Articles

Interaction of Thrombin and Antithrombin. Reaction Observed by Intrinsic Fluorescence Measurements†

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ABSTRACT: The reaction of plasma antithrombin (AT) with thrombin is accompanied by a change in intrinsic protein fluorescence. This signal can be used as a specific probe for progress of the reaction and allows continuous recording of kinetics under a variety of different concentrations of reactants. That the fluorescence change specifically monitors the reaction is shown in several ways: (1) Reaction of diisopropyl phosphofluoridate inhibited or N^{α}-*p*-tosyl-L-lysine chloromethyl ketone inhibited thrombin with AT does not exhibit a similar decrease in fluorescence. (2) For a range of protein concentrations over 2 orders of magnitude, the fluorescence data obtained in the absence of heparin are consistent with the established second-order behavior of the reaction, and the calculated rate constant is in good agreement with literature values. (3) Heparin increases the rate of fluorescence decrease, reflecting the acceleration of the antithrombin–thrombin reaction by this polysaccharide. (4) For experiments performed in the absence, or in the presence, of heparin, the decrease in fluorescence correlates well with the decline of thrombin activity, as measured with a small chromogenic anilide substrate. In experiments designed to demonstrate the value of this method for kinetic studies, reactions were carried out at constant heparin concentration and varying concentrations of the two proteins. Results provide qualitative support for the idea that, regardless of the mechanism of rate enhancement, heparin binds tighter to thrombin than to AT but heparin–thrombin is not the primary active species and may instead play a predominantly inhibitory role in the reaction. Experiments with trypsin (which reacts with antithrombin but shows little fluorescence change) and studies of the thrombin-catalyzed hydrolysis of small substrates suggest that the fluorescence perturbation may be due to the thrombin molecule and may be a general feature of thrombin active-site reactions.

The mechanism of reaction of the plasma inhibitor antithrombin (AT)[1] with proteases is understood in its broad outline; the inhibitor forms a 1:1 complex with the enzyme, and the glycosaminoglycan heparin is a positive effector of the rate of reaction but does not change the stoichiometry. Most researchers believe that a necessary condition for rate acceleration is the binding of heparin to AT [see Rosenberg & Damus (1973), Longas et al. (1980), Blackburn & Sibley (1980), Feinman (1979), and references cited therein], although some evidence in the literature suggests that the enzyme might be the target for heparin, at least in the reaction with thrombin [see Smith (1977), Hatton & Regoeczi (1977), Machovich et al. (1980), and references cited therein]. A major question in current research is whether the binding of heparin to either protein alone is sufficient or whether a ternary heparin–AT–enzyme intermediate is needed for rapid formation of the final, stable enzyme–AT complex. Several groups have presented evidence that such a species is required [see Holmer et al. (1979, 1981), Pomerantz & Owen (1978), Griffith (1982), and references cited therein], but other studies claim (Jordan et al., 1980) that any heparin–thrombin species are inhibitory. The resolution of such problems in mechanism depends on techniques for following the kinetics of reaction under a variety of different experimental conditions. Although several methods are currently available for monitoring the reaction, none are ideal. Assays for the loss of enzyme activity do provide a direct measure of the progress of the reaction (Jordan et al., 1980; Hatton & Regoeczi, 1977; Griffith, 1982), but they can be time consuming and somewhat cumbersome. The use of active-site titrants (Li et al., 1974, 1975; Evans et al., 1982; Olson & Shore, 1982) allows for continuous monitoring of the kinetics, but this method is indirect and depends on the fact that the active-site indicator is an inhibitor of the reaction, and corrections may have to be made for this effect. In this paper, we show that intrinsic protein fluorescence can be used as an alternative method for continuous recording of the kinetics of the thrombin–AT reaction. We show that the change in fluorescence is specific and gives results in agreement with traditional methods. To demonstrate that the method can be used to analyze the type of mechanistic problem described above, we provide a preliminary kinetic study of the effect of varying the concentration of reactants. In addition,

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[1] Abbreviations: AT, antithrombin; S-2238, D-phenylalanyl-pipecolyl-L-arginyl-*p*-nitroanilide; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; TAME, N^{α}-*p*-tosyl-L-arginine methyl ester; BAEE, *N*-benzoyl-L-arginine ethyl ester; DipF, diisopropyl phosphofluoridate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; PEG, poly(ethylene glycol).

