the enzyme is a tetramer and that it is sufficient to block one of the monomers to inactivate the complex. These results are in agreement with previously published data which demonstrated evidence for fluorescence energy transfer between purified reconstituted (Ca^{2+})ATPase monomers (Vanderkooi et al., 1977) and with equilibrium centrifugation of catalytically active (Ca^{2+})ATPase soluble in detergent (Le Maire et al., 1976). An alternative explanation for the observed results is that the interaction of DCCD with the enzyme follows multiple routes: part of the ^{14}C label is released as a diacylurea derivative following a nucleophilic attack of the intermediary complex (by an adjacent amino group of an amino acid side chain?) and part of the DCCD is covalently bound as a result of a rearrangement conversion of the intermediary complex (Carraway & Koshland, 1972).

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Antithrombin Reactions with α- and γ-Thrombins†

Tien-ling Chang, Richard D. Feinman,* Bryan H. Landis, and John W. Fenton II

ABSTRACT: Human α-thrombin with high clotting activity and its proteolyzed derivative γ-thrombin with virtually no clotting activity reacted in an essentially identical manner with antithrombin. The two enzyme forms bound proflavin with similar constants and showed identical behavior with small substrates. No significant differences were found for the antithrombin reactions (measured by proflavin displacement or active site titration) with respect to kinetics, extent of reaction, or effect of added heparin. The enzyme–antithrombin complexes could not be dissociated with sodium dodecyl sulfate ($NaDodSO_4$) but the $NaDodSO_4$-denatured complexes were dissociated by hydroxylamine treatment. The γ-thrombin–antithrombin complex has an approximate molecular weight of 75000 by disc gel electrophoresis as compared with 100000 for the α-complex, consistent with the polypeptide structures of the two proteins. The γ-thrombin–antithrombin complex did not inhibit clotting catalyzed by α-thrombin. In addition, fibrinogen did not affect the reaction of γ-thrombin with antithrombin or antithrombin–heparin. Thus, the antithrombin and antithrombin–heparin reactions do not involve the fibrinogen recognition sites which are destroyed by proteolytic conversion of α-thrombin to the noncoagulant γ form.

The enzyme responsible for the clotting of fibrinogen in blood, α-thrombin (EC 3.4.21.5), can be converted by proteolysis to a form, γ-thrombin, which retains esterase activity but has minimal clotting ability. The existence of multiple active forms is also known for other proteases such as trypsin and chymotrypsin, but the thrombin preparations have two unusual properties. First, α-thrombin's ability to clot fibrinogen represents high specificity for particular arginylglycine bonds. An enzymically active form such as γ-thrombin, which has lost this specificity, is clearly useful. Second, the amino acid residues responsible for enzymatic activity appear on three separate chains in γ-thrombin as a result of the α- to γ-proteolytic conversion, and is, thus, of interest in the study of enzyme mechanism. The structure and properties of the various human thrombin forms are summarized in Figure 1. The derivative γ-thrombin has been found to retain some of the biological activities of α-thrombin. These include activation of factor XIII (Credo et al., 1976; Lorand & Credo, 1977), factor D like activity in the alternate complement pathway (Davis et al., 1978), and reduced although qualitatively similar activity in initiating platelet reactions (Charo et al., 1977). Also, reaction of native and γ-thrombins was shown to be the same toward an affinity label for sites adjacent to the active site (exo-sites; Bing et al., 1977).

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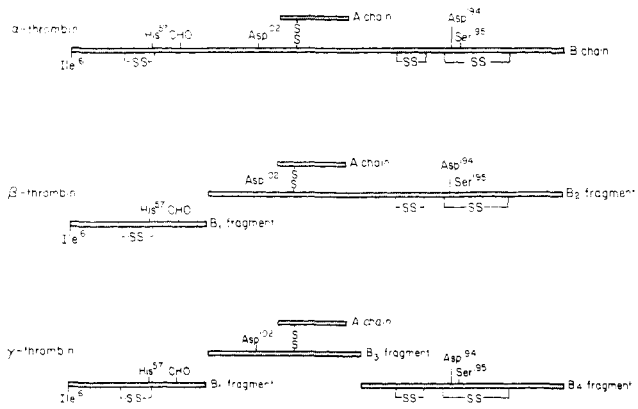

FIGURE 1: Human thrombins. The product of human prothrombin activation is α-thrombin. This form consists of a 36-residue A-chain and a 259-residue B-chain polypeptide joined by a disulfide bridge. Limited proteolysis (e.g., autolytic, tryptic) converts α-thrombin to β- and subsequently γ-thrombin with an accompanying loss of clotting activity (see text). These cleavages occur at approximately one-third and two-thirds from the B-chain amino terminus in the formation of β- and γ-thrombins, respectively. Human γ-thrombin is a unique enzymic form in that the B-chain fragmentation separates the functional residues comprising the charge relay system (His-57, Ser-195, and Asp-102) and the activation salt bridge (Ile-16 and Asp-194). This form has no counterpart in the bovine thrombin system. Those residues which are expected—from comparison with pancreatic proteases—to participate in small-molecule substrate binding are located adjacent to the catalytic serine in the carboxy-terminal B4 fragment (Magnusson et al., 1975). Structures are based on sequence data of Butkowski et al. (1977) and Thompson et al. (1977) for α-thrombin. Cleavage sites for β- and γ-thrombins are tentative assignments from Fenton et al. (1977b). Numbers for amino acids refer to the homologous position in chymotrypsin. The correspondences between these numbers and the actual position in the thrombin peptide chain are: Ile-16, 1; His-57, 43; Ser-195, 205; Asp-102, 99; Asp-194, 204.

