Esterase and Clotting Activities Derived from Citrate Activation of Human Prothrombin*

(Received for publication, October 13, 1966)

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SUMMARY

When human prothrombin is activated in 25% (weight per volume) sodium citrate, the apparent clotting and esterase activities of thrombin develop at equal rates and reach maximum levels in about 16 hours. Following this, the esterase activity remains stable, but over 90% of the fibrinogen-clotting activity disappears from the system during the next 500 hours. Chromatography of the 16-hour system yields a single protein peak containing 40% of the protein and all of the clotting and esterase activity of the activation system. Chromatography of the 500-hour system yields two protein peaks, the major one having essentially only esterase activity and a minor one which has principally clotting activity. This results in almost a 2-fold purification of the esterase fraction over that obtained by chromatography of the 16-hour system.

Comparisons were made between the preparations having both clotting and esterase activity and those having only esterase or clotting activity. With respect to the elution volume on gel filtration, electrophoresis, and sedimentation properties, no differences were observed. All preparations behaved as essentially single components having an estimated molecular weight of 30,000 g, with no evidence of dissociation to enzymatically active smaller molecules. Titration experiments with phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate showed a striking difference between the preparations, however. In addition, the clotting preparation had a profound affect on the citrate activation of prothrombin, while the esterase preparation did not.

The evidence thus obtained indicates that thrombin is similar to certain other estero-proteolytic enzyme systems in which the molecule can be modified so as to alter its relative activity toward ester and protein substrates. This is discussed in relation to various interpretations concerning the nature of the active center on the thrombin molecule, as well as the implications these findings may have on the factors believed necessary for the inception of blood coagulation.

In 1954, Sherry and Troll reported (1) that thrombin (EC 3.4.4.13) catalyzed the hydrolysis of certain synthetic arginyl esters, particularly α-N-p-tosyl-arginine methyl ester, in addition to its well known clotting (proteolytic) activity. They also demonstrated that these two enzymatic properties of thrombin were closely associated with each other under a number of experimental conditions. Subsequently, it has been shown that thrombin may catalyze the hydrolysis of a variety of esters and peptide bonds (2-4), some at rates exceeding that for TAMe (5). Furthermore, it is known that TAMe inhibits the clotting of fibrinogen by thrombin (1) and that certain other organic compounds, apart from inhibiting fibrinogen clotting, also competitively inhibit the TAMe and nitrophenyl esterase activities of the enzyme (4). On the basis of these and other studies, it now seems to be clearly established that the same active site on the enzyme molecule is responsible for the esterolytic and clotting activities of thrombin (6, 7), and that this site is similar in amino acid sequence to that found for other estero-proteolytic enzymes (8).

There are, however, a number of experimental situations in which the esterase and clotting activities of human and bovine thrombin can be dissociated from one another. This has been reported to occur: (a) following the acetylation of thrombin, or upon activation of acetylated prothrombin (9); (b) during the binding of thrombin by purified preparations of α₂(17 S) macroglobulin (10) or certain fibrinogen derivatives (11); (c) during the interaction of thrombin with "antithrombin" (9); and (d) following the activation of prothrombin in 25% citrate solution (9, 12). Under these conditions, the clotting activity of thrombin either disappears or is substantially altered in comparison with esterase activity.

At least two interpretations of these experimental findings have been proposed. Vorand (4) has suggested that while the catalytic center on the thrombin molecule must be the same for its two enzymatic activities, perhaps secondary binding sites could be altered so that the activity of the enzyme toward large fibrinogen molecules would be substantially less than that toward small synthetic esters. A similar explanation, based on steric effects, has been proposed by Waugh, Baughman, and Miller (2). Such an interpretation is analogous to that found for the

* This research was supported by Research Grant H-07703 from the National Heart Institute, United States Public Health Service. A preliminary report of this study was presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1966.

1 The abbreviations used are: TAMe, α-N-p-tosyl-arginine methyl ester; C1N, N-carbobenxoxyl-tyrosine p-nitrophenyl ester; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; STI, soybean trypsin inhibitor.
uncoupling of the activities of other well known serine proteolytic enzymes (i.e. carboxypeptidase, trypsin, pepsin, etc.). On the other hand, Lakí and Gladner (6) have suggested that the catalytic site of thrombin may be dependent upon a monomer-dimer transformation, and Landaburu and Seegers (13) have hypothesized that monomer thrombin has esterase activity, whereas dimer thrombin has clotting activity. This interpretation implies that the esterolytic and clotting activities of thrombin are associated with different sites, or at least parts of the site are associated with 2 different molecules.