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we present evidence that the major contributor to the fluorescence change is the thrombin molecule, and this fluorescent perturbation may accompany all reactions involving the active site of the enzyme.

Materials and Methods

Antithrombin. Human antithrombin was prepared according to a modification of the method of Thaler & Schmer (1975) as previously described (Chang et al., 1979). For some experiments, antithrombin samples generously provided by the American Red Cross were used.

Thrombin. Human α-thrombin was the generous gift of Dr. John W. Fenton II of the New York State Department of Health, Albany, NY, and typically had 2300 NIH units/mg.

Heparin. High-affinity heparin was the generous gift of Dr. Erik Holmer of Kabi, Stockholm, Sweden. It was purified from porcine mucosal heparin (AB Vitrum, Stockholm, Sweden) according to the method of Andersson et al. (1976). This material had an average molecular weight of 12000 and a specific anticoagulant activity of 280 IU/mg.

Chemicals. All chemicals used were reagent grade or better. The chromogenic substrate S-2238 (D-phenylalanyl-pipecolyl-L-arginyl-*p*-nitroanilide) was purchased from AB Kabi, Greenwich, CT.

Measurements of Protein and Heparin Concentrations. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Antithrombin concentrations were also determined by absorbance measurements by using $E_{1\%} = 6.5$ (Nordenman et al., 1977). Heparin concentrations were estimated by measuring the uronic acid content of the glycosaminoglycan by using the method of Bitter & Muir (1962). Inolex heparin lot 0405F011 was used as the standard in these assays.

Assays of Biological Activity. The active concentrations of stock solutions of thrombin were determined by active-site titration with pNPGB according to the method of Chase & Shaw (1969). Measurements of thrombin activity by the clotting test were as described earlier (Wong et al., 1982). Antithrombin active concentrations were assayed by the inhibition of thrombin activity as measured by pNPGB.

The kinetics of the antithrombin–thrombin reaction (with or without heparin) were monitored by using S-2238 in the following manner. Antithrombin was incubated with heparin in 0.05 M Tris, 0.15 M NaCl, and 0.1% PEG 8000, pH 8.3, 25 °C, for 5 min. Thrombin was then added to the incubation mixture to a final volume of 300 μL. After a variable amount of time, 500 μL of 0.60 mM S-2238 and 0.20 mg/mL Polybrene were added for 30 s followed by 200 μL of 75% acetic acid to stop the hydrolysis of the substrate. Levels of residual thrombin activity in the reaction mixture were determined by reading the OD_{405} value and comparing them to a standard curve.

Preparation of DipF- and TLCK-Thrombin. One milliliter of thrombin (88.5 μM) was diluted with 2 volumes of 0.30 M sodium phosphate and 0.75 M NaCl, pH 7.7. To this solution was added 10 μL of DipF (0.52 M in anhydrous 2-propanol) or 50 μL of TLCK (0.78 M) for 2 h at room temperature followed by a second addition of the same volume of each inhibitor. After another 2 h at room temperature, the solutions were incubated overnight at 4 °C and then dialyzed against 3 × 1000 mL of 0.05 M sodium phosphate and 0.75 M NaCl, pH 6.5. The final preparations exhibited no activity as measured by pNPGB titration and less than 1% activity as measured by the clotting test. Control thrombin samples which were not treated with inhibitors but were subjected to the other steps of the procedure retained full activity as measured by both assays, indicating little loss of thrombin activity by autolysis.

Fluorescence Measurements. Fluorescence intensity measurements were made on a Perkin-Elmer MPF-44A spectrofluorometer equipped with a differential corrected spectra unit (reference solution was Rhodamine B) and a thermostated cell holder. Unless otherwise indicated, all measurements were made at 25 °C, and all solutions were in 0.05 M Tris, 0.15 M NaCl, and 0.1% PEG 8000, pH 8.3. Intrinsic protein fluorescence was read at an excitation wavelength of 278 nm and an emission wavelength of 335 nm. For the antithrombin–thrombin reaction in the absence of heparin, long incubation times were generally required. Therefore, so that any possible photodecomposition of the protein samples could be avoided, fluorescence readings were taken only at 1–2-min intervals, with the samples exposed to light only 5–10 s during the actual reading. In the presence of heparin, incubation times were considerably shorter, and continuous monitoring of the reaction was possible. Nevertheless, the excitation slit width was reduced to 0.9 mm as a precautionary step (the emission slit width was usually 6 mm). All fluorescence readings were normalized to measurements of a standard tryptophan solution. Any instrument drift in the base line was detected by alternating between reading the sample solution and reading the reference solution. Finally, the fluorescence data were recorded on a Perkin-Elmer Model 056 recorder and stored on $5^1/_4$ in. floppy disks by an Apple II Plus microcomputer equipped with an IMI (State College, PA) Adalab analog to digital converter interface. Data stored by the latter method can then be retrieved and analyzed by the computer.

