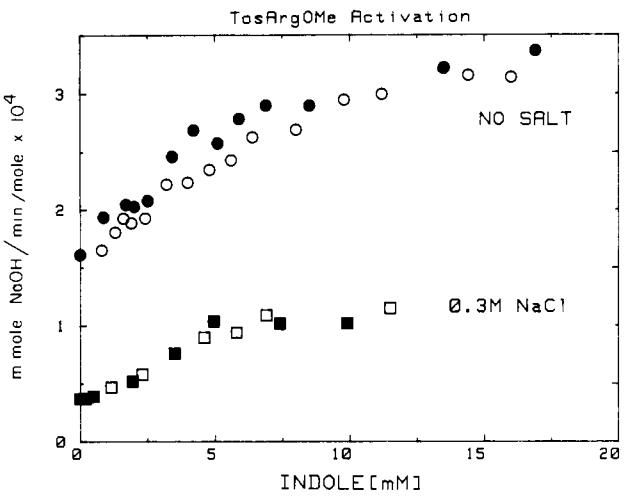

FIGURE 1: Relative rate constants of Tos-Arg-OMe hydrolysis by human α- or γ-thrombin vs. indole concentration. The open symbols (○, □) refer to α-thrombin, while the filled symbols (●, ■) refer to γ-thrombin in the absence (upper curve) or presence (lower curve) of 0.3 M NaCl. Conditions were 0.4 mM Tris-HCl, pH 8.1, 27 ± 0.5 °C, in 1 mM Tos-Arg-OMe. Enzyme concentration was 50 nM.

conditions. The solubility of skatole (1-methylindole) limited us to concentrations below 4 mM; yet the (extrapolated) maximum extent of activation was comparable to that for tryptamine although the apparent K_{actn} was somewhat larger. A similar solubility problem with 2,5-dimethylindole limited our experiments to 0.22 mM, where, however, a 1.5-fold rate activation was observed. Both enantiomers of tryptophan (D and L) effected a 1.7-fold (maximum) rate activation (compared to a 2-fold increase by indole). Lastly, we examined the trypsin activator tosylamide, which activated thrombin-catalyzed Tos-Arg-OMe hydrolysis by ~7% at 3 mM activator vs. 8% for trypsin under similar conditions (Howard & Mehl, 1965).

Tos-Arg-OMe Esterase Activity: *Inhibition.* In contrast to the indole analogues above, two tryptophan metabolites, serotonin (5-hydroxytryptamine) and indoleacetic acid, were found to be inhibitors of α-thrombin esterase activity. The inability of tryptamine or serotonin to totally inhibit the enzyme at saturating ligand concentrations strongly suggested that both inhibitor and substrate bind simultaneously to thrombin. While we have not planned more extensive kinetic measurements with these two inhibitors, their inability to totally inhibit the enzyme at saturating concentrations of inhibitor suggests a partial inhibition model where an enzyme–substrate–inhibitor complex can turn over substrate with reduced affinity (partial competitive inhibition) or with a reduced rate constant (partial noncompetitive inhibition) relative to that for the enzyme–substrate complex alone [see Segel (1975)]. In any case, these models or even more complex possibilities have the common property that both inhibitor and substrate bind *simultaneously* to thrombin. The nucleotide ATP, on the other hand, completely inhibited the enzyme at saturating concentration, suggesting a simple inhibition model.

Fibrinogen Clotting Activity. Figure 2 shows the change in clotting activity caused by the indole analogue tryptamine (○) and serotonin (□). The effects on the fibrinogen clotting rate were first a slight *activation* which was overcome by an *inhibition* at concentrations above ca. 7–9 mM. While it was impossible to derive an accurate fit to a combined activation/inhibition model, the results demonstrated the existence of two sites with apparent binding constants in the ca. 3–7 and >10 mM ranges, respectively. Results similar to those with tryptamine were found with the 5-fluoro analogue where the

