The Interaction of Thrombin and Heparin. Proflavine Dye Binding Studies†

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ABSTRACT: The proflavine dye binding technique was used to study the interaction of heparin and thrombin. The major conclusion, that heparin binds tightly to thrombin but *not* at the active site, was based on spectral properties of enzyme-heparin-proflavine solutions. Support for this idea comes from several observations. (1) Heparin is a poor inhibitor of the hydrolysis of tosylarginine methyl ester. The pattern of inhibition at 0.1 ionic strength is hyperbolic competitive suggesting formation of heparin-enzyme complexes which can react with substrates. (2) Heparin does not protect thrombin from inactivation by phenylmethylsulfonyl fluoride. (3) The inactive phenylmethylsulfonyl-thrombin binds heparin. Approximately 3–4 heparins bind to each thrombin molecule, and binding is tight with an average constant of 10^{-7} M. Ternary thrombin-fibrinogen-heparin complexes which were proposed to explain heparin inhibition of fibrinogen hydrolysis (Abildgaard, U. (1968), *Scand. J. Haematol. 5,* 432) could not be detected.

The enzyme thrombin plays a key role in thrombosis and hemostasis. Its main function is the catalysis of the fibrinogen to fibrin conversion (review, Magnusson, 1971). Thrombin is subject to regulation by many circulating inhibitors or pharmacological agents. One of the most widely used clinical anticoagulants with antithrombin activity is the polysaccharide heparin. This substance, which affects several steps in the coagulation process (reviews, Jaques, 1967; Ehrlich and Stivala, 1973), inhibits thrombin reactions in plasma or purified systems. The exact nature of the thrombin-heparin interaction is not well understood but it is generally believed that *in vivo,* inhibition is strongly dependent on the presence of antithrombin-heparin cofactor (Abildgaard, 1968b; Rosenberg and Damus, 1973). This antithrombin forms a tight complex with thrombin which destroys active site activity; heparin enhances the rate of complex formation but not the equilibrium (Rosenberg and Damus, 1973) although whether this is mediated by prior binding to thrombin is not known. *In vitro,* heparin is a powerful inhibitor of the clotting of fibrinogen even in the absence of antithrombin-heparin cofactor (Lyttleton, 1954; Abildgaard, 1968a). The study of clotting inhibition is complicated by the fact that heparin binds not only to thrombin, but also to fibrinogen (Bernfeld, 1966) and possibly to the fibrinogen-thrombin complex (Abildgaard, 1968a). It also interferes with the association of fibrin monomers (Godal, 1961). As a first step in characterizing the effects of heparin, we have investigated the thrombin-heparin reaction using the proflavine dye binding technique. This method has been used for the study of several proteolytic enzymes (Bernhard *et al.,* 1966; Hess, 1971) and has recently been shown to be applicable to thrombin chemistry (Feinman *et al.,* 1973; Koehler and Magnusson, 1974). The acridine dye proflavine binds to the active site of proteolytic enzymes with an accompanying spectral shift. Displacement of the dye by substrates or inhibitors can then be used to follow events at the active site. In this communication we present further evidence that proflavine spectral changes do reflect active site reactions and we have used this method to show that heparin binding is *not* at the active site of thrombin. We have determined that 3–4 heparins bind to one molecule of thrombin. In addition, we found that thrombin-fibrinogen-heparin complexes of the type proposed by Abildgaard could not be detected.

Materials

Thrombin was prepared from Parke-Davis topical thrombin by the method of Glover and Shaw (1971). Specific activities were in the range of 1800–2000 NIH units/mg of protein. Several experiments were also carried out with purified human thrombin which was a gift from Dr. John Fenton (specific activity 2265 NIH units/mg) (Fasco and Fenton, 1973). This material gave similar results to the bovine enzyme.

Heparin was purchased from Sigma Chemical Co. or Calbiochem as sodium or lithium salt of porcine intestinal mucosal heparin and was used without further purification. Calculations were based on a molecular weight of 11,000 (Hilborn and Anastassiadis, 1971).

Proflavine sulfate (Mann) was recrystallized from methanol in the dark; *p*-tosyl-L-arginine methyl ester (Tos-Arg-OMe)[1] (Schwarz/Mann), *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride (pNPGB), and phenylmethylsulfonyl fluoride ($PhCH_2SO_2F$) (Calbiochem) were used without further purification.