The problem as to which of these interpretations is correct is further compounded by the recognition by some workers that a number of protein-clotting factors interact sequentially to produce a "thromboplastic" complex essential for the activation of prothrombin (14). Several of these factors exist as zymogens and are activated in a manner similar to prothrombin. In their activated form, they possess potent esterase activity and presumably proteolytic but not fibrinogen-clotting activity. Consequently, such activated factors would have "contaminated" most, if not all, thrombin preparations thus far examined since their properties are quite similar (15, 16). Whether such clotting factors represent distinct enzyme systems themselves (14) or are "derivatives" of prothrombin and thrombin (9) is a controversial issue recently reviewed by Kline (17). Thus, the possibility that thrombin might exist in a form having clotting rather than esterase activity or both has rather important implications concerning not only the active center of the enzyme itself but also the events which lead to the inception of blood clotting.

In the present work, we have studied the alterations which occur in the clotting and esterase activity of human prothrombin following its activation and prolonged exposure to 25% sodium citrate. Under these conditions, over 90% of the clotting activity relative to TAME esterase disappears from the system. This alteration in thrombin activity was further investigated by ion exchange chromatography, gel filtration, sedimentation-velocity analysis, electrophoresis, and titration experiments with certain organic inhibitors. The data obtained, although subject to several interpretations concerning the nature of the active center on the thrombin molecule, indicate that the enzyme can be modified so as to alter its relative activity toward different substrates.

**Experimental Procedure**

**Preparation**—Prothrombin was isolated from freshly collected human blood by a method previously described (18). Fifteen preparations having specific activities ranging from 1600 to 2200 National Institutes of Health (two-stage units mg⁻¹ of protein) were used in this study. On the basis of analytical measurements presented elsewhere (19, 20), these products probably contained from 65 to 85% active material.

Prothrombin was converted to thrombin by activation in 25% (w/v) trisodium citrate solution at 24 ± 1° (20) under conditions as aseptic as possible. Polyethylene or siliconized surfaces were used to handle the thrombin solutions wherever practicable to prevent the loss of enzyme activity due to adsorption by glass.

Soybean trypsin inhibitor (crystallized three times) was obtained from Worthington. Unless otherwise stated, all other reagents were of analytical grade.

**Assay Procedures**—Prothrombin was assayed by the two-stage procedure, and thrombin by the National Institutes of Health method at 28°, with National Institutes of Health bovine thrombin (Lot No. 3B) as reference (0, 20). Seegers has reported the NIH unit to be 1.25 times the Iowa unit (0); however, three independent laboratories have reported values which average 1.7 ± 0.1 for this conversion factor (21-23).

Thrombin esterase activity was determined at 28° by the method of Ehrenpreis and Scheraga (24) with the aid of a thermostatic recording pH-stat (E. H. Sargent and Company) with TAME (Sigma) as substrate. The rate of TAME hydrolysis was determined from the slope of the zero order curve obtained for the initial 10 to 15% hydrolysis of the substrate, as calculated from the NaOH uptake. The unit of TAME esterase activity is defined as the number of 0.1 μM H⁺ titrated min⁻¹ ml⁻¹ of 0.01 M TAME ml⁻¹ of sample at pH 8.0, 28°, Γ/2 = 0.15 (12, 24). At 37° and 0.04 M TAME, which are the conditions under which the original Sherry and Troll unit is defined (1), the rate of substrate hydrolysis was found to be about 2-fold greater. This difference and the lack of agreement between the TAME esterase unit and the NIH clotting unit have been discussed elsewhere (12). In some experiments the method of Martin, Golubow, and Axelrod (3) was used to determine the esterase activity of thrombin against CTN (Sigma) by measurement of the rate of appearance of p-nitrophenol at 400 μM (Beckman model DU spectrophotometer).