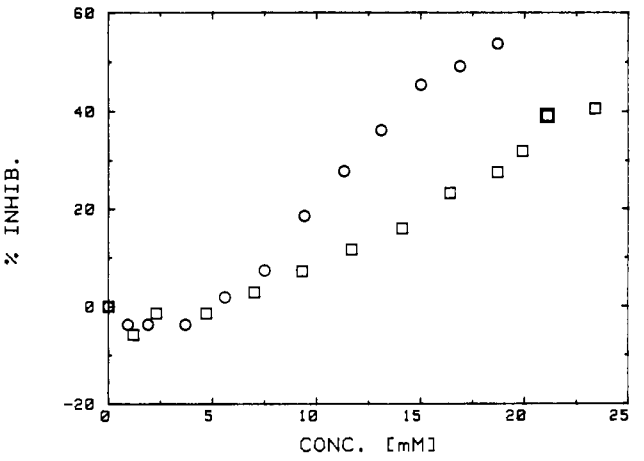

FIGURE 2: Percent inhibition of α-thrombin clotting activity vs. increasing concentrations of tryptamine (○) or serotonin (□). Conditions were 0.024 M sodium phosphate, pH 7.0, 0.1 M NaCl, and 0.66% (w/v) PEG 6000, 37.2 °C. Fibrinogen concentration was 0.67 mg/mL. Clotting times were converted to clotting units from a standard curve. The final activities are plotted as the percent of the initial clotting activity units before addition of the extrinsic ligand. Note that *negative* values of percent inhibition correspond to rate activation.

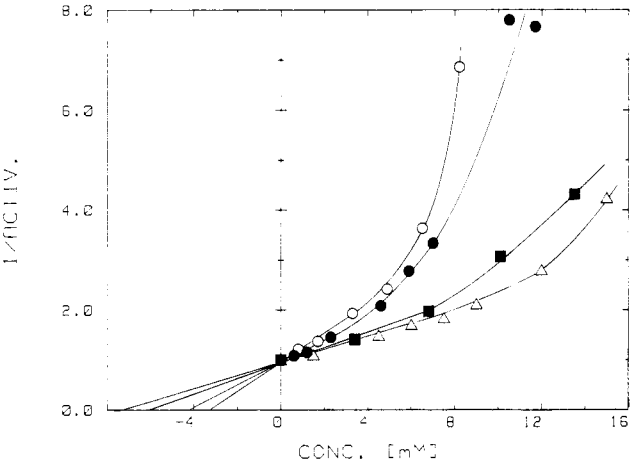

FIGURE 3: Dixon plots of inhibition of α-thrombin clotting activity. The inhibitors tested were ATP (○), ADP (●), PP_i (■), and UDP (△). Conditions were identical with those in Figure 2. The activity data are corrected for ionic strength effects using NaCl as the standard.

activation was even more pronounced (L. J. Berliner and H. Tsunematsu, unpublished results).[2]

[2] The reaction for thrombin-catalyzed clotting of fibrinogen is a two-step process, hydrolysis followed by fibrin polymerization. It is not totally valid to construe changes in clotting time to alterations in enzyme activity unless it is ascertained that the hydrolysis step is always rate limiting. Cases where the polymerization step becomes rate limiting have been reported, albeit under fairly high concentrations of group 1A salts (Landis et al., 1981) or with calcium (Credo et al., 1978). On the other hand, the inclusion of PEG 6000 in our clotting assay was chosen because of its pronounced effect on accelerating the rate of fibrin polymerization whereby thrombin-catalyzed hydrolysis is usually rate limiting (Fenton & Fasco, 1974). The ionic strengths of the effectors used in these studies do not even approach those concentrations utilized in Landis et al. (1981); furthermore, the clotting data for nucleotide phosphates were corrected for slight ionic strength effects, as noted in the text. Additional evidence for the relevance of the clotting data stems from the correlation, in every case, with kinetic effects on synthetic substrates (Tos-Arg-OMe or tosyl-ChromozymTH) or direct physical evidence of thrombin binding (proflavin or spin-labels). In preliminary experiments with the chromogenic tripeptide substrate, tosyl-ChromozymTH (tosyl-Gly-Pro-Arg-*p*-nitroanilide) (Claeson & Aurell, 1981), both indole and tryptamine were partial inhibitors. In the case of tryptamine, the approximate K_i fell in the same range as that where inhibition of clotting was observed (see Figure 2).