Fitting the Data to the Second-Order Rate Equation. For the simple second-order reaction, we have

$$A + B \rightarrow C \tag{1a}$$

where $A > B$ and C is the product formed by reacting A with B. If A, B, and C are all fluorescent at the same wavelength, it can be shown that the ratio of dF (the change in fluorescence at time t) to dF_{max} (the maximum fluorescence change) is related to the concentration of reactant B at that time to the initial concentration of B (B_0) by

$$dF/dF_{max} = (B_0 - B)/B_0 \tag{1b}$$

Rearranging and substituting $B_0/B = dF_{max}/(dF_{max} - dF)$ into the integrated second-order rate equation give

$$f = dF_{max}\{(A_0 - B_0)/[A_0 e^{(A_0 - B_0)k_2 t} - B_0]\} + f_{inf} \tag{1c}$$

where f is the fluorescence reading at time t, f_{inf} is the fluorescence reading at infinite time, and k_2 is the simple second-order rate constant. Equation 1c was fitted to the fluorescence data by a nonlinear least-squares regression routine to determine the values for k_2, dF_{max}, and f_{inf}.

Presentation of Fluorescence Data. Since fluorescence intensities alone are not absolute measures of sample concentration (or other sample parameters), readings at different instrument sensitivity settings made on different days cannot be directly compared. Therefore, for facilitation of the analysis of the data, fluorescence changes measured during the time course of the antithrombin–thrombin reaction (in the absence or presence of heparin) were normalized to the total or maximal change in fluorescence and presented as (dF_{max} –

