Characteristics of the Association between Prothrombin Fragment 2 and α-Thrombin†

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ABSTRACT: The esterolytic activity of bovine α-thrombin on the synthetic substrate *N*-α-*p*-tosyl-L-arginine methyl ester (TosArgOMe) is stimulated when the prothrombin activation fragment, prothrombin fragment 2, is added as previously reported by this laboratory (Heldebrant, C. M., and Mann, K. G. (1973), *J. Biol. Chem. 248,* 3642). A similar stimulation of β-thrombin is observed upon addition of prothrombin fragment 2. The binding constant of prothrombin fragment 2 to α-thrombin has been determined by the method of Gutfreund ((1972), Enzymes, Physical Principles, Wiley, New York, N.Y., pp 67–71). The dissociation constant is 7.7×10^{-10} M, and there is one molecule of prothrombin fragment 2 bound per molecule of α-thrombin. Prethrombin-2 competes for prothrombin fragment 2, so the enhancement of the esterolytic activity of α-thrombin by prothrombin fragment 2 was used as a probe to determine the dissociation constant for the binding of prothrombin fragment 2 to prethrombin 2. The dissociation constant for this association is 1.3×10^{-10} M. The kinetic parameters for the reaction of α-thrombin on TosArgOMe were determined in the absence and presence of prothrombin fragment 2 and are as follows: (a) in the absence of prothrombin fragment 2, $K_{m(app)} = 1.92 \times 10^{-4}$ M, and $k_{3(app)} = 35.8$ mol of TosArgOMe/mol of α-thrombin s^{-1}; (b) in the presence of prothrombin fragment 2, $K_{m(app)} = 1.76 \times 10^{-4}$ M, and $k_{3(app)} = 60.5$ mol of TosArgOMe/mol of α-thrombin s^{-1}. Thus, the stimulatory effect of bovine prothrombin fragment 2 on bovine α-thrombin is reflected in $k_{3(app)}$ and not in $K_{m(app)}$. In contrast to the stimulatory effect of prothrombin fragment 2 on the thrombin-catalyzed hydrolysis of TosArgOMe, it inhibits the activity of α-thrombin toward *N*-α-benzoyl-L-arginine ethyl ester and *N*-α-benzoyl-L-arginine *p*-nitroanilide. The inhibition of activity toward these substrates by prothrombin fragment 2 is also reflected in $k_{3(app)}$. Activity toward the nonspecific substrate *p*-nitrophenyl butyrate was completely inhibited by the addition of prothrombin fragment 2. Prothrombin fragment 2 has no effect on the inhibition of α-thrombin activity by the active-site serine inhibitors diisopropyl phosphofluoridate, phenylmethanesulfonyl fluoride, or *p*-nitrophenyl guanidinobenzoate. Inhibition by the active-site-histidine-modifying inhibitor, *N*-α-*p*-tosyl-L-arginine chloromethyl ketone, was enhanced by the addition of prothrombin fragment 2. Soybean trypsin inhibitor reduces the stimulation by prothrombin fragment 2, but only at high molar ratios. Prothrombin fragment 2 has no effect on the clotting activity of α-thrombin, nor inhibition of this activity by heparin, hirudin, or diisopropyl phosphofluoridate. Bovine prothrombin fragment 2 enhances the esterolytic activity of both human and bovine α-thrombin, but human prothrombin fragment 2 does not enhance the esterolytic activity of either human or bovine α-thrombin.

Thrombin is an essential proteolytic enzyme of the coagulation pathway which cleaves fibrinogen to yield the fibrin clot, activates factor V and factor VIII to more active forms, and catalyzes the formation of factor $XIII_a$ from precursor factor XIII. In addition, thrombin causes the aggregation of platelets and the formation of the platelet plug during the early stages of the hemostatic response. Previous reports have demonstrated that the activation of prothrombin, the precursor of thrombin, proceeds through a series of cleavages leading to the formation of thrombin (Stenn and Blout, 1972; Heldebrant and Mann, 1973; Heldebrant et al., 1973; Kisiel and Hanahan, 1973; Owen et al., 1974).

There are two peptides released during this activation: the amino-terminal peptide, prothrombin fragment 1,[1] and the penultimate amino-terminal peptide, prothrombin fragment 2. In characterizing the prothrombin molecule and its activation fragments, it has been observed that prothrombin fragment 1 is the calcium-dependent phospholipid binding portion of the prothrombin molecule (Gitel et al., 1973; Bajaj et al., 1975). Further, prothrombin fragment 1 has been shown to inhibit the conversion of prothrombin to thrombin by the