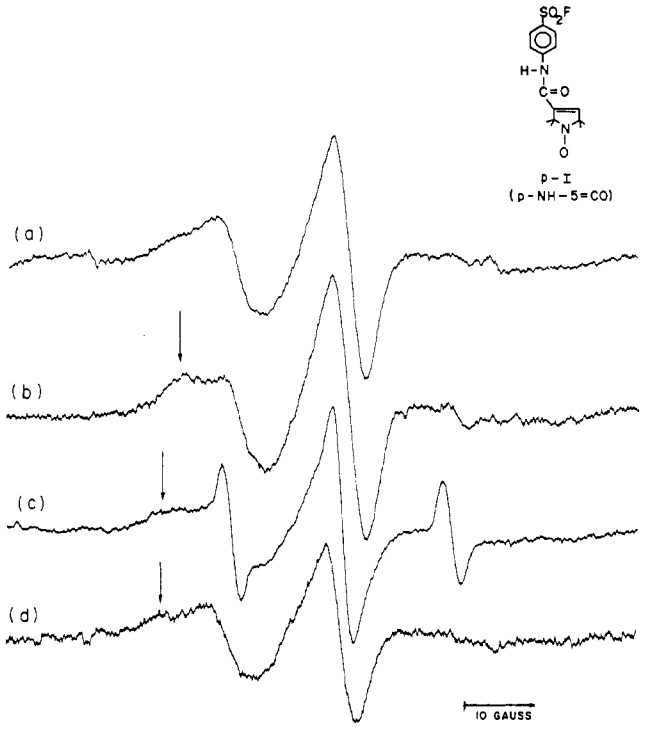

FIGURE 4: ESR spectra of spin-labeled human α-thrombin in the presence of various effectors: (a) none; (b) saturated indole ($\sim$10 mM); (c) 25 mM tryptamine; (d) 20 mM serotonin. Conditions were pH 6.5, 0.05 M sodium phosphate, 0.75 M NaCl, 27 $\pm$ 2 °C. ATP binding gave no detectable mobility change. The three narrow lines which are superimposed in the tryptamine-exposed enzyme (Figure 4c) were due to an accelerated desulfonylation side reaction. This had been observed before with thrombin as well as α-chymotrypsin (Wong et al., 1974; Berliner et al., 1981; Berliner, 1977).

Several nucleotides and related structural analogues were tested for their effect on thrombin-catalyzed fibrinogen clotting activity. The results, depicted as Dixon plots, are shown in Figure 3. In the cases of the nucleotides (ATP, ADP, and UDP), and inorganic PP_i, a biphasic inhibition was observed.[3] The data could not be fit to any simple two-site inhibition model without allowing for some cooperativity between these sites (Segel, 1975). Adenosine, on the other hand, inhibited clotting activity with simple hyperbolic behavior. While the adenine nucleotides (ATP and ADP) were the most potent inhibitors of this group, substitution by another base (e.g., UDP) or by pyrophosphate (PP_i) alone was sufficient to effect substantial inhibition of clotting. We also found that total phosphate buffer concentrations in the 50–100 mM range were inhibitory as well as ionic strengths (NaCl) above 100 mM.