An important physiologic reaction of thrombin is the interaction with the endogenous plasma coagulation inhibitors. Thrombin, as well as other coagulation enzymes, is known to react with the 65 000 molecular weight inhibitor antithrombin.[1] This inhibitor forms a tight, probably covalent complex with the active site of thrombin (Rosenberg & Damus, 1973; Owen, 1975). The glycosaminoglycan heparin is a positive effector of the reaction and is generally believed to effect rate enhancement via a conformation change in the antithrombin molecule (Rosenberg & Damus, 1973; Li et al., 1976), although it has also been proposed that the inhibited enzymes are the target for heparin (Sturtzebecher & Markwardt, 1977; Machovich et al., 1975–1977). The reaction of antithrombin with proteases can be followed by observing displacement of the acridine dye proflavin from the active site of the enzyme (Li et al., 1976, Feinman et al., 1977), and it is thus possible to study the kinetics of this reaction by continuous recording of enzyme inactivation. The results reported here show that α- and γ-thrombins have virtually identical kinetic behavior with respect to the antithrombin reaction and its activation by heparin. Thus, the modification in thrombin which abolishes clotting activity is at a locus separate from those sites involved in the antithrombin and antithrombin–heparin reactions.

Materials and Methods

Thrombin Preparations. Human α-thrombin was prepared from fraction III paste (gifts from Dr. Robert M. Silverstein and Dr. Fred Feldman of Armour Pharmaceutical Co., Kankakee, Ill.) and stored frozen in 0.75 M NaCl at −70 °C until used (Fenton et al., 1977a,b). Preparations of predominantly the γ-thrombin form were made by controlled passage of α-thrombin through trypsin–Sepharose 4B and similarily stored (Fenton et al., 1977b; Bing et al., 1977). Protein concentrations were based on an absorption coefficient of 1.83 mL mg^{-1} cm^{-1} at 280 nm in 0.10 M NaOH, and a molecular weight of 36 500 was assumed for all thrombin forms. Clotting assays were standardized against reference thrombin B-3 (kindly supplied by Dr. David L. Aronson, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.). Thrombin preparations were evaluated for enzymic purity as described elsewhere (Fenton et al., 1977a). The majority of the present experiments were performed with two α-thrombin preparations (2300 and 2600 US (NIH) clotting units/mg; 75.2 and 84.3% active by NPGB titration; 98.5% α-, 1.5% β-, 0.0% γ-thrombins and 99.4% α-, 0.6% β-, 0.0% γ-thrombin forms by electrophoresis of [^{14}C]DFP-labeled samples, respectively) and with three γ-thrombin preparations (1.3, 2.3, and 5.7 units/mg; 72.5, 75.0, and 76.5% active; and 0.0% α-, 15.0% β-, 85.0% γ-thrombin, 0.0% α-, 11.5% β-, 88.5% γ-thrombin, and 3.7% α-, 12.7, β-, 83.6% γ-thrombin forms, respectively).

Human antithrombin was purified by a modification of the procedure of Thaler & Schmer (1975). The final eluate of the Sepharose 4B–heparin-affinity column (0.02 M imidazole, 1.2 M NaCl, pH 6.5) was concentrated to a small volume with an Amicon ultrafiltration cell fitted with a PM 30 membrane. It was then dialyzed against 0.025 M Tris, 0.1 M NaCl, pH 7.0, and passed through a DEAE-Sephadex column equilibrated with 0.025 M Tris-HCl, 0.1 M NaCl, pH 7.0. Elution was carried out with 0.025 M Tris, 0.22 M NaCl, pH 7.0. The eluate was again concentrated with a PM 30 membrane. Ammonium sulfate precipitation was as described by Thaler & Schmer (1975). The final precipitate was dissolved in distilled water and extensively dialyzed against 0.01 M ammonium acetate and the 0.05 M sodium barbital, 0.1 M NaCl, pH 8.3 at 4 °C. The total protein concentration was determined by the method of Lowry et al. (1951). The concentration of active antithrombin was determined by the amount of thrombin which could be inactivated. Preparations were 90–98% active antithrombin and appeared to be a single band protein by NaDodSO$_4$ gel electrophoresis.