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[1] The nomenclature used in this paper is that which was agreed upon by the members of a Task Force on Blood Clotting Zymogens and Zymogen Intermediates of the International Society on Thrombosis and Hemostatis in the Vth Congress of this Society, held in Paris, France, in July 1975. Previous nomenclature used by several laboratories includes P for prothrombin (Stenn and Blout, 1972); intermediate 3 (Mann et al., 1971b; Heldebrant et al., 1973), fragment 1 (Kisiel and Hanahan, 1973; Esmon, et al., 1974), Fa (Stenn and Blout, 1972), A-fragment (Magnusson et al., 1974), and PR fragment (Reuterby et al., 1974) are equivalent to prothrombin fragment 1. Intermediate 1 (Mann et al., 1971b; Heldebrant et al., 1973; Kisiel and Hanahan, 1973; Esmon et al., 1974), P2 (Stenn and Blout, 1972), neoprothrombin-S (Magnusson, 1974) and prethrombin (Reuterby et al., 1974) are equivalent to prethrombin 1. Intermediate 4 (Heldebrant et al., 1973), fragment 2 (Kisiel and Hanahan, 1973; Esmon et al., 1974), Fb (Stenn and Blout, 1972), S-fragment (Magnusson et al., 1974), and O-fragment (Reuterby et al., 1974) are equivalent to prothrombin fragment 2. Intermediate 2 (Mann et al., 1971b; Heldebrant, et al., 1973; Kisiel and Hanahan, 1973; Esmon et al., 1974), P3 (Stenn and Blout, 1972), neoprothrombin-T (Magnusson et al., 1974), and prethrombin E (Reuterby et al., 1974) are equivalent to prethrombin 2.

prothrombinase complex, presumably by competing for the binding sites on the complex, to which the amino-terminal portion of the prothrombin molecule is attached during activation. Prothrombin fragment 2 also binds calcium, and has been shown to be the factor V binding portion of the prothrombin molecule (Bajaj et al., 1974; Mann and Fass, 1974; Esmon and Jackson, 1974). In the presence of prothrombin fragment 2, prethrombin 2 (the immediate precursor of α-thrombin) is activated at a much higher rate by the prothrombinase complex. This rate acceleration only occurs when factor V is present.

In addition to binding to factor V and prethrombin 2, prothrombin fragment 2 also binds to α-thrombin, enhancing the esterolytic activity of α-thrombin toward the synthetic substrate TosArgOMe[2] (Heldebrant and Mann, 1973; Mann et al., 1973). Using this enhancement of thrombin activity, we will determine the dissociation constants for the association of prothrombin fragment 2 with α-thrombin and with prethrombin 2.

The kinetic parameters have been calculated to determine if the change in α-thrombin activity is due to an alteration in $K_{m(app)}$ or a change in the catalytic mechanism. The effect of prothrombin fragment 2 on the activities of α- and β-thrombin toward other synthetic substrates and inactivation of thrombin by various inhibitors will also be presented.

Experimental Section

Materials

Ac-globulin was obtained from Difco, Detroit, Mich. NIH standard thrombin, Lot B-3, was obtained from the National Institutes of Health. Gum acacia was obtained from Scharr and Company, Chicago, Ill., and purified by the techniques described in the NIH clotting procedure.[3] Rabbit brain thromboplastin was prepared by methods previously described (Quick et al., 1935; Hurn and Barker, 1945). Sulfopropyl Sephadex C-50 was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Acrylamide, *N,N'*-methylenebis(acrylamide), imidazole, *N,N,N',N'*-tetramethylethylenediamine, and 2-mercaptoethanol were obtained from Eastman Organic Chemicals, Rochester, N.Y. *N*-α-*p*-Tosyl-L-arginine methyl ester and sodium dodecyl sulfate were purchased from Schwarz-Mann, Orangeburg, N.Y. *p*-Nitrophenyl butyrate, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, diisopropyl phosphofluoridate, tris(hydroxymethyl)aminomethane, fibrinogen (82.7% clottable), soybean trypsin inhibitor, and hirudin were from Sigma Chemical Co., St. Louis, Miss. Fibrinogen, STI, and hirudin were used without further purification. *N*-α-Benzoyl-L-arginine *p*-nitroanilide was a product of Bachem Inc., while phenylmethanesulfonyl fluoride was obtained from Pierce Chemical Co., Rockford, Ill., and *p*-nitrophenyl guanidinobenzoate was from Cyclo Chemicals, Los Angeles, Calif.

Proteins. Bovine α- and β-thrombin[4] were purified from Parke-Davis Topical Thrombin on sulfopropyl Sephadex C-50 by a modification (Mann et al., 1973) of the method of Lundblad (1971). α- and β-thrombin purified by this procedure were single components in a nonreduced state when examined by urea–sodium dodecyl sulfate electrophoresis. Reduction showed that the α-thrombin was pure, but the β-thrombin contained about 10% α-thrombin. β-Thrombin was used without further purification. Bovine α-thrombin used in these experiments had a specific activity of 2500–3000 NIH clotting units/mg and hydrolyzed 34–50 μmol of TosArgOMe min^{-1} mg^{-1} of α-thrombin. Bovine β-thrombin had a specific activity of 100–200 NIH units/mg and hydrolyzed 34–50 μmol of TosArgOMe min^{-1} mg^{-1} of β-thrombin. Human α-thrombin was made from the prothrombin present in factor IX concentrates (American Red Cross, lot no. 5) as previously reported (Downing et al., 1975). The human α-thrombin produced was purified on sulfopropyl Sephadex C-50 by the method used routinely for bovine thrombin. The human α-thrombin had a specific activity of 3800 NIH units/mg and hydrolyzed 34 μmol of TosArgOMe min^{-1} mg^{-1}. Bovine prethrombin 2 and prothrombin fragment 2 and human prothrombin fragment 2 were prepared as previously described (Heldebrant, et al., 1973) and each was a single band on urea–sodium dodecyl sulfate gel electrophoresis, both with disulfide bonds intact and reduced.