ESR Spin-Label and Proflavin Displacement. Berliner et al. (1981) and Berliner & Shen (1977a) have shown that a group of three structurally isomorphous fluorosulfonyl spin-label analogues were sensitive to ligands which bind to the apolar (indole) binding site. An increase in spin-label immobilization was observed which was due either to an orientation shift of the spin-label or to a ligand-induced protein conformation change (Berliner & Shen, 1977a; Berliner, 1977). Figure 4 shows ESR spectra for the active Ser directed irreversible inhibitor spin-label *p*-I (*p*-NH5=CO) with α-thrombin. Upon exposure to saturated indole (Figure 4b), a distinct broadening occurred (denoted by the arrow in Figure 4b), which differed from that with the spin-labeled enzyme alone (Figure 4a). The addition of either 25 mM tryptamine (Figure 4c) or 20 mM serotonin (Figure 4d) produced similar broadening effects (see arrows). The nucleotide ATP had no effect on spectrum 4a at concentrations up to 25 mM. Similar results to those in Figure 4a–d were observed with the spin-label *p*-V (*p*-CO-5NH), which binds at the same thrombin locus as did label *p*-I above (Berliner et al., 1981). On the other hand, the two labels *m*-III (*m*-CO-6OH) and *m*-V (*m*-CO-5NH), which bind in the region which differentiates α- and γ-thrombin (Berliner et al., 1981), were totally unaffected by saturating concentrations ($\sim$20 mM) of any of the effectors noted above (indole, tryptamine, serotonin, and ATP), as well as by adenosine or pyrophosphate at comparable or higher concentrations.

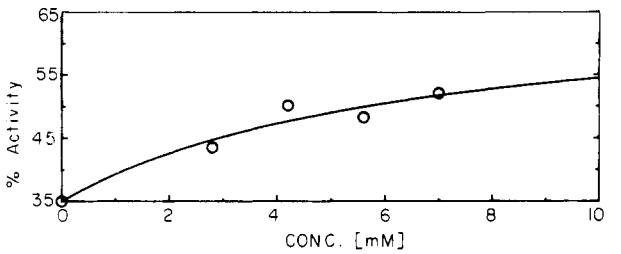

FIGURE 5: Clotting activity of α-thrombin in the presence of constant ATP concentration (3.5 mM) with increasing indole concentrations. A control containing neither ATP nor indole corresponds to 100% activity. The solid line is a theoretical fit to K_D(app) = 6.5 mM in 3.5 mM ATP. All other conditions were identical with those in Figure 2.

The dye proflavin binds to the thrombin active site with an optically detectable absorption at 468 nm, which may be measured by difference spectroscopy (Koehler & Magnusson, 1974). The competitive binding of another ligand is observed spectrally by the decrease in the thrombin–proflavin complex absorption difference spectrum. This was shown previously for indole (Berliner & Shen, 1977a) and for benzamidine (Koehler & Magnusson, 1974). We measured thrombin–proflavin difference spectra for the effectors ATP, tryptamine, and serotonin and calculated dissociation constants (K_D) on the basis of the methods described in Berliner & Shen (1977a). From these results (see also Table II), we obtained K_D(ATP) = 2.8 $\pm$ 1.0 mM. It was apparent that the adenosyl moiety of ATP was required since pyrophosphate (up to 34 mM) did not alter the thrombin–proflavin difference spectrum. Proflavin displacement was also observed for tryptamine or serotonin with dissociation constants of $\sim$6.9 and $\sim$2.2 mM, respectively.

Our initial ESR studies were performed in 0.75 M NaCl (pH 6.5, 0.05 M sodium phosphate) in order to stabilize the relatively high concentrations (10^{-4} M) of protein required for ESR sensitivity. We have followed these conditions wherever possible in our correlative physical studies, such as the proflavin experiments above. On the other hand, the kinetic studies (esterase, clotting, amidase) necessitated using much lower salt concentrations on substrate solubilities, and the catalytic rates were severely decreased at 0.75 M NaCl. Proflavin displacement at lower NaCl concentrations (0.1 M), pH 6.5, by indole and tryptamine yielded dissociation constants in the same range (3.4–9.4 mM) as those found at 0.75 M NaCl.