Heparin (Inolex Co; sodium salt) was used without further purifications or was purified to fraction A by cetylpyridinium chloride (Roden et al., 1972). Heparin concentrations are given in approximate molar value based on specific activity and approximate average molecular weight of 11 000 determined by osmometry.

Fibrinogen of bovine origin was purchased from Calbiochem and was further purified by the method of Blomback & Blomback (1956). The final product was 96% clottable.

Chemicals. *p*-Nitrophenyl *p'*-guanidinobenzoate hydrochloride (pNPGB)[2] was purchased from Vega-Fox Biochemicals. Tosylarginine methyl ester (Tos-Arg-OMe) was purchased from Schwarz/Mann Biochemical Co. Proflavin sulfate (Schwarz/Mann) was recrystallized from methanol in the dark. *N*-α-Benzoylphenylalanylvalylarginine *p*-nitroanilide was purchased from Ortho (Raritan, N.J.). Other

[1] Antithrombin is also referred to in the literature as antithrombin III, antithrombin–heparin cofactor, heparin cofactor, and Xa inhibitor (XaI).

[2] Abbreviations used: Bz-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester; Dip-F, diisopropyl phosphofluoridate; pNPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; PEG, poly(ethylene glycol); Bz-Phe-Val-Arg-*p*-nitroanilide, N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide; Tos-Arg-OMe, N^{α}-tosyl-L-arginine methyl ester; AT, antithrombin.

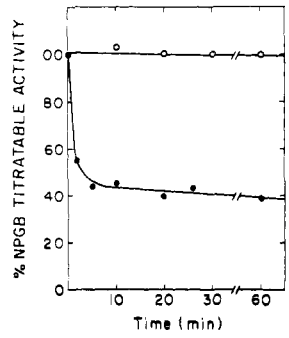

FIGURE 2: Inactivation of γ-thrombin by antithrombin. Antithrombin (2.4×10^{-6} M) was incubated with γ-thrombin (4.3×10^{-6} M) in 0.05 M sodium barbital, 0.1 M NaCl, pH 8.3, 25 °C. Samples at indicated times were titrated with 5×10^{-5} M pNPGB (solid circles). Control (no antithrombin): open circles.

chemicals were reagent grade and were used without purification.

Kinetic Methods. Reactions of enzymes with Bz-Arg-OEt and Bz-Phe-Val-Arg-*p*-nitroanilide were followed spectrophotometrically at 253 and 410 nm, respectively. Kinetic constants were determined by double-reciprocal plot using a weighted least-squares fit by a Wang calculator. The reactions with antithrombin using the proflavin method and stop-flow spectrophotometry were as described previously (Feinman et al., 1977). The extent of the antithrombin reactions were determined by active site titration with pNPGB (Chase & Shaw, 1969) or by proflavin difference spectra (Li et al., 1974). Proflavin–enzyme dissociation constants were determined by titration of fixed concentrations of dye with enzyme (Bernhard et al., 1966; Li et al., 1974). Clotting tests were performed on the Hyland Clotek machine, in the presence of low concentrations of PEG 6000 (Fenton & Fasco, 1975; Fenton et al., 1977a).

Effect of Fibrinogen on the γ-Thrombin–Antithrombin Reactions. In these experiments the loss of proflavin from the active site of γ-thrombin as antithrombin binds was followed spectrophotometrically. The reaction mixture contained 3.4×10^{-6} M γ-thrombin and 1.2×10^{-7} M heparin in 1.98 mL. Reaction was initiated by the addition of 20 μL of antithrombin solution to a final concentration of 3.9×10^{-6} M. Before the antithrombin addition, 20 μL of fibrinogen was added to final concentrations of 0.8 to 9×10^{-7} M. The same concentrations of fibrinogen were added to the reference solutions which were identical with the sample except that enzyme was omitted. Heparin was used in these experiments to accelerate the reaction before a clot formed; although γ-thrombin has only 0.1% of normal clotting activity, a clot will form at very high enzyme concentrations.

Results

Antithrombin Reactions with γ-Thrombin. The inactivation of γ-thrombin by antithrombin is shown in Figure 2. Enzyme activity was determined by titration with the active site titrating agent pNPGB (Chase & Shaw, 1969). The molar concentration of active sites lost was equal to the antithrombin added.

The γ-thrombin preparations studied contained 10–15% β- and 80–90% γ-thrombin forms, but <5% parent α-thrombin. These preparations had less than 0.2% of the clotting activity of pure α-thrombin. Preincubation of antithrombin with γ-thrombin, however, protected the clotting activity of α-thrombin from the effect of the inhibitor. This was true both in the presence and absence of heparin. The behavior of γ-thrombin in the clotting test is summarized in Table I.