Methods

Spectral measurements were made on a Beckman Acta III spectrophotometer. Steady-state kinetic experiments were performed on a pH-Stat (Radiometer titrator Model 11, ABU-1b auto-burette, SBR-2c recorder, a Model 28 pH meter, siliconized constant-temperature reaction vessel). Stop-flow experiments were performed on an Aminco-Morrow apparatus using operational amplifiers as a photometer.

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[1] Abbreviations used are: Tos-Arg-OMe, tosylarginine methyl ester; pNPGB, *p*-nitrophenyl-*p*-guanidinobenzoate hydrochloride; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; $PhCH_2SO_2$-thrombin, phenylmethylsulfonyl-thrombin.

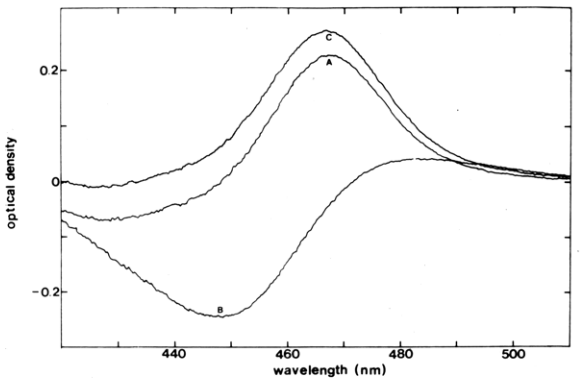


FIGURE 1: Visible difference absorption spectra of proflavine solutions. Reference cuvet contains 3.0×10^{-5} M proflavine in 0.01 M Tris-HCl (pH 7.4). Sample is an identical solution of proflavine containing: (A) thrombin, 2.3×10^{-5} M; (B) heparin, 3.0×10^{-5} M; (C) thrombin, 2.3×10^{-5} M and heparin, 3.0×10^{-5} M.

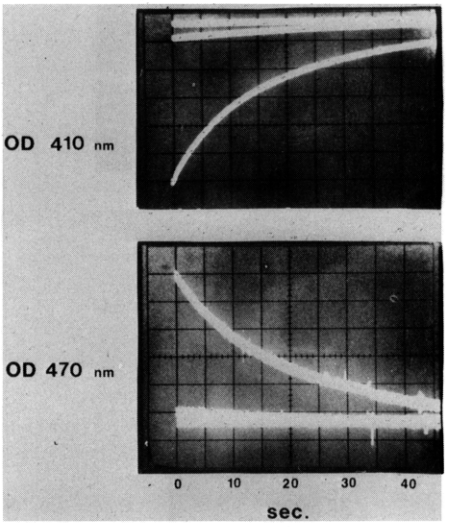


FIGURE 2: Acylation of thrombin by pNPGB observed by proflavine release (470 nm) and *p*-nitrophenol production (410 nm); 0.1 M sodium Veronal buffer (pH 8.3). Final concentrations: pNPGB, 2.0×10^{-5} M; thrombin, 2.2×10^{-5} M. Both syringes contained 1.0×10^{-4} M proflavine.

A Philbrick Model 4351 logarithmic amplifier allowed direct recording of optical densities on a Tektronix storage oscilloscope. The measured dead time of the instrument is 2.5–3.0 msec.

Analysis of Kinetic Data. The kinetic parameters described in this paper are based on the acyl-enzyme hypothesis (review, Bender and Kezdy, 1965) shown in eq 1. Under

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} E\text{-acyl} \xrightarrow{k_3} E + P_2 + P_1 \quad (1)$$

steady-state conditions, the parameters $K_{m(app)}$ and $k_{cat} = V_{max}/E_0$ are determined according to eq 2. In stop-flow ex-

$$E + S \overset{K_{m(app)}}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \quad (2)$$

periments where $E \cong S$ two steps are measured, and if $k_2 \gg k_3$ as is the case for substrates used here, the second step corresponds to k_3. The first step is the apparent second-order reaction of E and S to form acyl-enzyme which has rate constant $= k_2/K_s$. The relations between parameters in eq 1 and 2 are