**Gel Filtration**—This procedure was carried out on columns (1 x 60 cm and 1 x 40 cm) of beaded Sephadex G-100 (Pharmacia) equilibrated with either 0.5 M NaCl or 0.1 M sodium citrate (20). For estimates of relative molecular weight by this technique, void volumes (V₀) of the column and elution volumes (Vₑ) of the protein peaks were determined, and their ratio (Vₑ:V₀) was compared with a number of other essentially globular proteins as described elsewhere (20).

**Ion Exchange Chromatography of Activation Mixtures and Recovery of Fractions**—Thrombin activity was isolated from citrate activation mixtures by chromatography on columns (1 to 2.5 x 10 to 50 cm) of 200 to 400 mesh Rexyn CG51 (Fisher Chemical Company) or Bio-Rex-70 (Bio-Rad Laboratories) in a manner similar to that of Miller and Copeland (21). Samples to be chromatographed were dialyzed (20) or diluted to ionic strength of 0.1 and applied in volumes ranging from 10 to 25 ml to a column previously equilibrated with 0.1 M sodium acetate, pH 7.1 ± 0.1 at 24 ± 1°. Most of the protein of the samples appeared in the initial hold up volume and was washed through the column with 20 to 50 ml of solvent. After this, thrombin activity was recovered by eluting the column with 0.1 M sodium acetate containing increasing concentrations of NaCl or CaCl₂ with an apparatus which produces a continuous linear salt gradient (25). In each experiment the slope of the gradient was monitored by chloride analysis (26) of the 1-ml effluent fractions collected (Gilson Medical Electronics volumetric fractionator). By adjustment of the volumes and NaCl concentration in the mixing device above the column (25), gradients having a slope of 4 to 30 μeq NaCl ml⁻¹ of effluent were obtained at flow rates of 2 to 5 ml min⁻¹. Knowledge of the slope of the gradient was important since ionic strength has considerable influence on the rate of fibrinogen clotting by thrombin as well as on its esterolytic activity (27), and serious analytical errors are encountered unless this effect is taken into account. Consequently, in these experiments, the effluent fractions were diluted to an ionic strength of 0.16 before enzymatic analysis. This resulted in recoveries exceeding 88% of the clotting and esterase activities applied to the column.
Protein was analyzed chemically or by absorbance measurements at 280 m\(\mu\) (20). Fractions separated on the gel or chromatography columns were recovered by pooling the effluent fractions, desalting on Sephadex G-25 columns equilibrated with water (18), or by rapid dialysis (20), and then freeze-drying. In some experiments an acetone precipitation technique was used (21).

Biochemical Measurements—Sedimentation velocity analyses were performed in a Beckman Spinco model E analytical ultracentrifuge at 20°. Sedimentation coefficients (in Svedberg units) were calculated as previously described (20). Diffusion coefficients were estimated from the optical measurements by the method of Ehrenberg (28).

Electrophoretic analysis was carried out on cellulose acetate strips (Gelman Instrument Company, Sepaphore III) at pH 8.6 (Veronal buffer, \(\Gamma/2 = 0.075\)) with minor modifications of a procedure used previously (18). In some experiments, undyed strips were cut into 0.5-cm transverse segments eluted with 0.15 m NaCl at 0° for 12 hours and assayed for CTN esterase and clotting activity by the methods noted above.

Titration of Thrombin Activity with PMSF and DFP—For these experiments, the clotting and esterase activity peaks separated on the chromatography columns were pooled and diluted with phosphate buffer to a protein concentration of from 0.15 to 0.06 mg ml\(^{-1}\), \(\Gamma/2 = 0.3\), pH 7.0. Aliquots of 1 ml were incubated with 10 \(\mu\)l of various concentrations of PMSF (Sigma) dissolved in 2-propanol for up to 4 hours and then, following appropriate dilution, assayed for enzymatic activity. Titration experiments with DFP (Mann) were similar, except a phosphate buffer of pH 7.6 was used. The final pH of each system was checked before and after incubation with inhibitor with the use of a Radiometer AME-1 pH meter. Appropriate controls in which 2-propanol alone was added to the thrombin solutions were run concomitantly.

For comparison purposes, an \(\alpha\)-chymotrypsin preparation (crystallized three times, Worthington) was titrated with PMSF and DFP in a similar manner. Chymotrypsin esterase activity was measured in the pH-stat with \(N\)-acetyl-L-tyrosine ethyl ester (Calbiochem) as substrate at pH 8.0, as outlined by Laskowski (29).