Table I: Effect of Antithrombin on the Activity of α- and γ-Thrombin in the Clotting Test[a]

expt	conditions	clotting time (s) 0 min[b]	30 min[b]	60 min[b]
1	α(native)-thrombin + buffer	4.6	4.8	4.4
2	γ-thrombin + buffer	>300		>300
3	α(native)-thrombin + antithrombin	4.6	70.7	98.4
4	γ-thrombin + antithrombin	>300		>300
5	α(native)-thrombin + antithrombin (preincub. with γ for 60 min)	5.2	5.3	5.3
6	γ-thrombin + antithrombin (preincub. with α for 60 min)	88.2	92.8	86.6

[a] For each experiment, the clotting time for thrombin (test solution) was measured at 0, 30, or 60 min after mixing with antithrombin (experiments 3–6) or buffer (experiments 1 and 2). In experiments 5 and 6, the antithrombin was first preincubated with the indicated thrombin before mixing with the test solution. In each case, the mixture of antithrombin and test thrombin were maintained at 25 °C in 0.025 M sodium phosphate, 0.1 M NaCl, 0.66% PEG 6000, pH 7.0. The clotting was measured as described in Materials and Methods at 37 °C with the same buffer. [b] Time after mixing thrombin with antithrombin or buffer (min).

Complex formation between γ-thrombin and antithrombin was also demonstrable with $NaDodSO_4$ disc gel electrophoresis. On $NaDodSO_4$ electrophoresis γ-thrombin showed three bands corresponding to B_1, B_3, and B_4 chains (Figure 1). Mixtures of AT and the enzyme showed a new band at approximately 75 000 molecular weight, consistent with complex formation between AT and B_4. Hydroxylamine addition to the system caused dissociation of the ($NaDodSO_4$-denatured) complex, analogous to results of Owen (1975) with the native enzyme.

Binding of Proflavin to γ-Thrombin. Mixtures of γ-thrombin and the acridine dye proflavin show the characteristic visible difference absorption spectrum of serine protease–proflavin complexes (Figure 3; Bernhard et al., 1966; Li et al., 1974; Koehler & Magnusson, 1974). The difference λ_{max} = 466 mn. Titration of proflavin with γ-thrombin gives a dissociation constant, $K_d = 8.5 \times 10^{-6}$ M. This is approximately the same as the constant determined for the native enzyme, $K_d = 7.0 \times 10^{-6}$ M. It should be mentioned that this is the first published value for the binding constant of proflavin to human α-thrombin and is quite a bit tighter than the binding of dye to the bovine enzyme where the value is $K_d = 3.0 \times 10^{-5}$ M (Koehler & Magnusson, 1974; Li et al., 1974). Addition of antithrombin to proflavin–γ-thrombin mixtures abolished the difference spectrum (Figure 3). Table II shows results of experiments, discussed below, in which γ-thrombin was found to be partially inhibited by antithrombin–heparin mixtures. The amount of thrombin inhibited was the same whether determined by proflavin absorption changes or by titration of remaining sites with pNPGB. Loss of enzyme–proflavin difference absorption, then, reflects loss of enzyme active sites.

Kinetics of AT–γ-Thrombin Reaction. The reaction of antithrombin with thrombin can be studied by continuous recording of the change in proflavin absorption (Li et al., 1976; Feinman & Li, 1977). In the absence of heparin or in the presence of low concentrations of heparin, the reactions can be recorded by conventional methods. Figures 4A–C show kinetic traces for reaction of antithrombin with α- and γ-

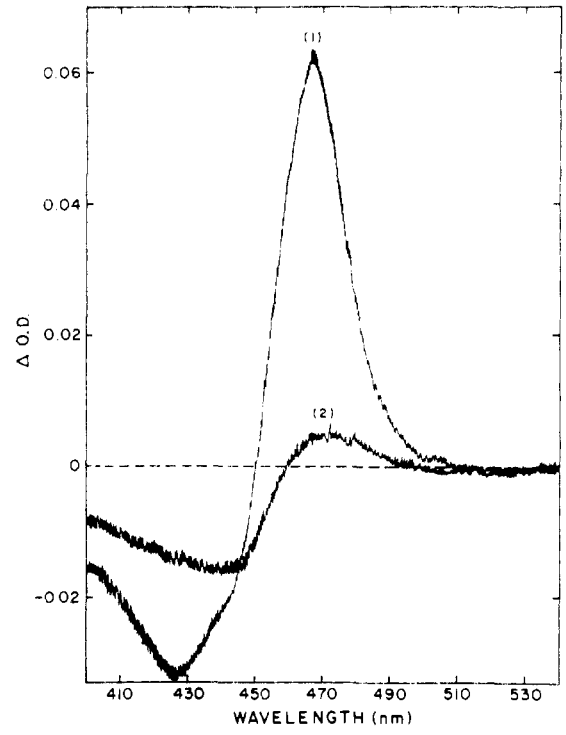

FIGURE 3: Proflavin difference spectra of solutions containing γ-thrombin and γ-thrombin–antithrombin complex. Reference solution: 3×10^{-5} M proflavin, 0.05 M sodium barbital, 0.1 M NaCl, pH 8.3. Sample solutions: (curve 1) same as reference plus 2.9×10^{-6} M γ-thrombin; (curve 2) same as reference plus 2.9×10^{-6} M γ-thrombin and 3.4×10^{-6} M antithrombin (45 min after incubation), 25 °C.