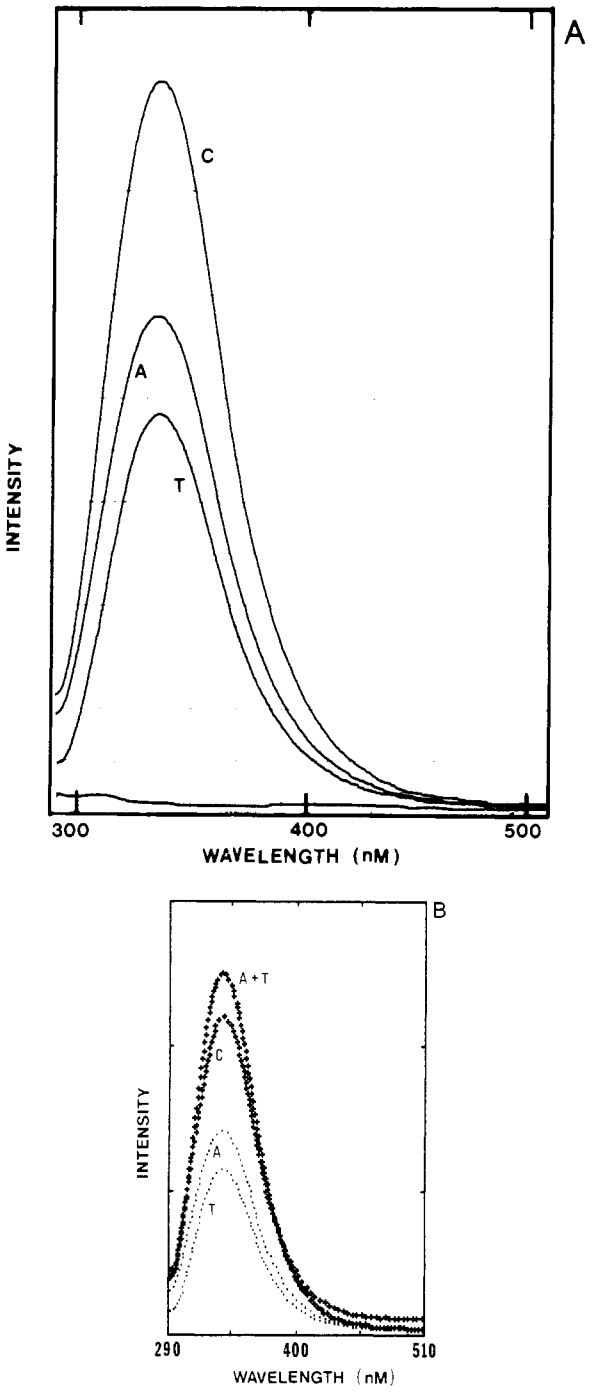

FIGURE 1: (A) Emission spectra of 0.25 μM thrombin (T), 0.50 μM antithrombin (A), and 0.25 μM thrombin + 0.50 μM antithrombin (C) after 30 min. The excitation wavelength is 278 nm. (B) Emission spectra from Figure 1A compared to the sum of spectra A and T (A + T). The sum A + T was computed over the entire wavelength region scanned.

$dF)/dF_{max}$. For each reaction, the value of dF_{max} was extrapolated from a linear least-squares fit of the data for the corresponding unaccelerated reaction to the equation

$$f = dF_{max}\{(A_0 - B_0)/[A_0e^{(A_0-B_0)k_2t} - B_0]\} + (f_0 - dF_{max}) \quad (2)$$

where slope = dF_{max}, y intercept = $f_0 - dF_{max}$, k_2 is the second-order rate constant determined previously under a wide range of reactant concentrations, and f_0 is f at $t = 0$.

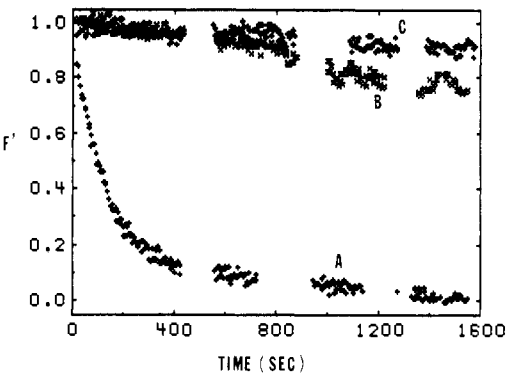

FIGURE 2: Reaction of 0.50 μM antithrombin with (A) 0.25 μM native thrombin, (B) 0.25 μM TLCK-thrombin, and (C) 0.25 μM DipF-thrombin. $F' = (dF_{max} - dF)/dF_{max}$. Excitation wavelength is 278 nm; emission wavelength is 335 nm. Interruptions in the trace are due to multiplexing with the tryptophan reference solution (see Materials and Methods).

Results

Thrombin–Antithrombin Reaction Is Accompanied by Changes in Intrinsic Protein Fluorescence. The emission spectra of antithrombin, thrombin, and the 1:1 complex formed by the two proteins are shown in Figure 1A. The excitation wavelength is 278 nm. The fluorescence emission peaks at 335 nm indicate that tryptophan residues are the major contributing fluorophores in all three protein species. Figure 1B shows the computed sum of the individual fluorescences of antithrombin and thrombin compared to the three spectra in Figure 1A. The figure shows that the fluorescence intensity of the complex is less than that of the computed sum. It is this decrease in fluorescence that we will show can be used to monitor the progress of the antithrombin–thrombin reaction.

Figure 2 (curve A) shows the time course of the fluorescence change when antithrombin and thrombin solutions are mixed. The idea that this change in fluorescence intensity is a specific signal for the antithrombin–thrombin reaction is supported by curves B and C, where AT was mixed with samples of thrombin that had been treated with the active-site inhibitors TLCK and DipF, respectively. It is clear from the figure that these inactive enzyme derivatives do not show the fluorescence change that accompanies the reaction of the native enzyme.