The determination of enzyme concentration, as well as an assumed molecular weight of 27,300 g, was based on the work of Gold and Fahrney (30).

RESULTS

\(\text{TAMe Esterase and Clotting Activity Following Citrate Activation of Human Prothrombin—}\)Fig. 1 presents an experiment in which activity measurements were performed at intervals after activation of human prothrombin in 25% (w/v) citrate solution. Initially, and for about 2 hours following activation, there is no detectable enzymatic activity. This lag phase is presumably due to the formation of an intermediate which has no significant prothrombin or thrombin activity (20).

After this interval, however, TAMe esterase and clotting activity develop in a manner suggesting autocatalysis, and subsequently reach maximum activities (about 75% of the potential two-stage activity (20)) in 16 to 24 hours. During this time, the ratio between the clotting and esterase activities is about 7.0 ± 0.5 (Fig. 1), there being no significant difference in the rate of appearance of the two activities. After maximum activation, there is a decline in clotting activity over the next 80 hours, although the TAMe esterase activity is not significantly altered. This apparent dissociation of the two enzyme activities was observed with all the preparations studied, a summary of which is presented in Fig. 2. These data show that prolonged exposure to citrate, more than 90% of the clotting activity relative to esterase activity progressively disappears over a period of 500 hours. When the experimental values of Fig. 2 were tested graphically for nearness of fit to first and second order rate equations, it was impossible to decide the kinetics of clotting inactivation.

Ion Exchange Chromatography of 16-Hour Activation Mixture—Fig. 3A presents the elution pattern of protein, fibrinogen-clotting activity, and TAMe esterase activity obtained following the chromatography of a prothrombin sample which was activated for 16 hours in citrate solution. As in seven similar experiments, approximately 60% (range, 55 to 64%) of the protein is not absorbed to the resin and appears in the initial hold up volume of the column (Peak I of Fig. 3A). No significant enzymatic activities are associated with this material. After the column is washed with acetate solution and a continuous linear NaCl gradient is initiated, (7 m NaCl per ml of effluent), two additional protein peaks are usually eluted. The first one (Peak II), although broad, is quite small and inactive. In some experiments it was not detected, while in others it was clearly separated from the following major protein peak, Peak
the elution pattern where it was overlapped by Peak II. This is apparent in Fig. 3, except that a steep elution gradient was used (approximately 25 mM NaCl per ml of effluent) in order to prevent spreading of the protein peaks and to increase the sensitivity of the clotting measurements. From the results obtained (Fig. 4), it is apparent that the activity peaks do not coincide with one another and must be associated with two different proteins, although only a single protein peak (Peak III of Fig. 4) was apparent. It should also be noted that the specific activity of the TAME esterase measurements (Fig. 4, inset) is about 70% greater than that obtained from chromatography of the 16-hour activation system (Fig. 3). Similar results (not shown) were also obtained when CTN esterase activity was measured.

The separation of the clotting and esterase activity peaks is more striking in the experiment depicted in Fig. 5, in which a shallow gradient (approximately 8 mM NaCl per ml of effluent) was used to elute a 500-hour activation mixture from the column.

Fig. 2. Change in the ratio of clotting to TAME esterase activity upon prolonged exposure of activation mixtures to 25% sodium citrate. The legend on the right-hand ordinate is the approximate percentage of clotting activity remaining in the system based on an enzyme ratio of approximately 7 at maximum activation for 16 hours. The different symbols used in this figure represent different activated prothrombin preparations.