Table II: Kinetic Parameters for Reaction of α- and γ-Thrombin with AT[a]

thrombin	heparin (μM)	2nd-order rate constant (10^3 M^{-1} s^{-1})	% enzyme inhibited: proflavin	% enzyme inhibited: NPGB titration
α		1.9	96.9	95.4
γ		2.0	93.2	93.3
α	3.5	510.0	67.2	71.2
γ	3.5	490.0	62.1	62.2
		app 1st-order rate constant (10^{-2} s^{-1})		
α	0.12	3.4	73.5	77.0
γ	0.12	3.8	74.1	
	0.035	1.5	79.3	

[a] Values are for reactions measured by proflavin displacement determined by conventional spectrophotometry (low values or no heparin) or in the stop-flow ([heparin] = 3.5 μM). Reactant concentrations were: α-thrombin, 2.8 μM; γ-thrombin, 2.9 μM; and antithrombin, 3.4 μM.

thrombin with no added heparin or at low heparin concentration. Figure 4D contains traces from rapid-mixing, stop-flow studies where high (about equimolar) concentrations of heparin were used. It is evident that the kinetic behavior of α- and γ-thrombins is virtually identical in this system. Kinetic behavior of antithrombin and thrombins is summarized in Table II. The reaction of antithrombin and thrombin alone followed second-order kinetics and the rate constants from reciprocal plots are the same. At low heparin concentrations, the reaction has an apparent first-order behavior (Li, Windwer, & Feinman, in preparation). The apparent first-order rate constants are identical for the two thrombin forms. Figure 5 shows that the first half-time of α- and γ-thrombin reactions is the same in the range of low (approximately $0.3–3.5 \times 10^{-7}$ M) heparin and varies inversely with the heparin concentration. In all experiments reported here, heparin was preincubated with antithrombin. Premixing thrombin with heparin did not appear to change the kinetics. Interpretation of such premixing experiments is now believed to depend on the analysis and fractionation of particular heparin samples (Owen, 1977) and these results will be discussed elsewhere (Li, Chang, & Feinman, in preparation).

Table III: Steady-State Kinetic Parameters for Reaction of Thrombins with Small Substrates

	Bz-Arg-OEt		Bz-Phe-Val-Arg-*p*-nitroanilide	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
α-thrombin	41	30	77	49
γ-thrombin	40	30	148	24

[a] Reactions were followed spectrophotometrically at 253 nm for Bz-Arg-OEt or 410 nm for Bz-Phe-Val-Arg-*p*-nitroanilide.

We also observed in these reactions that the addition of heparin reduced the total extent of thrombin inhibited by antithrombin, consistent with many other reports in the literature and recently proposed to be a consequence of heterogeneity of heparin (Owen, 1977, and references therein).

Reactions of γ-Thrombin and AT in the Presence of Fibrinogen. The exceedingly low specific clotting activities of γ-thrombin preparations allow reactions of this enzyme form to be studied in the presence of fibrinogen. We found that fibrinogen would not displace proflavin from γ-thrombin, and was without effect in the antithrombin reactions. Figures 4C and 5 show that γ-thrombin reacts with antithrombin at the same rate regardless of the presence or absence of fibrinogen. We also found that fibrinogen (up to 45 μM) did not affect the rate of hydrolysis of 2×10^{-5} M Bz-Phe-Val-Arg-*p*-nitroanilide by γ-thrombin, but a complete kinetic study of this system was not performed (Figure 6).

Reactions with Small Substrates. For comparison with protein substrate reactions, the steady-state kinetic parameters for two small synthetic substrates were determined. The observed values are summarized in Table III.

Discussion

The structural basis of the specificity of thrombin for fibrinogen remains a challenging problem in coagulation chemistry. The region of the fibrinogen molecule required for interaction with thrombin appears to be extensive (for review, see Scheraga, 1977), and a reasonable working hypothesis is that thrombin contains two functionally identifiable sites. The first is the classical active site and immediately adjacent area (probable sites for specificity for arginine and/or benzamidine binding). This first site would contain the residues involved in catalysis (His-57, Ser-195, Asp-102, Asp-194, Figure 1) and would be relatively nonspecific with regard to protein substrates. The second would be a region of greater biologic specificity presumably containing those structural features that recognize the fibrinopeptide region of fibrinogen. The proteolyzed product γ-thrombin provides presumptive support for this idea as well as a means of elaborating and further testing it. This derivative has virtually no clotting activity but has the same esterase activity as native thrombin for Bz-Arg OEt. A summary of the evidence that the active site and neighboring regions are unchanged by the α- to γ-thrombin conversion is as follows. Small molecule substrates and competitive inhibitors have the same behavior with both forms (Fenton et al., 1977b); there is no detectable difference in Ser-195 (205)