Methods

Protein concentration was determined by ninhydrin reaction (Moore, 1968) after alkaline hydrolysis (Fruchter and Crestfield, 1965). Crystalline bovine serum albumin (Pentex) was used as a standard. Alternately, protein concentration was determined by absorption at 280 nm. An extinction coefficient, $E^{1\%}_{1\,cm}$, of 19.5 was used for α-thrombin (Winzor and Scheraga, 1964) and prethrombin 2 (Heldebrant et al., 1973), and an $E^{1\%}_{1\,cm}$ of 12.5was determined for prothrombin fragment 2 by dry-weight analysis. All values were corrected for Raleigh scattering.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 6.25 M urea was performed by a modification (Downing et al., 1975) of the method of Swank and Munkres (1971). Gels were stained with Coomassie brilliant blue and destained electrophoretically.

Assays. All esterolytic assays using TosArgOMe, BzArgNAN, or BzArgOEt were conducted in the presence of 0.05 M Tris-HCl, pH 8.1, at a temperature of 25 °C. The hydrolysis of TosArgOMe was followed at 247 nm as described by Hummel (1959) using a $\Delta E_{247\,nm}$ of 409 M^{-1} cm^{-1}. Similarly, the hydrolysis of BzArgNAN and BzArgOEt were followed at 410 nm (Mole and Horton, 1973) and 252 nm (Foucault et al., 1974), respectively. The esterolytic activity toward NPB was assayed in 0.01 M sodium phosphate, pH 8.0 as previously described (Lundblad, 1973). Clotting activities were determined by a modification (Mann et al., 1971a) of the NIH procedure.

Determination of Dissociation Constants for Prothrombin Fragment 2 to α- and β-Thrombin. The dissociation constants for α- and β-thrombin were determined by the method of Gutfreund (1972). This method yields a linear plot when multiple sites (if present) are identical and there is no interaction between them. The parameters which must be known for this method are total enzyme concentration, C_E^0, and total ligand concentration, C_L^0, and a signal must be generated upon addition of a ligand. In the case of prothrombin fragment 2 binding to α-thrombin, the signal is the difference in esterolytic activity between thrombin alone and thrombin to which pro-

[2] The abbreviations used are: TosArgOMe, *N*-α-*p*-tosyl-L-arginine methyl ester; NPB, *p*-nitrophenyl butyrate; BzArgNaN, *N*-α-benzoyl-L-arginine *p*-nitroanilide; BzArgOEt, *N*-α-benzoyl-L-arginine ethyl ester; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; DFP, diisopropyl phosphofluoridate; PMSF, phenylmethanesulfonyl fluoride; NPGB, *p*-nitrophenyl guanidinobenzoate; STI, soybean trypsin inhibitor; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone.

[3] Minimum Requirements for Dried Thrombin (1946), Second Revision, National Institutes of Health, Bethesda, Md.

[4] Unless otherwise noted, all reference to α- or β-thrombin, prothrombin fragment 2, or prethrombin 2 refer to bovine material.

thrombin fragment 2 has been added. The ratio of liganded enzyme to free enzyme, R, is the observed signal divided by the maximum signal.

$$\frac{1}{(1-R)} = \frac{KC_L{}^0}{R} - nKC_E{}^0 \quad (1)$$

From eq 1 (Gutfreund, 1972), a plot of $1/(1-R)$ vs. $C_L{}^0/R$ yields a slope of K_{ass} and at the X intercept, $n = C_L{}^0/RC_E{}^0$. Thus, this plot yields the association constant and the number of sites per enzyme molecule.

Determination of the Association Constant for Prothrombin Fragment 2 and Prethrombin 2. A ratio of the association constants for α-thrombin and prethrombin 2 can be calculated since prethrombin 2 competes with α-thrombin for prothrombin fragment 2. For the equilibria 2 and 3, the ratio of association constants is given by eq 4.

$$E_1 + L \overset{K_1}{\rightleftharpoons} E_1L \quad (2)$$

$$E_2 + L \overset{K_2}{\rightleftharpoons} E_2L \quad (3)$$

$$\frac{K_1}{K_2} = \frac{C_{E_1L}}{C_{E_1}C_L} \times \frac{C_{E_2}C_L}{C_{E_2L}} = \frac{C_{E_1L}C_{E_2}}{C_{E_1}C_{E_2L}} \quad (4)$$

The total concentrations of the enzyme species are: $C_{E_1}{}^0 = C_{E_1} + C_{E_1L}$ and $C_{E_2}{}^0 = C_{E_2L} + C_{E_2}$. Also, $C_{E_1L} = RC_{E_1}{}^0$ from previous definition of R. Then substituting for C_E, C_{E_2} and C_{E_1L}, we obtain eq 5.

$$\frac{K_1}{K_2} = \frac{RC_{E_1}{}^0(C_{E_2}{}^0 - C_{E_2L})}{(C_{E_1}{}^0 - RC_{E_1}{}^0)\,C_{E_2L}} = \frac{R}{(1-R)} \times \frac{(C_{E_2}{}^0 - C_{E_2L})}{C_{E_2L}} \quad (5)$$

The total concentration of ligand is given by $C_L{}^0 = C_{E_1L} + C_{E_2L} + C_L$. If the concentration of free ligand, C_L, is negligible compared to enzyme-bound ligand, C_L can be eliminated from this equation.[5] Substituting into eq 5 for C_{E_2L} and C_{E_1L} we obtain eq 6.

$$\frac{K_1}{K_2} = \frac{R}{(1-R)}\left(\frac{C_{E_2}{}^0}{C_L{}^0 - RC_{E_1}{}^0} - 1\right) \quad (6)$$

Thus, knowing the association constant for enzyme 1, the total concentrations of ligand, enzyme 1, and enzyme 2, and the ratio of liganded enzyme 1 to total enzyme 1, we can obtain the association constant for enzyme 2.