Kinetics of the Antithrombin–Thrombin Reaction in the Absence of Heparin. The reaction of antithrombin and thrombin at low concentrations of protein is shown in Figure 3. Under these conditions, long incubation times were required, and the proteins were exposed to light for only short periods of time to avoid any possible photodecomposition of the samples. In Figure 3, the longer lines represent individual fluorescence readings of the reaction mixture with the shorter adjacent lines representing readings of the standard tryptophan solution (see Materials and Methods). By fitting these time points to eq 1c, we determined the second-order rate constant (k_2). Good fits to eq 1c were obtained for reactant concentrations from 10 nM to 1 μM (inset to Figure 3), indicating that the unaccelerated reaction, as monitored by fluorescence changes, follows the predicted second-order kinetics. In addition, the average value of $(1.14 \pm 0.05) \times 10^4$ M^{-1} s^{-1} determined for k_2 is in good agreement with values determined by using more conventional assay methods (Bjork & Nordenman, 1976; Jesty, 1979; Longas et al., 1980; Jordan et al., 1980; Griffith & Lundblad, 1981; Wong et al., 1982), further supporting the specificity of this spectral change.

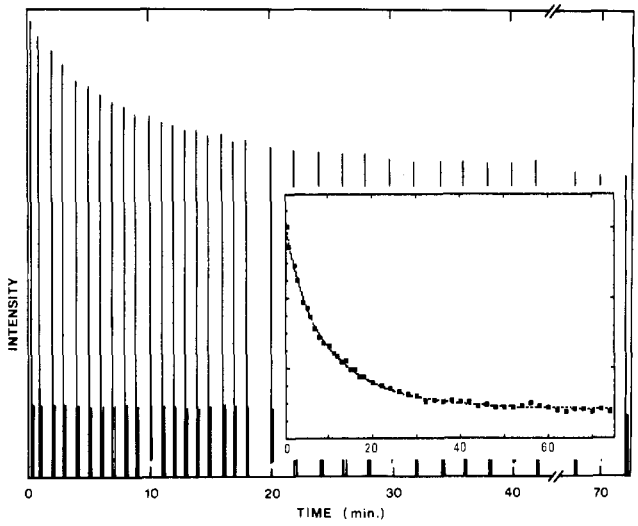

FIGURE 3: Reaction of 0.19 μM antithrombin with 0.097 μM thrombin. Longer lines represent fluorescence readings of the reaction (sample) mixture; shorter lines represent fluorescence readings of the standard (reference) tryptophan solution. (Inset) Nonlinear least-squares fit (---) of the same data (■) to eq 1c, shown on the same time scale. Excitation wavelength is 278 nm; emission wavelength is 335 nm.

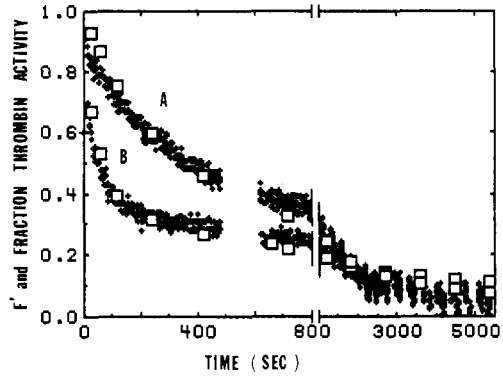

FIGURE 4: (A) Reaction of 0.17 μM antithrombin with 0.17 μM thrombin. (B) As in (A) except with 7.4 nM heparin added to the reaction mixture. (□) Reaction as monitored by using S-2238; (+) reaction as monitored by fluorescence (excitation wavelength 278 nm; emission wavelength 335 nm). $F' = (dF_{max} - dF)/dF_{max}$.

Kinetics of the Antithrombin–Thrombin Reaction in the Presence of Heparin. When high-affinity heparin was added to a reaction mixture containing antithrombin and thrombin, the rate of the intrinsic fluorescence change increased, reflecting the acceleration of the reaction by the glycosaminoglycan. Using low amounts of heparin so that the fluorescence from the antithrombin–heparin association does not contribute (Nordenman et al., 1978), it can be seen in Figure 4 that heparin does not alter the extent of the fluorescence change but does accelerate the initial drop in intensity. These results are in accord with the suggested "catalytic" nature of the heparin effect (Bjork & Nordenman, 1976; Carlstrom et al., 1977; Jordan et al., 1979; Feinman, 1979).