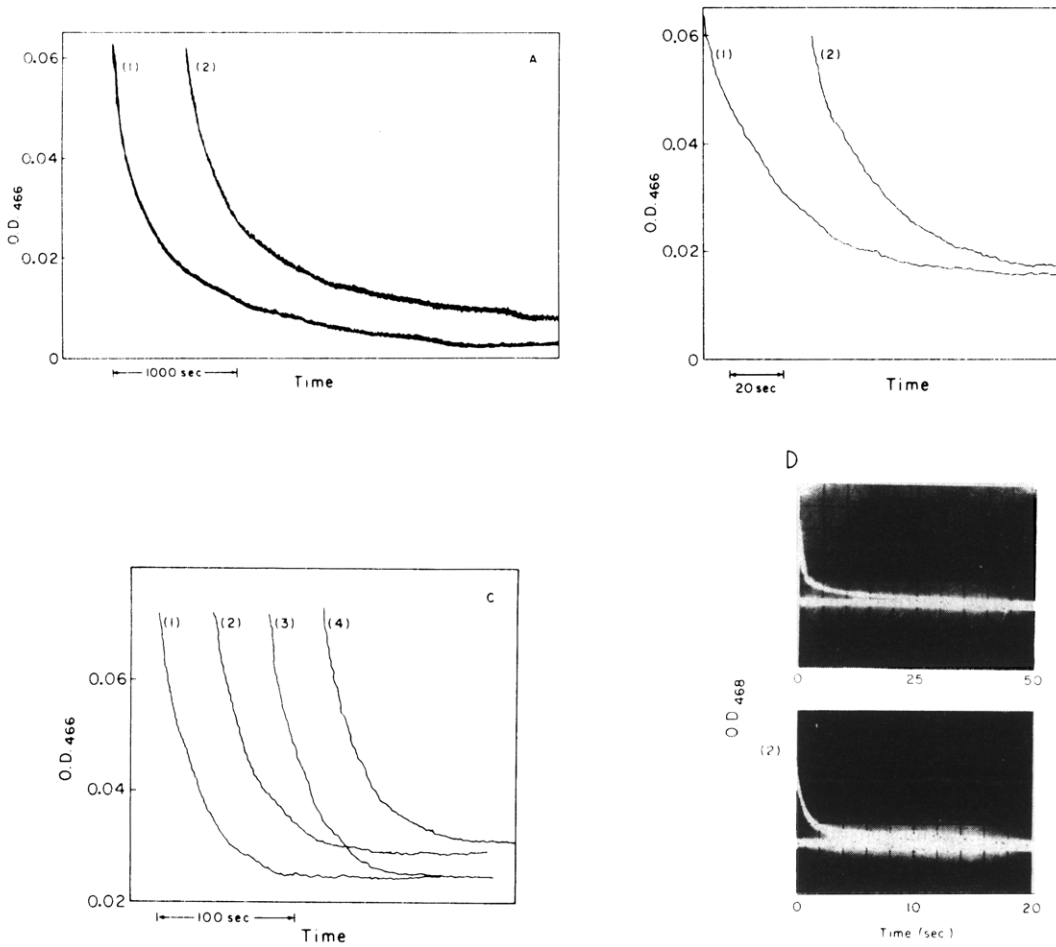

FIGURE 4: Kinetic studies of γ-thrombin–antithrombin and α-thrombin–antithrombin reactions, and the effects of heparin and fibrinogen. Reactions were carried out by observing the displacement of proflavin from the active sites of enzymes by antithrombin at 466 nm spectrophotometrically. (A) Antithrombin, 3.4×10^{-6} M, was added to 2.8×10^{-6} M α-thrombin (1) and 2.9×10^{-6} M γ-thrombin (2). (B) The effect of low concentrations of heparin on thrombin–antithrombin reactions. Antithrombin, 3.4×10^{-6} M, was premixed with 1.2×10^{-7} M heparin and then added to 2.8×10^{-6} M α-thrombin (1) and 2.9×10^{-6} M γ-thrombin (2). Both reference and sample solutions contained 3×10^{-5} M proflavin in 0.05 sodium M barbital, 0.1 M NaCl, pH 8.3 at 25 °C. (C) The effect of fibrinogen on γ-thrombin–antithrombin reaction. Antithrombin, 3.9×10^{-6} M, and varying concentrations of fibrinogen were added to a solution of 3.4×10^{-6} M γ-thrombin and 1.2×10^{-7} M heparin. Fibrinogen concentrations were: (1) zero; (2) 0.8×10^{-7} M; (3) 1.5×10^{-7} M; (4) 9×10^{-7} M. (D) Stopped-flow kinetics: reactions of thrombin, γ-thrombin, and antithrombin–heparin. (1) α-Thrombin (5.3×10^{-6} M) in one syringe was mixed with a solution of antithrombin (4.9×10^{-6} M) and heparin (6.9×10^{-6} M) in the other syringe. Both syringes contained 0.05 M sodium barbital, 0.05 M NaCl, pH 8.0, and proflavin (4.2×10^{-5} M). (2) γ-Thrombin (4.0×10^{-6} M), antithrombin (4.2×10^{-6} M), heparin (4.7×10^{-6} M), and proflavin (3×10^{-5} M) in 0.05 sodium M barbital, 0.1 M NaCl, pH 8.3. Reaction as described in 1.