Evidence for Interactions between the Indole and ATP (or PP_i) Subsites. Indole and ATP differed in their effects on esterase and clotting activity, yet both displaced proflavin. In order to check whether their mutual ability to release proflavin was in fact indicative of a mutal overlap between the indole and ATP sites themselves, we devised a "competitive binding"

[3] While we did not examine PP_i above 14 mM concentrations, there is a tendency in the Dixon plot toward biphasic inhibition behavior at higher concentrations. It is clear from the ATP and ADP results (Figure 3) that the adenosyl moiety lends more binding specificity to the thrombin–nucleotide interaction.

clotting experiment which is described as follows: Clotting reaction mixtures were prepared which contained sufficient ATP or PP_i to cause ca. 40–60% inhibition. A reaction mixture was then "titrated" with increasing indole concentrations, which resulted in a progressive increase in clotting activity. Figure 5 shows an example at a fixed concentration of ATP (3.8 mM) with varying indole concentrations measured in 0.024 mM sodium phosphate, 0.1 M NaCl, and 0.66% (w/v) PEG 6000, pH 7.0, 37 °C. In all the cases with PP_i, a complete restoration of clotting activity was observed at 4–5 mM indole. In the experiments with ATP (e.g., Figure 5), only partial restoration of activity (ca 50%) occurred. Since the second ATP site also contributes to the clotting inhibition, it was not unexpected that full clotting activity was not restored at saturating indole concentrations. In fact, the increase in clotting activity appeared to be due to a specific activation of the clotting rate by indole, which was similar to the activation of clotting at low concentrations of tryptamine or serotonin as seen in Figure 2. Preliminary experiments with indole alone show up to 15–25% observed rate activation at 3–5 mM indole (B. G. Conery and L. J. Berliner, unpublished results). Similar results were also obtained with 5-fluoroindole (L. J. Berliner and H. Tsunematsu, unpublished results).

Discussion

It is evident from these studies that human thrombin contains multiple (binding) recognition sites, each related to specific thrombin functions (see Table II). The qualitative differences between each group of thrombin effectors allow some general classification of binding modes (subsites) and their properties.

Apolar (Indole) Ligands. The apolar (indole) site discovered by Berliner & Shen (1977a) appears to be identical in both the α and γ forms of human thrombin, based on the similarities in activation of Tos-Arg-OMe esterase activity as well as from previous physical measurements (spin-labels) by Berliner et al. (1981). While the obvious *differences* between α- and γ-thrombin are (1) the lack of clotting (fibrinogen binding) activity of γ-thrombin, (2) those conformational differences mapped in solution (Berliner et al., 1981) and by molecular modeling (Furie et al., 1982), and (3) a reduced structural stability of γ-thrombin toward denaturants (Bauer et al., 1981), this work and other evidence point to a general *similarity* in their overall structure. For example, the intrinsic fluorescence parameters for α- and γ-thrombin were identical (Berliner & Shen, 1977b). When labeled with the active serine-directed fluorophores dansyl fluoride or *p*-nitrophenyl anthranilate, both α- and γ-thrombin derivatives have identical energy transfer fluorescence parameters (quantum yield, energy transfer efficiency, λ_{em}^{max}) for each fluorophore (Berliner & Shen, 1977b). γ-Thrombin will substitute for the α form in several physiological reactions (Fenton et al., 1979) with the single exception of fibrinogen clotting activity. It is probable then that differences in α- and γ-thrombin structure may be restricted to limited region(s) of the molecule.

The indole analogues studied here were divided into two general classes on the basis of their effects on thrombin-catalyzed Tos-Arg-OMe esterase activity. The activators indole, skatole, and 2,5-dimethylindole are all nonpolar molecules while tryptamine and D- or L-tryptophan contain positively charged side chains in common. If Tos-Arg-OMe activation arises from a unique interaction between the enzyme and the indole skeleton, it follows that an adjacent subsite must exist which binds the primary ammonium group of tryptamine or D- or L-Trp. This model becomes more complex, however, when one attempts to rationalize the *inhibitory* behavior of serotonin, where a hydroxyl group has been introduced at the 5-position of tryptamine, or of indoleacetic acid, which is a tryptamine analogue in the sense that the positively charged ammonium group is replaced by a (negatively charged) carboxylate.[4]