$$k_{cat} = k_2k_3/(k_2 + k_3) \cong k_3$$

$$K_{m(app)} = [k_3/(k_2 + k_3)]K_s \quad (3)$$

or dividing these two equations

$$k_{cat}/K_{m(app)} = k_2/K_s \quad (4)$$

Equations 3 and 4 were used to test the applicability of eq 1 to the reactions studied. The first-order rate constants for k_3 determination, the second-order constants for acylation reactions, and steady-state parameters were calculated with appropriate weighted least-squares analysis on a Model 700 Wang calculator. The hyperbolic inhibition constant was determined graphically by plotting slopes of Lineweaver-Burk plots *vs.* concentration of inhibitor. K_I (see eq 6) was determined from the following equation (using K_s and K_s' from the intercept and final value): slope $= (K_IK_sK_s' + K_sK_s'[I])/(K_IK_s' + K_s[I])$.

Results

Thrombin Reactions with Substrates. The difference absorption spectrum of thrombin–proflavine *vs.* proflavine is shown in Figure 1, curve A. The decrease in absorption at 470 nm when dye is displaced can be used to follow the kinetics of thrombin reactions. The acylation of thrombin by an approximately equimolar amount of *p*-nitrophenyl-*p'*-guanidinobenzoate (pNPGB) in the presence of proflavine is shown in Figure 2. When this active site titrant (Chase and Shaw, 1969) reacts with thrombin, formation of acyl-enzyme (and release of nitrophenol, P_1 in eq 1) is much more rapid than deacylation. This acylation reaction can be followed in two ways: appearance of nitrophenolate ion (410 nm) and desorption of dye (470 nm). It can be seen in Figure 2 that the time course of the reaction is the same for both cases. The second-order rate constant k_2/K_s for acylation (see Methods) is 5×10^3 M^{-1} sec^{-1}. When this is compared with the same parameter determined in the absence of proflavine (by 410 absorption), an inhibition constant of 3.1×10^{-5} M can be calculated. In Table I this value is compared with binding and inhibition constants determined by other methods. It is clear from Figure 2 and Table I that changes in proflavine absorption reflect events taking place at the active site.

The reaction of the specific ester substrate tosylarginine methyl ester with thrombin, followed by the proflavine method, is shown in Figure 3, curves A and B. The kinetic curves show the formation of an intermediate, consistent with the acyl-enzyme mechanism. The second step in the reaction (the presumed k_3 of eq 1) is equal to k_{cat} from steady-state measurements, as expected for this theory. The

TABLE I: Binding and Inhibition Constants for Thrombin–Proflavine Interaction.

Method	K (M)
(1) Spectrophotometric titration	2.1×10^{-5} [a] $0.9\text{–}2.1 \times 10^{-5}$ [b]
(2) Inhibition of steady-state hydrolysis of Tos-Arg-OMe	2.0×10^{-5} [a] $0.8\text{–}5 \times 10^{-5}$ [b]
(3) Inhibition of acylation by pNPGB	3.1×10^{-5}

[a] Feinman *et al.*, 1973. [b] Koehler and Magnusson, 1974.

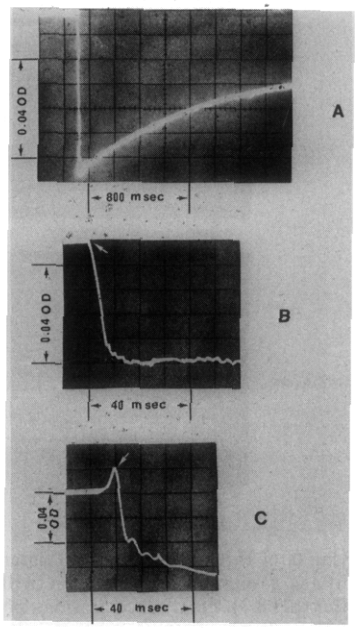


FIGURE 3: Reaction of thrombin with Tos-Arg-OMe in the stopped-flow followed by proflavine desorption and rebinding. Vertical axis is optical density at 470 nm. 0.1 M sodium phosphate buffer (pH 7.5). Final concentrations: Tos-Arg-OMe, 2.0×10^{-5} M; thrombin, 2.4×10^{-5} M. Both syringes contained 6.0×10^{-5} M proflavine. (A) 200 msec/division; (B) 10 msec/division (trace A on a faster time scale); (C) 10 msec/division. Final concentration of heparin 1.0×10^{-4} M. Arrow indicates where flow stops.

second-order rate constant for the first step, $k_2/K_s = 1.3 \times 10^7$ M^{-1} sec^{-1} (pH 8.3, 0.1 M Veronal buffer). This is much higher than the value expected from the acyl-enzyme hypothesis, $k_{cat}/K_{m(app)} = 2.4 \times 10^5$ M^{-1} sec^{-1} measured under the same conditions.