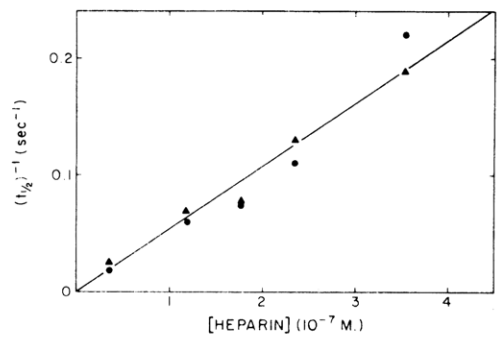

FIGURE 5: The effect of heparin concentration on α- and γ-thrombin antithrombin reaction. A mixture of 3.4×10^{-6} M antithrombin and varying concentrations of heparin was added to 3.0×10^{-6} M α-thrombin (▲) or 3.2×10^{-6} M γ-thrombin (●). The decrease of absorbance at 466 nm due to dye displacement was recorded spectrophotometrically. The first half-time of the reaction was measured and plotted against heparin concentrations. All reactions were carried out with 3×10^{-5} M proflavin, 0.05 M sodium barbital, 0.1 M NaCl, pH 8.3.

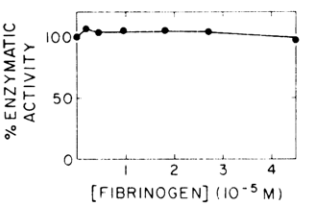

FIGURE 6: The effect of fibrinogen on the reaction of γ-thrombin with a small substrate. 4.4×10^{-8} M γ-thrombin was added to a solution of 2×10^{-5} M Bz-Phe-Val-Arg-*p*-nitroanilide and different concentrations of fibrinogen. Reference contained the same solution of the anilide substrate and fibrinogen except no enzyme was added. The rate of *p*-nitroaniline formation was measured spectrophotometrically at 405 nm. The reaction was carried out with 0.05 M Tris, 0.05 M imidazole, 0.125 M NaCl, pH 8.3 at 25 °C.

conjugated spin-label and fluorescent probes (Berliner & Shen, 1977); proflavin binding is essentially the same for the two enzymes (Results); the two thrombin forms show identical A- and B-chain labeling ratios with an exo-site affinity-labeling reagent (Bing et al., 1977).

The activity of γ-thrombin is not restricted to small molecules. This derivative can activate factor XIII (Credo et al., 1976; Lorand & Credo, 1977) and has factor D like activity in the alternate pathway of complement (Davis et al., 1978). These protein substrates, therefore, presumably do not require the fibrinogen binding site in thrombin. The reduced pro-

coagulant activity (factors V and VIII) of γ-thrombin may, however, be indicative of the loss of this specific biologic site (Fenton et al., 1977b). Platelets show a somewhat intermediary effect, responding in a qualitatively similar way to γ-thrombin but at a much reduced level from the native enzyme (Charo et al., 1977).

The observations that γ- and α-thrombin have essentially identical behavior with antithrombin are surprising, especially with regard to the effect of heparin. Heparin is generally believed to cause a conformation change in the antithrombin molecule so as to effect a more rapid rate, and it is reasonable to believe that this will involve facilitated binding of some part of the activated antithrombin molecule to some secondary site on thrombin (in addition to the active site). It has been suggested that such a secondary site might be identical with or part of the secondary fibrinogen site (Feinman & Li, 1977). This would seem not to be the case from results presented here. It would thus appear that antithrombin only reacts at the primary site (active site and immediately adjacent regions). In addition, if fibrinogen binds to γ-thrombin at all, it does not occlude this site since the reaction with antithrombin is unaffected by the presence of fibrinogen. The fact that tripeptide anilide hydrolysis is not reduced by fibrinogen would seem to indicate that the latter does not bind at all. These results do not mean that the binding of antithrombin to thrombin (or other proteases) does not involve sites other than the active center, but only that such regions of the thrombin molecule probably did not coevolve with antithrombin in the way that fibrinogen and fibrinogen-binding sites evolved in parallel.