Nucleotide Analogues. The strong biphasic inhibition of clotting which was caused by several nucleotides as well as by pyrophosphate (Figure 3) suggests that there exist at least two sites which have affinity for negatively charged phosphate groups; the second cooperatively linked site is also nucleotide specific. The inhibitory behavior of adenosine, on the other hand, was consistent witha single noncompetitive inhibition site. Also, adenosine binding did not appear to be competitive with ATP from clotting measurements with mixtures of each inhibitor (C. T.-L. Chang and L. J. Berliner, unpublished results).[5]

Some Generalizations on the Spatial Relationships between Thrombin Binding Interactions. The compounds discussed above were divided into two general groups or classes of ligands, based on both their structural similarities and their effects on thrombin function. It is also pertinent to examine, from other experiments, whether any relationship exists between these two classes. Does the displacement of proflavin by indole, tryptamine, serotonin, and ATP (but not PP_i) suggest that the adenosyl moiety of ATP overlaps the indole binding site on α-thrombin? The ESR spin-label results indicated that while the indole analogues (tryptamine and serotonin) affected the mobility of spin-labels which specifically monitor the binding of indole (Berliner et al., 1981), there was no ESR effect observed upon binding ATP, adenosine, or PP_i. Clotting experiments with mixtures of indole and ATP demonstrated that even at saturating indole concentrations, a partial (ATP induced) inhibition was still present (see Figure 5). This suggests that indole and (at least one) ATP bind to different sites which may or may not be conformationally linked. Lastly, the activation of Tos-Arg-OMe hydrolysis by several of these indole analogues required that the effector and the substrate molecule bind simultaneously. On the other hand, ATP inhibition of Tos-Arg-OMe activity may be accounted for by a simple competitive inhibition model (Segel, 1975). The observations above would suggest that indole analogue binding may induce an enzyme conformational change which is manifested in Tos-Arg-OMe activation, activation of clotting, spin-label mobility changes, and reduced proflavin binding. While the last two effects above could also be explained by mutual overlap of the indole analogue with the reporter group, it is clear in either model that the ATP (nucleotide) binding site(s) are spatially distinct from the indole (apolar) site(s).

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[4] As an alternative model for the case of serotonin, which contains a primary amine, it is possible that the inhibition is due to basic specificity pocket binding; i.e., apolar site binding was sterically obstructed by adding a 5-OH group to tryptamine. On the other hand, negatively charged indoleacetic acid would not bind in the basic pocket.

[5] Furthermore, α-thrombin covalently labeled by reductive alkylation of a protein amino group by the 2′- or 3′-carbon of dial–adenosine yielded a modified derivative with three adenosyl moieties per enzyme molecule (C. T.-L. Chang and L. J. Berliner, unpublished results). While the inhibitory behavior of exogenous adenosine with native α-thrombin was consistent with a single class of binding, it is obviously impossible at this juncture to assess the contribution of each adenosyl group toward the total observed inhibition. Similar experiments with dial–ATP-labeled thrombin showed that only one ATP site could be covalently labeled. The dial–ATP–thrombin had reduced (but not obliterated) clotting activity yet retained 100% Tos-Arg-OMe activity, suggesting that this ATP site was not the Tos-Arg-OMe inhibition site (C. T.-L. Chang, B. G. Conery, and L. J. Berliner, unpublished results).