Spectral Characteristics of Heparin and Thrombin. It is well known that heparin binds proflavine and other acridine dyes (Stone and Bradley, 1967; Bradley and Wolf, 1959). The difference absorption spectrum of heparin plus proflavine *vs.* dye alone is shown in Figure 1, curve B. Titration studies have shown that approximately 60 binding sites/molecule exist and binding is approximately stoichiometric at low dye/site (Stone and Bradley, 1967).[2] The spectrum in Figure 1, therefore, represents essentially pure bound dye and there is an average of about one dye per heparin molecule. The extinction coefficient for bound dye is 21,300 in agreement with the literature value of 21,000 (Stone and Bradley, 1967). Comparison of curves A and B in Figure 1 shows that spectra of the heparin–dye (ID) and thrombin–dye (ED) complexes are quite different; λ_{max} for the ED difference spectrum (468 nm) is near the isosbestic point for the ID difference spectrum (472 nm). When low concentrations of thrombin are added to solutions containing heparin and proflavine, spectra are obtained which can be characterized as the sum of spectra due to the ID and ED interaction. When the concentration of thrombin becomes large (approximately equimolar with respect to heparin) the spectrum in curve C, Figure 1 is obtained: the ID spectrum appears to be completely lost and the ED spectrum persists or is somewhat enhanced. Thus, while heparin apparently

[2] Heparin exhibits several spectral changes or metachromasia in its association with dyes (Bradley and Wolf, 1959; Wollin and Jacques, 1973 and reference therein). The spectrum shown in Figure 1 is the α spectrum which appears when the dye/sites ratio is low and no dye–dye associations take place.

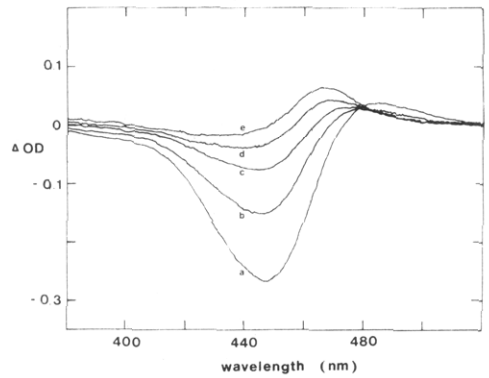


FIGURE 4: Titration of heparin–dye solutions with thrombin; 0.01 M Tris-HCl (pH 7.4). Proflavine = 3.0×10^{-5} M, heparin = 1.7×10^{-5} M, thrombin concentrations (10^{-6} M): (a) none, (b) 1.08, (c) 2.15, (d) 3.21, (e) 5.25.

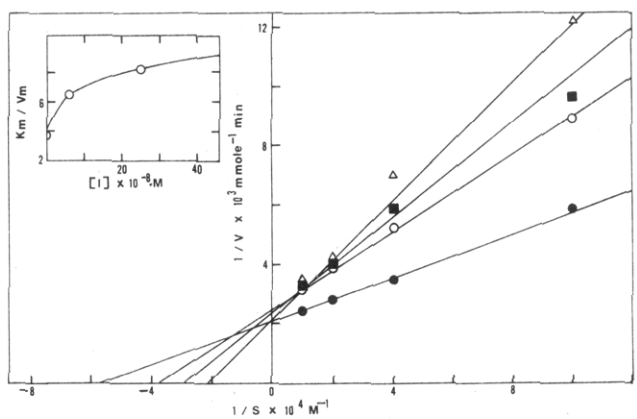


FIGURE 5: Steady-state kinetics of thrombin-catalyzed hydrolysis of Tos-Arg-OMe. pH-Stat reactions 0.1 M NaCl (pH 8.3). Concentrations of heparin (I): (●) none, (○) 6.3×10^{-8} M, (■) 2.5×10^{-7} M, (△) 1.0×10^{-6} M. Inset: slopes as a function of heparin concentration.