It should be mentioned that the fact that the antithrombin reaction in the presence of heparin is the same for the modified and native thrombin is further suggestive evidence that antithrombin and not the enzyme is the target for heparin, a question which is still considered open. This is consistent with the fact that antithrombin and heparin–antithrombin react with other coagulation factors (Harpel & Rosenberg, 1976). These results are, however, somewhat in conflict with those of Machovich et al. (1977) who studied the interaction of plasma inhibitors with bovine β-thrombin. This form is a less proteolyzed derivative than γ-thrombin and retains about 10% clotting activity (Kingdon et al., 1977). They measured the loss of this residual activity when reaction with antithrombin took place. The β-thrombin was somewhat less susceptible to antithrombin than α, but, more striking, there was a drastic difference in the response to heparin added to their system. It is possible that the results reported by Machovich demonstrate the effect of heparin on β-thrombin in the clotting test. Measuring the active-site behavior, for example, by active ester titration, might explain the discrepancy between that report and our results.

The behavior of antithrombin with the two forms of thrombin is in contrast with that of hirudin, the anticoagulant protein from leeches (Bagdy et al., 1976, and references therein). Hirudin has a molecular weight of ~7000 and forms an extremely tight noncovalent complex with α-thrombin. The complex with γ-thrombin is two to three orders of magnitude weaker (Landis et al., 1978). Thus, hirudin would seem to bind to fibrinogen recognition sites and these results are consistent with hirudin being a thrombin-specific inhibitor, which forms a noncovalent complex, whereas antithrombin is a relatively nonspecific protease inhibitor, forming covalent complexes.

Antithrombin is the major coagulation inhibitor of the plasma and the only component whose concentration can be related to thrombotic tendencies (Harpel & Rosenberg, 1976, and references therein). The demonstration that γ-thrombin has behavior identical with that of native enzyme suggests that, if any proteolyzed forms of thrombin exist in the circulation, they may be of importance in reducing the antithrombin content. Machovich et al. (1976, 1977) has similarly suggested that β-thrombin which, in his work, reacted with antithrombin but with a reduced rate may be of biologic importance. The type of autolytic degradation required to produce these derivative forms is encountered in handling concentrated preparations but autolytic conversion of α- to the derivative β- and γ-thrombins might conceivably occur in vivo at sites of high local enzyme concentrations. Also, homologous conversions might be carried out by trypsin-like enzymes of various origins. In this regard, although brief exposure of α-thrombin to platelets does not cause conversion to derivative enzyme (Martin et al., 1976), enzyme species resembling the derivative forms have been observed upon incubating thrombin with tissue cultures (Zetter et al., 1977). Whether these derivatives do, in fact, accumulate in plasma and may thereby reduce endogenous anticoagulant activity remains to be determined.

One important implication of the present studies is that the use of an impure thrombin preparation will lead to low antithrombin assays when measured with the clotting test. Since any proteolyzed forms of thrombin will also react with antithrombin, the apparent inhibition of the clotting power of a thrombin preparation will be reduced compared with the actual molar concentration of inhibitor in the antithrombin sample under test. Ideally, well-characterized α-thrombin preparations should be used, or the ability of antithrombin to neutralize titratable active sites should be measured as well as clotting inhibition.

In summary, an important biological function of thrombin, reaction with antithrombin and heparin–antithrombin, is unaffected by the proteolysis that leads to loss of clotting activity.

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Yeast Mitochrondrial Deoxyribonuclease Stimulated by Ethidium Bromide. 1. Purification and Properties†

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ABSTRACT: A deoxyribonuclease (EtdBr DNase), which is about 25 times more active on double-stranded DNA, in which EtdBr is intercalated has been purified from yeast isolated mitochondria. This enzyme appears to be located in the mitochondrial membrane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified fraction yielded six major bands whereas, in native conditions, it migrates as a single band. In addition to the EtdBr DNase, this fraction contains two other DNase activities, both of which are inhibited by EtdBr at the same concentration which is used to measure the EtdBr DNase activity. One is detected on double-stranded DNA at acid pH and the other on single-stranded DNA at neutral pH. The three activities cosediment in a sucrose gradient and have similar rates of heat inactivation. The level of stimulation of the EtdBr DNase depends on the amount of intercalated EtdBr per nucleotide, the maximal activity being reached when all the intercalated sites are occupied. More detailed studies on the mechanism of this stimulation are described in the accompanying paper (Jacquemin-Sablon, H., et al. (1979) *Biochemistry 18* (following paper in this issue)). The enzyme, which cuts one strand at a time in a covalently closed circular PM2 DNA molecule, is classified as an endonuclease. Possible involvement of the EtdBr DNase in the process of "petite" induction by EtdBr was investigated by measuring the enzyme level in two mutants resistant to EtdBr mutagenesis. This enzyme was present at a normal level in both strains.

The DNA intercalating drug ethidium bromide[1] displays biological properties in several organisms. Smith et al. (1971) reported that, in mammalian cells, it rapidly inhibits mitochondrial DNA synthesis and induces a change in its superhelix density. However, there was no significant nicking or degradation of this DNA (Smith, 1977). Similar effects have been observed in the kinetoplastic DNA of trypanosome (Riou & Delain, 1969; J. Benard, G. Riou, & J. M. Saucier,

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[1] Abbreviations used: EtdBr, ethidium bromide; mtDNA, mitochondrial DNA; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin.

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