III, which had all of the enzyme activities of the unchromatographed activation mixture associated with it. Peak III appeared to be relatively homogeneous, at least beyond the area in the elution pattern where it was overlapped by Peak II. This is apparent in Fig. 3B where it is shown that the specific activity measurements and the clotting-esterase ratio are nearly constant with effluent volume. In nine experiments the average specific activities of these enzyme measurements were 3933 (range, 3200 to 5200) NIH clotting units per mg of protein and 450 (range, 334 to 506) TAME esterase units per mg of protein. This represents a 2- to 2.5-fold purification over that of the original unchromatographed activation mixture and, with respect to clotting measurements, a specific activity of thrombin comparable to that of Miller and Copeland (21) with the use of their correction factor (1.8) for the difference in the TRY and NIH unit. Attempts to determine if Peak III could be resolved into more than one protein component were unsuccessful. Pooling of the Peak III effluent tubes (noted by the horizontal line in Fig. 3A) followed by rechromatography gave an elution pattern for a single, homogeneous protein eluted at the same salt concentration (approximately 300 mM) as in Fig. 3A. Columns 50 cm in length gave elution patterns similar to 10-cm columns, although recoveries of less than 50% of the material applied were obtained. Similar observations have been made by Miller and Copeland (21). No difference in chromatographic behavior of Peak III was noted if the columns were run at 5° or if CaCl₂ were used as the salt gradient. Varying the protein applied to the column from 4 to 20 mg gave essentially identical results. It was also observed that if the Peak III fraction was rechromatographed immediately or after storage at -20° for 1 week, it appeared as a single protein component with no evidence of any Peak I or II material. However, if it were allowed to stand at 24° for several days, during which time a significant loss of clotting activity was apparent, appreciable amounts of Peak I protein reappeared on chromatography.

Prothrombin alone (10 mg per ml) or when "activated" in 25% citrate solution containing STI (2 mg per ml) was not adsorbed to the exchange columns and appeared in the initial hold up volume with no protein peaks eluted with the salt gradient.

Ion Exchange Chromatography of 500-Hour Activation Mixture—Fig. 4 shows the chromatographic elution pattern of a prothrombin preparation which had been activated for 500 hours. The conditions of this experiment were similar to those of Fig. 3, except that a steep elution gradient was used (approximately 25 mM NaCl per ml of effluent) in order to prevent spreading of the protein peaks and to increase the sensitivity of the clotting measurements. From the results obtained (Fig. 4), it is apparent that the activity peaks do not coincide with one another and must be associated with two different proteins, although only a single protein peak (Peak III of Fig. 4) was apparent. It should also be noted that the specific activity of the TAME esterase measurements (Fig. 4, inset) is about 70% greater than that obtained from chromatography of the 16-hour activation system (Fig. 3). Similar results (not shown) were also obtained when CTN esterase activity was measured.

Fig. 3. Chromatography of a 16-hour citrate-activated prothrombin preparation. A 19-ml sample of diluted activation mixture containing 14.1 mg of protein (specific activity, 1879 clotting units and 190 TAME esterase units per mg) was placed on column (1.5 × 10 cm) of Bio-Rex-70 equilibrated with 0.1 M sodium acetate. The column was then washed with 40 ml of solvent and eluted with a continuous linear gradient of NaCl-acetate of increasing ionic strength. Analytical values obtained on the 1-ml fractions collected are presented in A and the specific activities are present in B. The slope of the NaCl gradient in this experiment was approximately 7 mM per ml of effluent. Estimated recoveries were 92% for protein, 86% for TAME esterase, and 88% for clotting activity.
Under these conditions, an additional fraction (Peak IV) containing 5% of the protein of the elution pattern and all of the residual clotting activity is clearly separated from Peak III, which contains 16% of the protein and only esterase activity. In five chromatography experiments with different 500-hour activation mixtures, the average specific activity of Peak III was 770 (range, 683 to 889) TAMe esterase units per mg, while Peak IV had a range of 70 to 1600 clotting units per mg.

In one experiment, a 500-hour activation mixture was chromatographed under the conditions shown in Fig. 4; Peak III was isolated and immediately rechromatographed under the conditions of the experiment shown in Fig. 5. With the exception of missing Peaks I and II, results identical with those given in Fig. 4 were obtained. These two peaks were then pooled and immediately rechromatographed for a third time with the experimental conditions of Fig. 4. With the exception of the missing inactive peaks, only one peak, Peak III, emerged from the column with an enzyme pattern identical with that in Fig. 4. These experiments served to confirm the fact that the separation of the two enzymatic activities and their association with two different protein peaks was markedly dependent upon the slope of the salt gradient used to elute the column. In five experiments, with the use of a shallow NaCl gradient, the esterase peak activity was eluted from the column at about 300 ± 50 mM, while the clotting activity peak was eluted at 450 ± 50 mM. Thus, it appeared that the residual clotting protein which remained in the 500-hour citrate activation system had altered chromatographic properties when compared to the 16-hour system in which both activity peaks were eluted at 300 mM.