Correlation of the Fluorescence Change with Loss of Thrombin Activity. In order to further demonstrate that the intrinsic fluorescence change accompanying the inactivation of thrombin by antithrombin is a true measure of the kinetics of the reaction, we measured the loss of enzyme activity by using the standard rate assay techniques. In parallel experiments performed under similar conditions and reactant concentrations, the kinetics of the inhibitor–protease reaction were monitored by changes in fluorescence intensity or by loss of enzyme activity as measured by using the chromogenic substrate S-2238. As shown in Figure 4, the loss in thrombin activity is essentially superimposable on the decrease in fluorescence intensity both in the absence (Figure 4A) and in the presence (Figure 4B) of heparin.

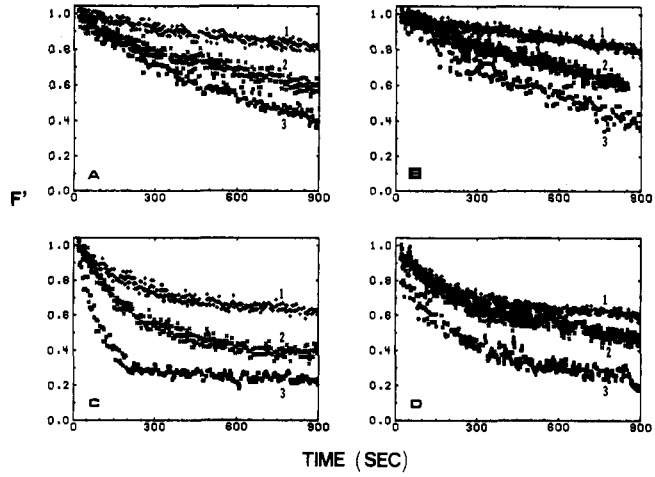

FIGURE 5: (A) (1) Reaction of 25 nM antithrombin with 25 nM thrombin in the absence of heparin; (2) as in (1) except [antithrombin] = 50 nM; (3) as in (1) except [antithrombin] = 100 nM. (B) (1) Reaction of 25 nM antithrombin with 25 nM thrombin in the absence of heparin; (2) as in (1) except [thrombin] = 50 nM; (3) as in (1) except [thrombin] = 99.9 nM. (C) As in (A) except 1.1 nM heparin was included in all reaction mixtures. (D) As in (B) except 1.1 nM heparin was included in all reaction mixtures. $F' = (dF_{max} - dF)/dF_{max}$. Excitation wavelength is 278 nm; emission wavelength is 335 nm.

Use of the Fluorescence Change for Studying the Kinetics of the Heparin-Accelerated Antithrombin–Thrombin Reaction. In order to study the action of heparin in greater detail, the concentrations of antithrombin and thrombin were lowered so that the unaccelerated reaction contributes little to the total fluorescence change within the times of our measurements (i.e., the initial unaccelerated rate was relatively slow). In addition, under these conditions, the kinetics of the heparin-enhanced reaction can be observed on a conventional time scale. As indicated in the introduction, a major concern in antithrombin research is the relative contributions to the reaction of the various heparin–protein species. Under these conditions, it is possible to determine the effects of varying the concentrations of the reactants on the kinetics of the reaction. Figure 5C shows that when thrombin and heparin were maintained at fixed concentrations, increasing the antithrombin concentration caused marked increases in the rate of reaction. These accelerations in rate are heparin dependent; much smaller increases in rate were observed when antithrombin concentrations were raised in the absence of heparin (Figure 5A). Moreover, since the AT–thrombin reaction is bimolecular, one would expect to observe similar rate increases if the concentration of thrombin is likewise increased. Indeed, Figure 5B demonstrates that increasing the thrombin concentration at fixed AT levels in the absence of heparin produced results which essentially duplicated those obtained when the antithrombin concentration was varied (Figure 5A). However, for the heparin-enhanced reaction, this is surprisingly not the case. As shown in Figure 5D, increasing the concentration of thrombin at fixed antithrombin and heparin levels did not cause the corresponding increase in the rate of the reaction