Results

The Effect of Prothrombin Fragment 2 on the Esterolytic Activity of α- and β-Thrombin toward TosArgOMe. Previous studies in our laboratory demonstrated that prothrombin fragment 2 increases the esterolytic activity of α-thrombin toward the substrate TosArgOMe. In order to examine the nature of this binding between prothrombin fragment 2 and α-thrombin, a saturation experiment was performed, as in Figure 1 (closed circles), in which the increase in the esterolytic activity of α-thrombin is plotted against increasing concentrations of prothrombin fragment 2. This curve represents TosArgOMe assays containing a constant concentration of α-thrombin (0.01 mg/ml) and varying concentrations of prothrombin fragment 2. In each assay, prothrombin fragment 2 was added to a cuvette containing 3 ml of 1.04×10^{-3} M TosArgOMe, 0.05 M in Tris, pH 8.1. α-Thrombin was then added, and the change in absorbance at 247 nm was recorded. Addition of prothrombin fragment 2 to an assay which already contained α-thrombin showed no lag before the accelerated rate was observed, indicating that the interaction between prothrombin fragment 2 and thrombin is rapid, as would be expected if the interaction is one of simple binding. Although prothrombin fragment 2 has been shown to bind calcium (Mann et al., 1973; Bajaj et al., 1975), calcium is not required for stimulation of α-thrombin by prothrombin fragment 2 (Mann et al., 1973).

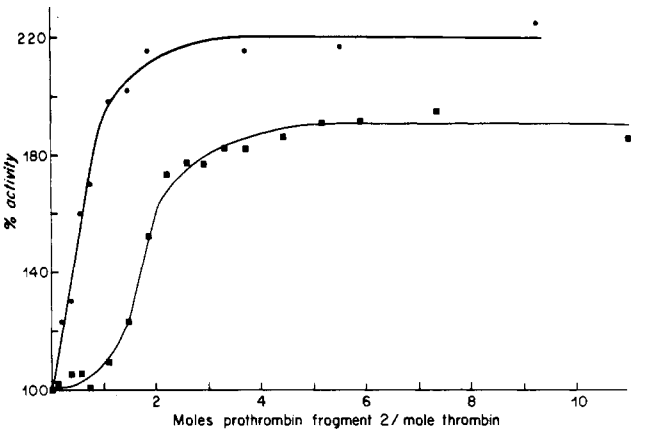

FIGURE 1: Enhancement of the esterolytic activity of α-thrombin upon addition of prothrombin fragment 2 and the effect of prethrombin 2 on the enhancement of the esterolytic activity of α-thrombin by prothrombin fragment 2. Relative percent esterolytic activity is plotted as a function of the molar ratio of prothrombin fragment 2 to α-thrombin, ●; α-thrombin, ■; α-thrombin plus prethrombin 2 at a molar ratio of 1.0:1.0.

To determine whether prothrombin fragment 2 or α-thrombin has proteolytic activity toward each other, prothrombin fragment 2 was incubated with α-thrombin at the saturating molar ratio of 1.8:1. After incubation for 10 min in 0.05 M Tris, pH 8.1, at 25 °C, there was no change in either the α-thrombin or the prothrombin fragment 2, as judged by urea–sodium dodecyl sulfate electrophoretic mobility with disulfide bonds intact or reduced, indicating that the observed stimulation is not the result of proteolytic modification of either component.

In other control experiments, prothrombin fragment 2 showed no esterolytic activity toward TosArgOMe when thrombin was not added to the assay; prothrombin fragment 2 did not alter the activity of TPCK-treated trypsin on TosArgOMe, nor did addition of bovine serum albumin in place of prothrombin fragment 2 produce an alteration of α-thrombin esterolytic activity toward TosArgOMe (Heldebrant and Mann, 1973). Similar experiments with β-thrombin yielded a saturation curve similar to the curve for α-thrombin.

Competitive Binding of Prothrombin Fragment 2 to Prethrombin 2. In order to examine the possibility that prethrombin 2 may compete with α-thrombin for prothrombin fragment 2 present in the system, we conducted a saturation experiment similar to that in the previous section. In this experiment, prethrombin 2 was included at an equimolar concentration with the α-thrombin. Figure 1 (closed squares) shows the enhancement of the esterolytic activity of α-thrombin toward TosArgOMe by prothrombin fragment 2 in the presence of prethrombin 2. The closed squares represent various concentrations of prothrombin fragment 2 included in assays containing 0.01 mg/ml of α-thrombin and 0.01

[5] As will be shown later, the association constant for α-thrombin and prothrombin fragment 2 is very large. This dictates that the concentration of free prothrombin fragment 2 is very small when the concentration of α-thrombin is in large excess of the concentration of prothrombin fragment 2.