Finally, the tryptamine effects on clotting (initial activation followed by inhibition) should be discussed in light of the strong activation of Tos-Arg-OMe activity and the ability to displace proflavin and to alter spin-label mobility. Tryptamine is an aromatic amine like the thrombin inhibitors benzamidine, benzylamine, and 5-amidinoindole, which inhibit by binding to the basic specificity pocket (Geratz & Tidwell, 1977). The clotting inhibition by tryptamine (see Figure 2) may correspond to binding in the basic specificity pocket while the initial activation phase (Figure 2) corresponded to binding at the Tos-Arg-OMe activation (apolar) site. The apolar binding was much stronger than basic pocket binding (see K_{actn}, K_D, and K_i values in Table II). Inhibition of Tos-Arg-OMe activity due to tryptamine binding in the basic pocket was not evident in the esterase activity studies of Figure 1 since, e.g., the effective concentration range of an inhibitor which competes with Tos-Arg-OMe would have to exceed 30-fold its K_i value at the 1 mM Tos-Arg-OMe concentration used in this experiment (Curragh & Elmore, 1964). On the other hand, Bz-Arg-OMe did not display substrate activation in the presence of these effectors under the same conditions (B. G. Conery and L. J. Berliner, unpublished results). If one builds a model of Bz-Arg-OMe and Tos-Arg-OMe and superimposes their common Arg-OMe moieties, the former substrate is essentially linear with the benzoyl group position relatively fixed in space while the latter is bent at the tosyl linkage. Presumably, the tosyl moiety binding site, which is the trigger for substrate activation (see tosylamide results, Table II), is situated at some angle "up" or "down" from the (linear) Arg-OMe binding locus. A further consideration which must be taken into account is that for Bz-Arg-OMe deacylation is rate limiting, while for Tos-Arg-OMe the rate-determining step is influenced significantly by acylation (Curragh & Elmore, 1964).

Physiological Significance of These Interactions. The three principal metabolites, serotonin, ADP, and ATP, which are involved in platelet secretion and aggregation phenomena were all effectors of thrombin function at concentration levels in the 1.0–5.0 mM range under the conditions of these experiments. It is known that the levels of these metabolites in circulating normal blood are low (e.g., the general concentration range for ADP stimulation of platelet aggregation is nominally in the micromolar range). However, the levels of these metabolites during platelet-induced secretion might be quite high near the platelet surface, depending upon its secretory diffusion properties and the metabolite concentration gradient. Thus, it is conceivable that *local* concentrations of these metabolites may be sufficiently high to alter certain thrombin functions and to effect thrombin–platelet interactions. It is quite likely that the recent reports of thrombin inhibition by pyridoxal phosphate (Griffith, 1979) and diphosphoglycerate (Delprincipe et al., 1981) are related to the nucleotide binding phenomena outlined in this work.

We know that thrombin has multiple functions in both coagulative and noncoagulative events besides those platelet-related interactions. The various ligand binding phenomena presented in this work are interesting in that their effects on thrombin function should be invaluable to the understanding of its physiological interactions at the molecular level. More detailed physical and chemical studies are in progress to elucidate the distance and conformational relationships between the subsites discussed above.

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Registry No. *p*-I, 52413-11-3; Tos-Arg-OMe, 901-47-3; ATP, 56-65-5; ADP, 58-64-0; UDP, 58-98-0; PP_i, 14000-31-8; P_i, 14265-44-2; adenosine, 58-61-7; indole, 120-72-9; tryptamine, 61-54-1; skatole, 83-34-1; 2,5-dimethylindole, 1196-79-8; D-tryptophan, 153-94-6; L-tryptophan, 73-22-3; tosylamide, 70-55-3; serotonin, 50-67-9; indole-3-acetic acid, 87-51-4; proflavin, 92-62-6; thrombin, 9002-04-4.