binds to thrombin (loss of ID spectrum), this binding does not seem to displace proflavine from the active site. The loss of dye–heparin spectrum further suggests that proflavine does not bind to heparin when it is attached to thrombin (see Discussion). This allows titration of the heparin with low concentrations of thrombin (Figure 4). This is not a true titration since displaced dye can bind to sites on other heparin molecules. Also, heparin–dye associations become complex as the number of sites is decreased (Stone and Bradley, 1967). For these reasons, it is not possible to determine true binding constants. However, from the point of saturation, the number of heparin molecules bound per molecule of thrombin is 3–4. The average binding constant can be estimated from the fact that the fraction bound is roughly stoichiometric at the concentrations used. If it is assumed that more than 5×10^{-6} M free heparin could be detected (as ID), the average binding constant per site is about 10^{-7}–10^{-8} M. The results were approximately the same regardless of ionic strength (0.01–0.10 M Tris-HCl) although the net optical density changes at high ionic strength were much smaller due to the greatly reduced heparin–dye association under such conditions.

Heparin Inhibition of Tos-Arg-OMe Hydrolysis. We measured the inhibition of the thrombin-catalyzed hydrolysis of Tos-Arg-OMe at 0.1 M ionic strength by heparin; the results are shown in Figure 5. It can be seen that heparin is a poor inhibitor which would be expected if, in fact, it did

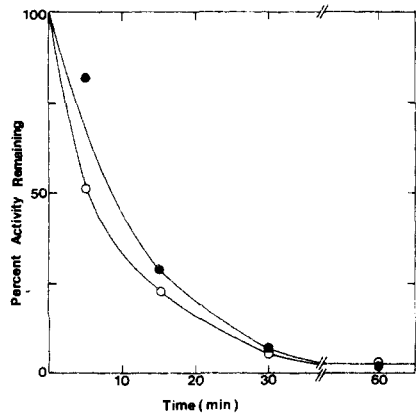


FIGURE 6: Inactivation of thrombin by $PhCH_2SO_2F$ in the presence and absence of heparin. Thrombin, 5.9×10^{-4} M in 0.01 M phosphate (pH 8.27), was treated with $PhCH_2SO_2F$. Activity was assayed by removing samples and measuring rates of hydrolysis of 6.5×10^{-4} M solutions of Tos-Arg-OMe. Heparin concentration: (●) none, (○) 6.8×10^{-4} M.

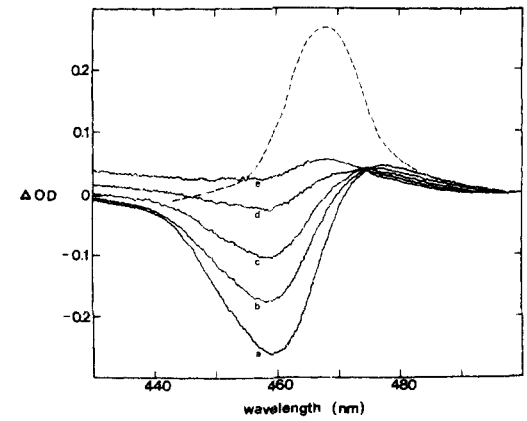
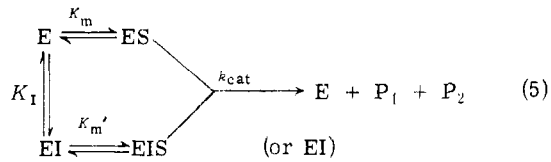


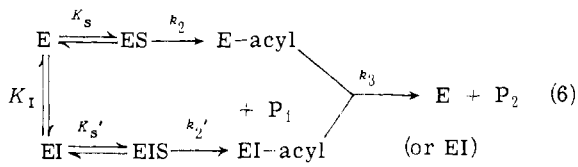
FIGURE 7: Titration of heparin-dye solutions with $PhCH_2SO_2$-thrombin; 0.01 M Tris-HCl (pH 7.4), proflavine = 3.0×10^{-5} M, heparin = 3.3×10^{-5} M. $PhCH_2SO_2$-thrombin concentrations (10^{-6} M): (a) none, (b) 4.6, (c) 6.9, (d) 12.0, (e) 24.0. The dotted line is redrawn from Figure 1C and shows the spectrum expected for native thrombin at the same enzyme concentration as curve e.

not bind at the active site. The mode of inhibition is hyperbolic competitive up to a heparin concentration of 10^{-5} M. The K_I is 3×10^{-8} M. A simple interpretation of this inhibition pattern is that there are two forms of the enzyme, with and without bound heparin. The heparin-enzyme has a poorer K_m and the same V_{max} (eq 5). If the acyl-enzyme



(5)

mechanism is actually operative for Tos-Arg-OMe either K_s or k_2 could be affected by heparin binding, and k_{cat} (= k_3) should be unchanged (eq 6) (see Discussion). At low ionic strength the extent of inhibition is similar but the pattern is complicated and does not fit any common mechanism.