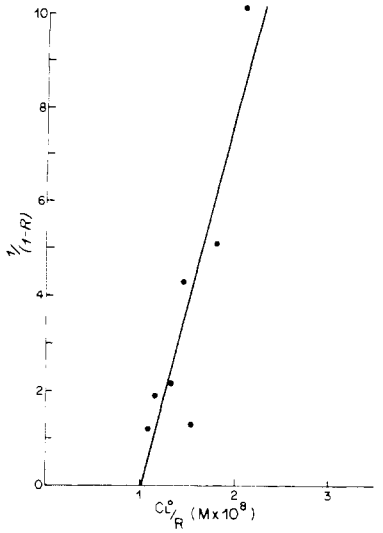

FIGURE 2: $1/(1 - R)$ as a function of C_L^0/R. Values were calculated as described in the experimental section. Data used was that presented in Figure 1, closed circles.

mg/ml of prethrombin 2. It is apparent from this figure that the prothrombin fragment 2 is associated with prethrombin 2 until saturation of the prethrombin 2 is achieved, which occurs at approximately 1 mol of prothrombin fragment 2 per mol of α-thrombin. Addition of more prothrombin fragment 2 results in enhancement of the esterolytic activity of the α-thrombin present with maximal stimulation at 2 mol of prothrombin fragment 2 per mol of α-thrombin. This plot suggests that prothrombin fragment 2 binds to prethrombin 2 with greater affinity than to α-thrombin. Association of prothrombin fragment 2 to prethrombin 2 is also observed during the purification of these proteins over various chromatographic media, making them difficult to separate. Prethrombin 2 has no esterolytic activity by itself, nor does addition of prothrombin fragment 2 to prethrombin 2 result in esterolytic activity toward TosArgOMe.

Determination of Dissociation Constants and Number of Binding Sites for the Association of Prothrombin Fragment 2 and α-Thrombin. Due to the size and limited supply available of prothrombin fragment 2, approaches in which the amount of free ligand is the known variable, such as equilibrium dialysis or gel filtration, were judged impractical for our system. In the thrombin–prothrombin fragment 2 esterolytic system, the concentration of free enzyme and the concentration of liganded enzyme is known since thrombin gives a constant initial rate of TosArgOMe hydrolysis and addition of prothrombin fragment 2 increases this rate of hydrolysis up to a maximum value at which point the thrombin is saturated with prothrombin fragment 2.

The equations giving the dissociation constant, then, must be transformed to include free and liganded enzyme rather than free and enzyme-bound ligand. According to Gutfreund (1972), this transformation results in eq 1.

Figure 2 shows a plot of $1/(1 - R)$ vs. C_L^0/R. From this plot, the dissociation constant between prothrombin fragment 2 and α-thrombin was calculated to be 7.7×10^{-10} M and the number of binding sites was calculated to be 1.17, indicating that one prothrombin fragment 2 molecule is bound to each molecule of α-thrombin. A similar experiment with β-thrombin yielded a dissociation constant of 4.3×10^{-8} M, and again one molecule of prothrombin fragment 2 is bound per molecule of β-thrombin.

TABLE I: Inhibition of α-Thrombin Activity on NPB by Prothrombin Fragment 2

Molar Ratio Prothrombin Fragment 2/α-thrombin	% Activity with Substrate	
	TosArgOMe	NPB
0.0	100	100
1.17	140	74
2.34	150	0

Determination of the Dissociation Constant between Prethrombin 2 and Prothrombin Fragment 2. Once the dissociation constant between prothrombin fragment 2 and α-thrombin was established, the dissociation constant for the interaction between prothrombin fragment 2 and prethrombin 2 could be determined. Figure 1 indicates that prethrombin 2 and α-thrombin compete for the prothrombin fragment 2 present. Using this competition between prethrombin 2 and α-thrombin, a ratio of dissociation constants can be calculated as Methods, resulting in eq 6. The dissociation constant for prethrombin 2, as calculated from eq 6 for several experiments, was 1.3×10^{-10} M. Note that this dissociation constant is smaller than the dissociation constant for α-thrombin. This difference in dissociation constants explains the sigmoidal curve seen in Figure 1. As was the case for α-thrombin, the dissociation constant is very small, indicating a very tight association between prethrombin 2 and prothrombin fragment 2.

Interaction Between Human α-Thrombin and Human Prothrombin Fragment 2. The esterolytic activity of human α-thrombin was only slightly affected by human prothrombin fragment 2. A 40-fold molar excess of human prothrombin fragment 2 gave a 50% increase in the rate of TosArgOMe hydrolysis by human α-thrombin. This observation prompted us to conduct experiments in which components from the two species were mixed. Human prothrombin fragment 2 had no significant effect on either human or bovine α-thrombin. Bovine prothrombin fragment 2, on the other hand, enhanced the esterolytic activity of both human and bovine α-thrombin. This finding indicates that the structural feature(s) required for this phenomena are present in thrombin from both sources, but absent in the human prothrombin fragment 2. Results such as these exemplify the inherent danger of mixing proteins from different species.