Gel Filtration Analysis of Activation Products—Fig. 6 (A, B, and C) depicts the protein and enzyme elution patterns of 16-, 214-, and 500-hour citrate activation mixtures after Sephadex G-100 gel filtration. From the results shown, it is evident that in spite of differing column conditions and clotting-esterase ratio (C:E) of the samples applied, both activities emerge from the columns at the same relative V_t:V_o ratio. Further, the C:E ratio of the peak tubes from each of the three experiments is about the same as that expected from the values obtained on the original activation mixtures (Fig. 2). Comparison of the specific activity of the TAMe esterase analyses for the peak tubes gives values of approximately 240, 500, and 825 TAMe units per mg of protein, respectively, for the 16-, 214-, and 500-hour samples after gel filtration. This apparent purification...
and the protein elution pattern of the system was equivalent to thrombin preparation which had been “activated” for 16 hours in sodium citrate containing ST1. Under these conditions, no detectable clotting or TAMe esterase activity was measurable, and the protein elution pattern of the system was equivalent to the sum of the prothrombin and ST1 preparations run individually. In comparing this experiment with those in Fig. 6 (A, B, and C), it can readily be seen that with conversion to thrombin, the activation system becomes more heterogeneous. The unactivated prothrombin preparation has a \( V_e:V_o \) of 1.3 for the major protein peak (and activity peak (20)) and a minor protein contaminant at the \( V_o \) (Fig. 7A). After activation, the protein and activity peaks of thrombin are shifted to \( V_e:V_o \) of 1.65 to 1.71 (Fig. 6, A, B, and C), and additional protein fragments are seen at greater elution volumes. These peaks are not present in prothrombin preparations in which activation has been inhibited by ST1 (Fig. 7A). The relationship of these activation products is more clearly delineated in the experiments shown in Fig. 7, B and C, in which the major protein peaks isolated after ion exchange chromatography were subjected to gel filtration analysis. Fig. 7B represents Peak III isolated by chromatography of a 16-hour activation mixture (Fig. 3). The Sephadex pattern of this material shows a single protein and enzymatic peak at \( V_e:V_o = 1.7 \), which corresponds to the major protein peak of the unchromatographed activation mixture (Fig. 6A). Fig. 7C represents Peak I pooled from several chromatographic experiments of 500-hour activation mixtures. The protein elution pattern of this material shows two major protein peaks corresponding to \( V_e:V_o = 2.0 \) and 2.8. The other proteins which are evident from the \( V_e:V_o = 25 \) may be contaminants or unconverted proteins of the original prothrombin preparations.

From a comparison of the \( V_e:V_o \) ratios of the activation fragments isolated above with similar data obtained with a series of essentially globular proteins of known molecular weight (20), the following molecular weight estimates were obtained: prothrombin \( (V_e:V_o = 1.3) \), 69,000 to 70,000; Peak I activation fragments \( (V_e:V_o = 2.0 \) and 2.8), 20,000 and less than 10,000; and Peak III or Peak III + IV for the esterase and clotting activity of thrombin \( (V_e:V_o = 1.65 \) to 1.71), 30,000 ± 3,000. These values are comparable to those obtained previously on unchromatographed activation mixtures analyzed by gel filtration (20).

Sedimentation Analyses—The sedimentation patterns of the chromatographic fractions isolated from citrate activation mixtures, as well as prothrombin, are presented in Fig. 8, and their sedimentation coefficients in relation to protein concentration are diagrammed in Fig. 9.

At an ionic strength of 0.15, pH 7.0, the human prothrombin preparations used in these studies usually sediment as single components (Fig. 8A). However, the dependence of \( s \) on protein concentration was found to be due to the fact that with prolonged exposure to citrate, more of the protein breaks down to inactive fragments of smaller molecular size, which have greater elution volumes than the enzymatically active protein observed at maximum activation for 16 hours.

Fig. 8. Tracings of schlieren sedimentation patterns of human prothrombin and thrombin preparations following chromatographic isolation from citrate activation mixtures. Photographs were taken at times indicated after the rotor reached full speed. Rotor speed was 30,780 rpm, except in D which was 29,630 rpm. Phase plate angle was 45° and protein concentration was 4 to 7 mg per ml in phosphate-NaCl buffer, pH 7.0, \( l/2 = 0.15 \). Specific activities of preparations studied are presented in the figure. Sedimentation is from left to right.