(6)

If proflavine binds to the active site of thrombin even with heparin bound, substrate should displace it in the usual way. Thus, it should be possible to follow the reaction of Tos-Arg-OMe with thrombin by the proflavine method in the presence of heparin. Figure 3, curve C, shows reactions in the stop-flow followed by dye displacement. It can be seen that the total amount of dye which is bound to the active site (= total released in the acylation step) is the same, or slightly more than that bound in the absence of heparin. The rate of acylation is only slightly reduced—by about 20%. On a slower scale, it was observed that k_3 is unchanged.

Binding of Heparin to $PhCH_2SO_2$-Thrombin and Other Proteins. Phenylmethylsulfonyl fluoride ($PhCH_2SO_2F$) inactivates thrombin and other serine proteases by reaction at the active site serine (Fahrney and Gold, 1963; Lundblad, 1971). Figure 6 shows the reaction of $PhCH_2SO_2F$ with thrombin in the presence and absence of heparin. Heparin does not protect against inactivation by $PhCH_2SO_2F$. The resulting phenylmethylsulfonyl derivative ($PhCH_2SO_2$-thrombin) binds a negligible amount of proflavine (presumably due to 2–3% residual activity in our preparation). When $PhCH_2SO_2$-thrombin is added to heparin-proflavine solutions the difference absorption spectrum (*vs.* free dye) is almost completely abolished (Figure 7). The small absorption can be attributed to the presence of 2–3% thrombin which did not react with $PhCH_2SO_2F$.

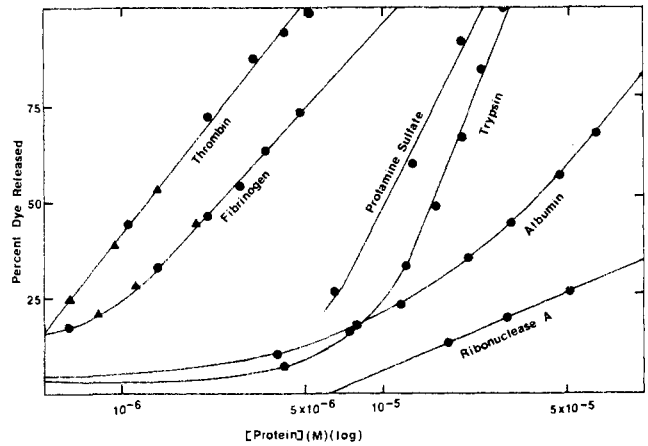


FIGURE 8: Ability of proteins to displace dye from heparin. All solutions contained heparin 1.7×10^{-5} M and proflavine 3.0×10^{-5} M in 0.01 M Tris-HCl buffer (pH 7.4). Proteins were added stepwise and dye released was determined by measuring ΔOD at 446 after each addition (see Figure 4). Protamine solutions showed some precipitation of heparin-protamine complexes. Thrombin experiments were performed in the absence of fibrinogen (●) or with 1.0×10^{-6} M fibrinogen added (▲). For fibrinogen experiments, thrombin was absent (●) or 9.5×10^{-7} M (▲).

The binding of other proteins to heparin was also measured by their ability to displace proflavine and the results are shown in Figure 8. The same problems of interpretation of the thrombin titration apply to these experiments. However, the qualitative observation can be made that for a single concentration of dye and of heparin, thrombin is more effective at causing release of proflavine from heparin than several other proteins.

Since the hydrolytic step in the fibrinogen-fibrin conversion is inhibited by heparin (Abildgaard, 1968a) it is possible to use proflavine displacement to measure the binding of

heparin to thrombin in the presence of fibrinogen, and to fibrinogen in the presence of thrombin. Figure 8 (triangles) shows results of stepwise addition of one protein to heparin–dye solutions containing the other. The titrations are the same in both cases. Thus, displacement of dye from heparin by thrombin and fibrinogen are independent of each other.