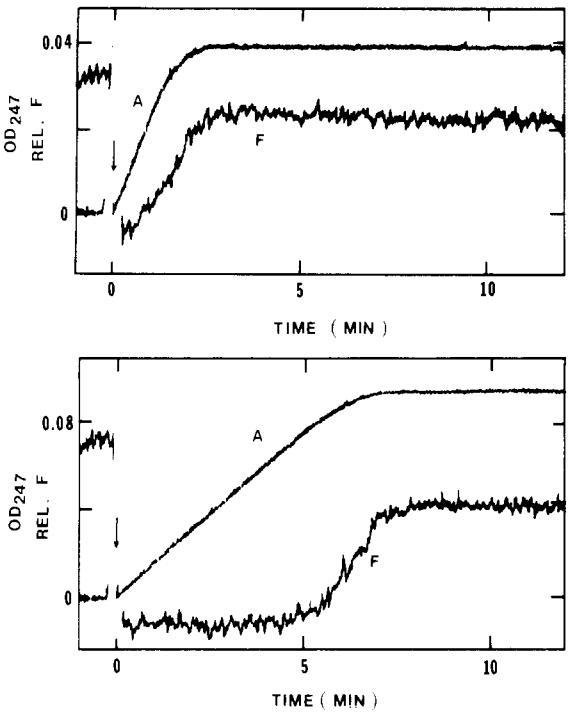

FIGURE 6: (Top panel) (F) Kinetics of reaction of 36 μM TAME with 0.10 μM thrombin as monitored by fluorescence (excitation wavelength 278 nm; emission wavelength 335 nm). (A) Kinetics of hydrolysis of 36 μM TAME by 0.10 μM thrombin as measured at OD_{247}. (Bottom panel) Same as in top panel except [TAME] = 180 μM (note: the initial rate of hydrolysis of TAME is actually the same in both top and bottom panels; the difference shown in the figure is due to different scales on the OD_{247} ordinate).

that was observed when only the antithrombin concentration was raised (Figure 5C).

Use of the Fluorescence Change as a General Method for Studying Thrombin Reactions. When the reaction of trypsin with antithrombin was monitored by intrinsic protein fluorescence measurements, only minimal changes were observed. This suggested that the major component of the fluorescence change in the AT–thrombin reaction may be attributed to the thrombin molecule. It was of interest, then, to determine if the perturbation of intrinsic protein fluorescence is characteristic of thrombin reactions in general. The reaction of thrombin with small substrates provides a good first choice for these studies since many substrates are available which do not fluoresce at the same wavelengths as proteins. Initial attempts to use substrates such as pNPGB or S-2238, however, were unsuccessful due to complete or partial quenching of the thrombin fluorescence. This effect was found to be nonspecific since these substrates also quenched the fluorescence of tryptophan alone. On the other hand, ester substrates such as N^{α}-*p*-tosyl-L-arginine methyl ester (TAME) or *N*-benzoyl-L-arginine ethyl ester (BAEE) only minimally quenched the fluorescence of tryptophan. The addition of either of these substrates induced a biphasic change in the intrinsic fluorescence of thrombin. As shown in Figure 6 for the reaction of thrombin with TAME, there is an initial drop in fluorescence intensity when substrate is added, followed by an eventual recovery of the intensity to the levels recorded in the absence of substrate (the incomplete recovery of fluorescence is most likely due to minor quenching effects by the substrate). The duration of the first phase corresponded exactly to the hydrolysis of the substrate; i.e., the thrombin fluorescence remained in the first phase of decreased intensity only as long as the enzyme was actively hydrolyzing substrate. In addition, the life of the first phase increased with increasing substrate concentration (top panel vs. bottom panel of Figure 6). These results indicate that this phase in the perturbation of thrombin fluorescence provides a direct visualization of the E–S and/or acyl enzyme intermediates in the reaction of the enzyme with substrate. The initial drop in fluorescence reflects the formation of the E–S and/or acyl enzyme intermediates, while the recovery of fluorescence reflects the eventual destruction of these intermediates following substrate depletion.

Discussion

The results presented here demonstrate that the intrinsic protein fluorescence of a mixture of thrombin and antithrombin is correlated with the concentration of active enzyme in the solution. Analysis of kinetics using this parameter as an indicator gives results consistent with reports in the literature, and with experiments performed in parallel by using traditional methods. That the decrease in fluorescence is actually due to changes in the thrombin molecule is suggested by the fact that minimal fluorescence changes are seen when trypsin is allowed to react with antithrombin, a reaction that has been shown to follow the same general scheme (Wong et al., 1982). In addition, other thrombin reactions involving the active site also show this change in fluorescence, and most significantly, it is possible (Figure 6) to show a perturbed fluorescence in a steady state during the turnover of substrate under the usual conditions of Michaelis–Menten kinetics.