The Effect of Prothrombin Fragment 2 on the "NonSpecific" Esterolytic Activity of α- and β-Thrombin. Early experiments dealing with the interaction between prothrombin fragment 2 and α-thrombin in which the esterolytic activity of α-thrombin toward the synthetic substrate TosArgOMe was enhanced upon addition of prothrombin fragment 2 suggested that the effect of prothrombin fragment 2 on α-thrombin may be a change in specificity. To test this hypothesis, the "nonspecific" substrate NPB was used in place of TosArgOMe to determine whether the "nonspecific" esterolytic activity of α-thrombin was altered by addition of prothrombin fragment 2. In contrast to the results obtained with TosArgOMe, prothrombin fragment 2 resulted in a decrease in the hydrolytic activity of α-thrombin toward NPB.

Table I shows the decrease in the hydrolytic activity of α-thrombin toward the nonspecific substrate NPB upon addition of prothrombin fragment 2. In this experiment, various concentrations of prothrombin fragment 2 were added to concentrations of α-thrombin and NPB of 0.09 mg/ml and 1×10^{-3} M, respectively. Prothrombin fragment 2 completely

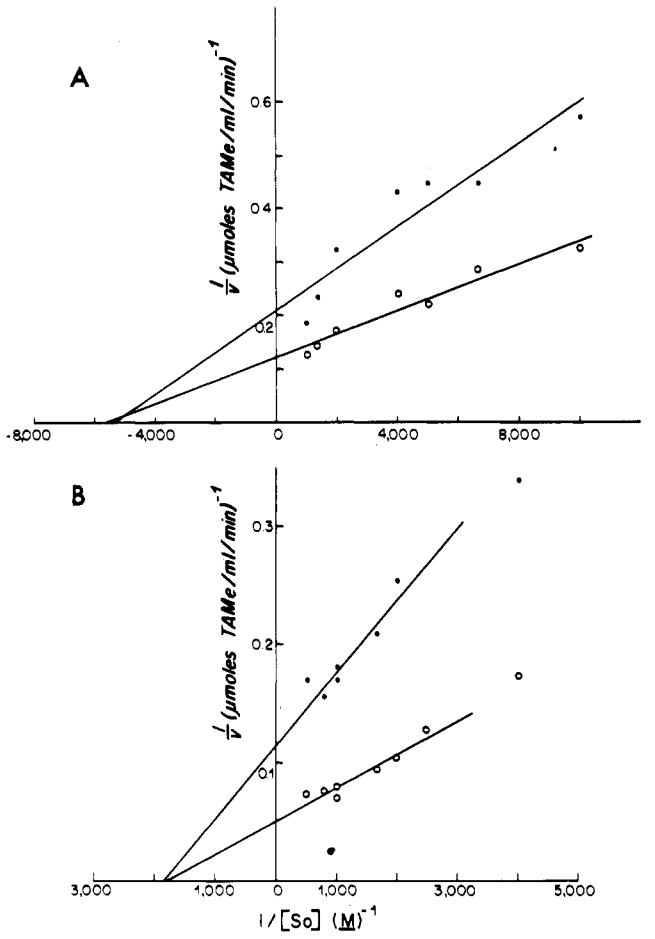

FIGURE 3: (A) Lineweaver-Burk plot for α-thrombin on the substrate TosArgOMe, ●; α-thrombin, ○; prothrombin fragment 2 plus α-thrombin at a molar ratio of 1.4:1.0. (B) Lineweaver-Burk plot for β-thrombin on the substrate TosArgOMe, ●; β-thrombin, ○, prothrombin fragment 2 plus β-thrombin at a molar ratio of 1.3:1.0.

inhibited the activity of α-thrombin toward NPB when present at a molar ratio of 2.3:1. A similar decrease in activity toward NPB was observed when prothrombin fragment 2 was added to β-thrombin at similar molar ratios.

Determination of $K_{m(app)}$ and $k_{3(app)}$ for α- and β-Thrombin on TosArgOMe. To further examine the association between prothrombin fragment 2 and α-thrombin, the kinetic parameters for the reaction of α-thrombin and the substrate TosArgOMe in the presence and absence of prothrombin fragment 2 were examined.

Figure 3A presents Lineweaver-Burk plots for α-thrombin on the substrate TosArgOMe in the presence and absence of prothrombin fragment 2. In this experiment, a constant concentration (0.09 mg/ml) of α-thrombin was added to buffer containing various concentrations of TosArgOMe ranging from 10^{-4} to 10^{-3} M. The data for α-thrombin in the absence of prothrombin fragment 2 is plotted as the closed circles in Figure 3A. In similar experiments in which prothrombin fragment 2 was added at a molar ratio, prothrombin fragment 2 to α-thrombin of 1.4:1, the data obtained is shown in Figure 3A. A similar experiment was conducted with β-thrombin in which the concentration of β-thrombin was held constant at 0.1 mg/ml final concentration and the TosArgOMe concentration was varied between 2.5×10^{-4} M and 2×10^{-3} M. These data are presented in Figure 3B, in which the closed circles represent β-thrombin alone, and the open circles represent prothrombin fragment 2 added to β-thrombin at a molar ratio of 1.3:1. A summary of the kinetic constants for α- and β-thrombin in the presence and absence of prothrombin fragment 2 is given in Table II.