Discussion

Use of Proflavine Method with Thrombin. It is clear from this and other work (Feinman *et al.*, 1973; Koehler and Magnusson, 1974) that proflavine binds to the active site of thrombin, and that spectral changes on binding and desorption can be used to follow reactions with substrates and inhibitors—similar to the results with other proteolytic enzymes (Bernhard *et al.* 1966; Hess, 1971). In summary, the evidence includes the similarity of binding and inhibition constants (Table I), lack of binding by $PhCH_2SO_2$–thrombin, and the direct observation that the kinetics of acylation by pNPGB are the same whether observed by spectral changes due to dye desorption, or product appearance. In addition, Tos-Arg-OMe hydrolysis shows kinetics qualitatively similar to predictions from the acyl-enzyme theory. In this regard, it is surprising that quantitative agreement with the acyl-enzyme hypothesis (eq 4) is not found. Although this theory has been supported by direct observation with rapid kinetic methods for many substrates of proteases (Hess, 1971; Feinman and Bernhard, in preparation), in some cases departures from the model have been observed (Bernhard and Gutfreund, 1970). These latter results were interpreted in terms of additional intermediates in the reaction scheme. The data presented here are not sufficient to decide whether this, or some other explanation, will apply to the Tos-Arg-OMe results. Work is continuing on this problem in our laboratory. From the identity of the second step with k_{cat}, it is reasonable to believe this second step is deacylation. Thus, for interpretation of our results with heparin we have assumed the first step represents acylation and contains ES complex formation although we do not assign any specific interpretation as to rate-determining step, etc.

Interaction of Thrombin and Heparin. Our interpretation of the results presented here is that thrombin binds 3–4 heparin molecules and that none of these bind at the active site. The basic observation supporting this idea comes from Figure 1 which shows that, in mixtures of thrombin, heparin, and proflavine, the spectrum resembles that for enzyme and dye alone. The most likely explanation is that thrombin binds heparin and dye is displaced from the latter (ID spectrum is lost). The heparin cannot bind at the active site or the ED spectrum would have been abolished due to displacement of dye. (All substances known to interact at the active site of serine proteases displace proflavine.) The reduction in ID spectrum cannot simply be due to competition by thrombin for the dye since, at the concentrations in Figure 1, for example, only 25% of the dye is bound to thrombin and, hence, any free heparin would bind to the available dye. The additional evidence that heparin binds thrombin but not at the active site can be summarized as follows. (a) Inhibition of Tos-Arg-OMe hydrolysis is hyperbolic competitive consistent with eq 6. (b) Tos-Arg-OMe hydrolysis can be followed in the stopped-flow by observing displacement of proflavine even in the presence of heparin. The amount of dye displaced is the same as that seen in the absence of heparin, although the acylation rate is somewhat reduced. (c) Heparin does not protect thrombin against inactivation by $PhCH_2SO_2F$. (d) $PhCH_2SO_2$–thrombin (which does not bind proflavine) causes release of dye from heparin (Figure 7).

Qualitatively our conclusion seems well supported. It is, however, difficult to quantitate the binding of heparin. The major problem is that the dye–heparin interaction is somewhat complex (Stone and Bradley, 1967). Our estimate from the binding studies is in the same range, 10^{-7}–10^{-8} M, as that determined from Tos-Arg-OMe inhibition. We can, thus, make the qualitative interpretation that binding is tight.

The exact nature of the heparin–thrombin association is not known but heparin–protein interactions are generally considered to be electrostatic. Such interactions are usually highly dependent on ionic strength and it is significant that binding of heparin is not drastically reduced in higher ionic strength. In this regard, even at 0.1 M salt, 10^{-6} M heparin will completely abolish clotting of a purified fibrinogen solution (Feinman and Williams, unpublished). Figure 7 shows that thrombin has higher selectivity than several other proteins for heparin, although this may not signify "specificity" in the sense of highly evolved sites for heparin.