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pH Studies toward the Elucidation of the Auxiliary Catalyst for Pig Heart Aspartate Aminotransferase†

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ABSTRACT: The pH variation of the kinetic parameters for aspartate aminotransferase has been measured in order to determine which enzyme residues participate in auxiliary catalytic and binding roles. When the initial rate is obtained by varying concentrations of α-ketoglutarate and several different fixed concentrations of aspartate, competitive inhibition by both substrates is observed, indicative of E–PLP–α-ketoglutarate and E–PMP–aspartate dead-end complexes (E = enzyme, PLP = pyridoxal phosphate, and PMP = pyridoxamine phosphate). Inhibition by aspartate is significantly enhanced at high pH as a result of deprotonation of the amino group of aspartate, while α-ketoglutarate inhibition is stronger when an enzyme group with a pK of 6.4 is protonated. The maximum rate is pH independent from 5.5 to 10.0. The V/K for α-ketoglutarate decreases below a pK of 5.8 and above a pK of 9.2, while the V/K for aspartate decreases below a pK of 6.4 and above pK values of 9.2 and 10.0. The K_i for inhibition by maleate, competitive with aspartate (representing maleate binding to E–PLP), exhibits a pH dependence identical with α-ketoglutarate substrate inhibition, while the K_i for inhibition by maleate, competitive with α-ketoglutarate (representing binding to E–PMP), increases above a pK of 9.3. The K_i for inhibition by α-methylaspartate competitive with aspartate and indicative of binding to E–PLP increases below a pK of 6.5 and above a pK of 9.3. The temperature dependence of V/K pK values yields ΔH_{ion} values of 5–6 kcal/mol for all groups. V/K values obtained in the presence of 30% DMF with cationic acid buffers suggest that all groups are cationic acids. In all probability the group with a pK of 9.2 reflected in both V/K pH profiles is the pyridinium moiety of the cofactor. The above data are consistent with a mechanism in which (1) the nitrogen of the Schiff base between the 6-amino of lysine-258 and PLP must accept a proton from the amino group of aspartate to start the first half-reaction, (2) lysine-258, which originally participates in the Schiff base, acts as a general base during the conversion of aspartate and E–PLP to oxalacetate and E–PMP, and (3) at the completion of the first half-reaction, the 6-amino of lysine-258 and the pyridoxamino group of PMP may be hydrogen bonded and this diamine system is positively charged.

Aspartate aminotransferase catalyzes the reaction

L-aspartate + α-ketoglutarate ⇌
oxalacetate + L-glutamate

This enzyme has been studied extensively by several groups [reviewed in Braunstein (1972)], and an abundance of information has been obtained concerning its physical, kinetic, and spectral properties. The native holoenzyme is isolated with pyridoxal phosphate bound as a Schiff base to the 6-amino moiety of lysine-258 (Braunstein, 1972). Velick & Vavra (1962) have shown that the enzyme catalyzes a ping-pong reaction; aspartate transfers its amino group to the cofactor (PLP)[1] and dissociates as oxalacetate prior to the addition of α-ketoglutarate, which accepts the amino group and dissociates as L-glutamate. During the course of conversion of aspartate to oxalacetate, pyridoxal phosphate is converted to pyridoxamine phosphate. At pH 7.3, α-ketoglutarate at high concentration is found to inhibit the reaction, and this inhibition is competitive with aspartate (Velick & Vavra, 1962). In addition, inhibition is observed by mono- and dianions (notably mono- and dicarboxylic acids), which are competitive vs. both aspartate and α-ketoglutarate (Velick & Vavra, 1962; Bonseb et al., 1975; Harruff & Jenkins, 1978).

Chemical modification studies have implicated various enzyme residues as being the auxiliary acid–base catalyst facilitating removal of the aspartate α proton. The most notable of these is lysine (Turano et al., 1963). In addition, histidine has also been implicated (Peterson & Martinez-Carrion, 1970). Recently, the three-dimensional structure has been obtained to 2.7–2.8 Å resolution for both the mitochondrial (Jansonius et al., 1981) and cytoplasmic (Arnone et al., 1981) enzymes. Structures for these enzymes are very similar, particularly with respect to the active site, and indicate that the only residues in close enough proximity to act as an acid–base catalyst (and still be consistent with the known stereochemistry of the reaction) are lysine-258 and tyrosine-70* (tyrosine-70* is located

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DMF, dimethylformamide; NADH, nicotinamide adenine dinucleotide, reduced; α-KG, α-ketoglutarate; Asp, aspartate.

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