Fluorescent methods are, in general, highly sensitive, and reactions with low reactant concentrations can be readily monitored. Because of the low concentrations, heparin-accelerated reactions can be observed on a conventional time scale. As demonstrated in Figures 4 and 5, addition of heparin at a heparin:antithrombin molar ratio of 0.04:1 to a mixture containing antithrombin and thrombin at equimolar concentrations causes a small acceleration of the reaction. This appears unusual since at similar heparin:antithrombin molar ratios drastic increases in reaction rates are observed when measured by clotting or small chromogenic substrate assays. However, in such assays, antithrombin is usually in excess of thrombin. Indeed, when much higher levels of antithrombin were used under our conditions, much greater rate accelerations were also observed (Figure 5C).

The utility of this method of following the thrombin–AT reaction is demonstrated by the study (Figure 5) in which the concentrations of protein reactants are varied. The rationale of the study is as follows. It is assumed, first, that the reaction is roughly "catalytic" in heparin; that is, the binding of heparin to the thrombin–AT complex is much weaker than the binding to either reactant, and the heparin concentration is, therefore, relatively constant in the early part of the reaction as the heparin recycles. The initial rate of reaction (neglecting the reaction rate in the absence of heparin which is relatively small under these conditions) is proportional to the concentration of active heparin–protein(s) species. The concentration of this intermediate, and, therefore, the rate of reaction, should go up if the concentration of the relevant reactant or reactants is increased, unless that species is already saturated or unless the added reactant participates in an inhibitory mechanism in addition to its effect in stimulating the rate. The observed result is that increasing the concentration of AT has a much greater effect on the rate of reaction in the presence of a small amount of heparin than a corresponding increase in the concentration of thrombin. These data suggest certain restrictions on any proposed mechanism for the reaction. First, the lack of stimulation by thrombin would suggest that thrombin–he-

parin by itself is not the active species. This is consistent with much work in the literature. In addition, the observation that one reactant increases the rate, while the other does not, indicates that one of the following must be true.

(1) If heparin–AT is the only active species in rate acceleration, then the data in Figure 5 indicate that its equilibrium formation is not saturated (hence, the increase in rate as AT is added). Furthermore, the thrombin–heparin species must be present in sufficient concentration to be inhibitory directly and/or indirectly by competing with AT for heparin (hence, little acceleration occurs at equimolar protein concentrations, and thrombin additions do not induce the increases in rate observed with AT). This is consistent with the analysis given by Jordan et al. (1979) and the observations of Pletcher & Nelsestuen (1982). In distinction to the interpretation in the latter work, however, the model suggested here argues that reaction of thrombin (with heparin–AT) is rate determining, but the lack of dependence on thrombin concentration is attributable to a competing reaction that depends on thrombin. On the other hand, Griffith (1982) has found saturable kinetics with respect to both AT and thrombin. More work will be needed to explain the difference between these conflicting observations.

(2) If a rapidly equilibrating AT–thrombin–heparin ternary complex is the active species, then one can make the interpretation that the thrombin–heparin association is completely saturated. In other words, the active species must be at a maximum for the given AT concentration, and thrombin cannot increase it further. Adding antithrombin, then, increases the rate by virtue of increasing the equilibrium concentration of the ternary complex. An inhibitory role for some thrombin species may also exist, depending on the relative binding constants of the two proteins for heparin.

In summary, the results suggest that the thrombin–heparin association is tighter than the AT–heparin association, and/or the mechanism is complicated by additional inhibition by some thrombin–heparin binary species. This observation bears on a somewhat confusing point in the literature. Those researchers who have argued for the primacy of the thrombin–heparin interaction have emphasized tighter binding of heparin to thrombin (Griffith, 1979), whereas those workers who have advocated AT as the target have found poorer binding of heparin to thrombin than to AT (Jordan et al., 1980), although, of course, there is no necessary requirement that the active species has the tightest binding constant. The current work suggests that, regardless of the mechanism, the thrombin association is tighter than the AT binding. In this regard, the best evidence supporting a ternary complex is the observation (Holmer et al., 1981) indicating that, for different enzymes, there is variability in the sensitivity to the molecular weight of the heparin used. This is explained by the requirement for binding of enzyme to heparin in the case of some proteases, such as thrombin, IXa, and XIa, but not others, such as Xa and XIIa where AT binding produces the maximal rate enhancement.

Acknowledgments

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