TABLE II: Kinetic Parameters of α- and β-Thrombin in the Presence and Absence of Prothrombin Fragment 2

	$K_{m(app)}$ (μM)	$k_{3(app)}$ (mol of TosArgOMe/mol of thrombin s^{-1})
α-Thrombin	192	35.8
α-Thrombin+ prothrombin fragment 2	176	60.5
β-Thrombin	500	46.2
β-Thrombin+ prothrombin fragment 2	544	107.3

The Effect of Prothrombin Fragment 2 on Inhibition of α-Thrombin by Covalent Protease Inhibitors. Several proteolytic inhibitors (DFP, PMSF, NPGB) which are known to function by covalently blocking the active-site serine residue in trypsin-like enzymes were examined with respect to the prothrombin fragment 2–α-thrombin intrraction. Prothrombin fragment 2 had no effect on the inactivation of α-thrombin by DFP or PMSF which acylate the active-site serine. For inhibition by DFP, α-thrombin (0.016 mg/ml) was incubated with 2×10^{-3} M DFP in 0.05 M Tris, pH 8.1. At various times, aliquots were withdrawn and assayed with TosArgOMe as described in the Experimental Section. A first-order rate was observed for inactivation of α-thrombin by DFP (data not shown). Addition of saturating concentrations of prothrombin fragment 2 resulted in no change in the rate of inactivation. Similar experiments using 5×10^{-4} M PMSF yielded a similar result, that is, addition of prothrombin fragment 2 did not change the rate of inactivation of α-thrombin (0.05 mg/ml) by 5×10^{-4} M PMSF. In experiments with a molar excess of NPGB, there was no change in either the initial burst nor the steady-state hydrolysis of NPGB by α-thrombin (0.05 mg/ml) upon addition of prothrombin fragment 2 at saturating levels. While conducting the DFP inhibition experiments, the observation was made that addition of DFP to a final concentration of 1×10^{-3} M to α-thrombin (0.016 mg/ml) in 0.05 M Tris, pH 8.1, containing 1.04×10^{-3} M TosArgOMe did not result in a decrease in the esterolytic activity of α-thrombin toward TosArgOMe. A similar observation that both TosArgOMe and benzamidine protect thrombin from inactivation by PMSF has previously been reported by Lundblad (1971).

In contrast to the active-site serine inhibitors, different results were obtained with the active site histidine inhibitor, TLCK. Figure 4 shows inactivation of α-thrombin by TLCK in the presence (open circles) and absence (closed circles) of prothrombin fragment 2. The open triangles represent a control which contained only α-thrombin at a final concentration of 0.05 mg/ml. TLCK was added, to a final concentration of 5×10^{-4} M, to α-thrombin (0.05 mg/ml) (closed circles) and to prothrombin fragment 2 plus α-thrombin (open circles). At the times indicated, esterolytic activities on the substrate TosArgOMe were measured. It is apparent from Figure 4 that prothrombin fragment 2 significantly changed the rate of inhibition by TLCK.

The Effect of Various Macromolecular Inhibitors on Prothrombin Fragment 2 Stimulation of α-Thrombin. Three macromolecular inhibitors (heparin, hirudin, and STI) which are known to interact with α-thrombin were studied in relationship to the prothrombin fragment 2–α-thrombin interac-

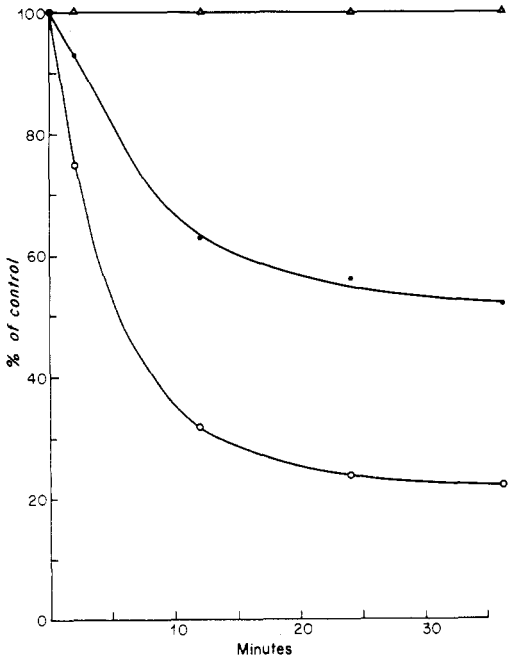

FIGURE 4: Increase in the rate of inhibition of α-thrombin by TLCK upon addition of prothrombin fragment 2, △; α-thrombin, ●; α-thrombin plus 5×10^{-4} M TLCK, ○; prothrombin fragment 2 and α-thrombin at a molar ratio of 5.2:1 plus 5×10^{-4} M TLCK.

tion. Saturating concentrations of prothrombin fragment 2 had no effect on inhibition of the clotting or esterolytic activities of 2 NIH units of α-thrombin at concentrations of heparin from 1 USP unit/ml to 50 USP units/ml. Prothrombin fragment 2 was added at molar ratios ranging from 3.6:1 to 300:1, but addition of prothrombin fragment 2 resulted in no change in the inhibition by heparin.

Hirudin was found to inhibit α-thrombin in a linear manner, that is, remaining α-thrombin activity was linearly dependent on the concentration of hirudin added. Addition of prothrombin fragment 2 at a molar ratio of 6.7:1 did not affect the inhibition of α-thrombin by hirudin. Prothrombin fragment 2 increases the esterolytic activity of α-thrombin toward TosArgOMe in the presence of less than saturating concentrations of hirudin, but this increase could be attributed solely to α-thrombin free of inhibitor at these concentrations. When saturation with hirudin was achieved, prothrombin fragment 2 no longer had any effect, and no esterolytic activity was observed.