From the results above, the inhibition of thrombin-catalyzed hydrolysis of fibrinogen by heparin cannot be due to blocking of the active site. Rather, it seems likely that heparin binds at a secondary fibrinogen binding site preventing ES complex formation. Abildgaard (1968a) has suggested that inhibition of thrombin-catalyzed release of fibrinopeptides was due to formation of thrombin–fibrinogen–heparin complexes. The titration of heparin by either thrombin or fibrinogen in the presence of the other (Figure 8) did not allow us to detect such a ternary complex. Additional binding of heparin in the range of 10^{-6} M would have been detected.[3] Abildgaard's original proposal was based on the observation that inhibition was increased by higher fibrinogen concentration. Inhibition in his system was measured as fraction of control at the same substrate concentration. The increased inhibition may then only be due to the effect of fibrinogen binding heparin as shown here and in other work (Bernfeld, 1966). Also, it is known that increasing concentration of fibrinogen can inhibit clotting even in the absence of heparin (Ehrenpreis and Scheraga, 1959; Shinowara, 1966; Feinman and Williams, unpublished). In any case, the substantial binding of heparin to both thrombin and fibrinogen is probably sufficient to account for the inhibition of fibrinogen hydrolysis and clotting in the absence of antithrombins. Although a fibrinogen–thrombin association in the presence of heparin is not excluded (see footnote 3), Figure 8 suggests in fact, that thrombin–heparin and fibrinogen–heparin do not form a complex at all.

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[3] We do not mean to imply that no ternary complexes exist, only that there is no *additional* binding of heparin which is specifically a result of fibrinogen–thrombin associations. Only a ternary complex where additional binding was involved could be used to explain enhanced inhibition as described by Abildgaard (see text).

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The Cooperative Binding of Two Calcium Ions to the Double Site of Apothermolysin†

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ABSTRACT: The binding of calcium ions to apothermolysin has been studied by gel filtration at 25°, with free calcium ion concentration ranging from 10^{-3} to 10^{-6} M; pH 9.00 ± 0.05. Two calcium ions were found to dissociate simultaneously in this concentration range. Analysis of the binding data reveals a linear Hill plot with slope 2.0 and a parabolic Scatchard plot, thus demonstrating that the binding of these two calcium ions is completely cooperative. From the maximum of the Scatchard plot the overall binding constant is determined to be 2.8×10^{9} M^{-2} under these conditions. We conclude that our binding data apply to the double-site [Ca(1)-Ca(2)] wherein the calcium ions are separated by 3.8 Å as described by B. W. Matthews and L. H. Weaver [*Biochemistry 13,* 1719 (1974)].

Thermolysin is known to bind four calcium ions (Feder *et al.,* 1971). This is confirmed by the three-dimensional structure of thermolysin at 2.3-Å resolution (Colman *et al.,* 1972), which reveals the presence of four calcium binding sites: Ca(1) and Ca(2), which are only 3.8 Å apart and form a so-called double site; and Ca(3) and Ca(4), which are quite distant from each other and from the double site. The four binding sites have been confirmed crystallographically by replacing the calcium ions with strontium, barium, and trivalent lanthanide ions (Colman *et al.,* 1972). Recently, a detailed study of the site conformational changes accompanying the replacement of the calcium by lanthanide ions (Matthews and Weaver, 1974) has provided a wealth of structural detail on the four binding sites.

Surprisingly, the calcium ion association constants of thermolysin in solution have not yet been determined, although an interesting calcium binding isotherm can be anticipated, especially from the structure of the double site to which an independent binding of the two calcium ions is highly unlikely. One of the problems in studying the binding of calcium ions to proteolytic enzymes is that their rate of autolytic degradation increases progressively with a decreasing amount of bound calcium ions. This problem has been recognized before in the case of thermolysin (Feder *et al.,* 1971) and columns equilibrated with 1,10-phenanthroline, as chelating agent for the active site zinc ion, have been used to suppress its autolytic degradation by formation of the apoenzyme. Unfortunately the high uv absorbance of 1,10-phenanthroline does not allow a convenient spectrophotometric determination of the resulting apothermolysin. We will describe here how the use of a non-uv absorbing zinc chelating agent, TEP,[1] allows an accurate estimation of the calcium binding isotherm for the two least firmly bound ions. The results are directly interpretable on the basis of the known X-ray structure of thermolysin.

Experimental Section

Materials. Thermolysin (crystallized, lot no. P-1512) was obtained from Sigma Chem. Co. and was used without further purification, after it was established that this material was homogeneous in gel filtration on Sephadex G-100

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[1] Abbreviations used are: TEP, tetraethylenepentamine; Tris, tris(hydroxymethyl)aminomethane.