STI has been reported to bind to α-thrombin in a 1:1 complex (Lanchantin et al., 1969). Concentrations of STI up to a 100-fold molar excess with respect to α-thrombin had no effect on the activity of the enzyme toward TosArgOMe. A similar experiment, utilizing the α-thrombin–prothrombin fragment 2 complex, demonstrated a slight inhibition in activity by STI. The results of this experiment indicate that either STI competes for an overlapping binding site on α-thrombin or that STI competes for prothrombin fragment 2. At 100-fold molar excess, STI inhibits α-thrombin activity toward fibrinogen (ca. 30%). Thus, STI appears to influence the interaction of α-thrombin with the relatively large-protein molecules of prothrombin fragment 2 and fibrinogen, but does not affect hydrolysis of the relatively small substrate TosArgOMe. This observation suggests that STI masks a portion of the substrate and/or prothrombin fragment 2 binding portion of the α-thrombin molecule, but this masked area is not in the immediate area of the catalytic site or TosArgOMe binding site.

Discussion

As a result of binding to α- and β-thrombin, prothrombin fragment 2 enhances the esterolytic activity of α- or β-thrombin toward the synthetic substrate TosArgOMe. Using this enhancement by prothrombin fragment 2 as a probe, dissociation constants between prothrombin fragment 2 and α-thrombin, β-thrombin, and prethrombin 2 have been determined. We have also shown 1:1 stoichiometric binding of prothrombin fragment 2 to α- and β-thrombin.

Examination of the apparent kinetic constants, $K_{m(app)}$ and $k_{3(app)}$, reveals that prothrombin fragment 2 exerts its effect on $k_{3(app)}$ but has little, if any, effect on $K_{m(app)}$. Thus, the change in activity is due to an increase in rate of catalysis and not in the binding affinity of thrombin for TosArgOMe. Substituting the substrate BzArgOEt or BzArgNAN for TosArgOMe yielded an inhibition rather than an enhancement of thrombin's esterolytic activity upon addition of prothrombin fragment 2. As is the case for TosArgOMe, prothrombin fragment 2 exerts its effect on $k_{3(app)}$ but has little effect on $K_{m(app)}$. These conflicting findings on benzoyl vs. tosyl derivatives are not presently understood.

Studies with serine protease inhibitors indicate that inhibition of thrombin by the serine reactive inhibitors DFP, PMSF, and NPGB is not affected by the addition of prothrombin fragment 2. However, none of these inhibitors contain either a tosyl or a benzoyl moiety.

Inhibition by the active-site histidine inhibitor, TLCK, was affected by the addition of prothrombin fragment 2. The reaction of TLCK with serine proteases is known to be a two-step mechanism resulting in irreversible acylation of the active-site histidine rather than the active-site serine. Since we found that, for TosArgOMe, $K_{m(app)}$ is unchanged, we believe that the stimulation of the reaction between TLCK and thrombin is a stimulation of k_2, the rate of acylation. These observations are similar to those seen for cholate and thrombin by Exner and Koppel (1973). It should be noted, however, that prothrombin fragment 2 is effective at near equimolar concentrations with thrombin, whereas concentrations of bile salts required are several orders of magnitude in excess of the thrombin present (Exner and Koppel, 1973). Therefore, if prothrombin fragment 2 and bile salts affect thrombin in a similar fashion, prothrombin fragment 2 must be much more effective because it binds to thrombin with much higher affinity. Also, bile salts are much smaller, most likely precluding a secondary interaction with another molecule such as factor V.

The magnitude (10^{-10} M) of the dissociation constants between prothrombin fragment 2 and α-thrombin or prethrombin 2 indicates that the affinity of prothrombin fragment 2 for α-thrombin and prethrombin 2 is very large. Moreover, with dissociation constants of this magnitude, it would seem that prothrombin fragment 2 is always associated with α-thrombin or prethrombin 2 in vivo. Since prothrombin fragment 2 binds to α-thrombin with such high affinity, it may be a major contaminant in most α-thrombin preparations. It may go unnoticed in gel electrophoresis experiments because it stains poorly and diffuses out of the gel quickly. Therefore, prothrombin fragment 2 may be, in fact, necessary for one or more of the functions of α-thrombin, such as cleaving factor VIII, factor V, or fibrinogen. Although our in vitro experiments do not indicate a requirement for prothrombin fragment 2 for the clotting activity of thrombin, we cannot at this point rule out this possibility in vivo. Prothrombin fragment 2 may also be involved in the reaction of thrombin with plasma inhibitors as well as the interaction between thrombin and platelets. As previously stated in the activation of prothrombin, prothrombin

fragment 2 accelerates the activation of prethrombin 2. Its affinity for α-thrombin suggests that after thrombin is generated from prethrombin 2, it remains associated with prothrombin fragment 2 and presumably to factor V and the rest of the prothrombinase complex. If that is the case, the prothrombinase complex would not turn over unless prothrombin or prethrombin 1 can compete with the prothrombin fragment 2-α-thrombin complex for the prothrombinase complex.

In the human system, there is some degree of difficulty in separating prothrombin fragment 2 from prethrombin 2 or α-thrombin as in the bovine system. This observation suggests that a similar association is present in the human system, but this association is not manifest in an enhancement of α-thrombin esterolytic activity.

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