![Fig. 7. Sephadex G-100 elution pattern of an inhibited activation system and fractions of an activation system separated by ion exchange chromatography. A, \( \square \), protein elution pattern of a prothrombin preparation (5 mg) containing ST1 (2 mg) dissolved in 1 ml of 0.25% sodium citrate for 16 hours before analysis; \( \triangle \), protein elution pattern of ST1 (2 mg per ml), and \( \lozenge \), protein elution pattern of prothrombin (5 mg per ml), without exposure to citrate. B, approximately 3 mg of Peak III isolated from a chromatography column experiment similar to Fig. 3. \( \square \), protein concentration; \( \bigcirc \), clotting activity; \( \bullet \), TAMe esterase activity. C, approximately 7 mg of a pool of Peak I fractions collected from chromatography columns in which activation mixtures greater than 16 hours were analyzed. \( \square \), protein concentration. Dimensions of gel columns were 1 × 40 cm in A and 1 × 60 cm in B and C.](http://www.jbc.org/content/754/5/2490/F6.large.jpg)
protein concentration (Fig. 9A) suggests that the preparations undergo a reversible dissociation in dilute solution. Although details are not available, similar observations have also been made with bovine prothrombin products (9, 32). Extrapolation of the data (Fig. 9A) to infinite dilution gives an \( s_{\text{wo}} = 3.6 \), which is close to an estimated \( s = 3.45 \) for prothrombin activity in native oxalated plasma determined in a partition cell (18). These values are substantially different from those obtained at high ionic strengths, in which case the preparations have an \( s_{\text{wo}} = 4.6 \) with no evidence of reversible dissociation.

The thrombin preparations examined in this study appear to be reasonably homogeneous, whether isolated from 16-hour (Fig. 8C) or 500-hour (Fig. 8D) activation mixtures, in spite of the fact that the latter was a pool of Peak III (approximately 75%) and Peak IV (approximately 25%) protein preparations. No appreciable concentration dependence of \( s \) was noted; the extrapolated points (Fig. 9B) gave a value of \( s_{\text{wo}} = 3.55 \). This is practically identical with that of prothrombin, noted above, with the same buffer system (Fig. 9A). Harmison, Landaburu, and Seegers (33) have reported \( s_{\text{wo}} = 3.76 \) for bovine thrombin, but their data demonstrated an unusual linear decrease in sedimentation rate with decreasing protein concentration.

From the optical measurements in three sedimentation experiments a value of \( D_{20,w} = 10.6 \pm 0.5 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1} \) was obtained. This is higher than \( D_{20,w} = 8.76 \) found for bovine thrombin (33), although comparable to a value of 11.0 obtained by Aronson and Menache (34) for "biologically" derived human thrombin. With \( s_{\text{wo}} = 3.55 \), \( D_{20,w} = 10.6 \), and assuming \( \bar{v} = 0.735 \) (29), the Svedberg equation (28) gives a value of 30,800 \text{ g} for the molecular weight of the human thrombin preparations studied. This is in reasonable agreement with an average of 30,000 ± 3,000 \text{ g} estimated from the gel filtration experiment noted earlier. From their sedimentation-diffusion experiments, Aronson and Menache (34) obtained a value of 31,800 \text{ g} for human thrombin obtained from biologically activated prothrombin.

In other experiments, no significant difference was noted in the sedimentation coefficient (corrected for salt redistribution) of thrombin preparations dissolved in buffers with ionic strengths of 0.15 and 0.50 at pH 7.0. \( Peak I \), which from the gel filtration experiments is heterogeneous (Fig. 7C), demonstrates considerable boundary spreading during sedimentation analysis (Fig. 8B). Estimates of the major component in this system gives an \( s_{\text{wo}} \) of about 2.0. No attempt was made to study \( Peaks II \) or \( IV \) by themselves, since the material isolated was insufficient for analysis.

Electrophoretic Analysis—Fig. 10 presents the protein pattern, clotting activity, and CTN esterase measurements for two thrombin preparations chromatographically isolated from a 16- and 500-hour activation mixture and subjected to cellulose acetate electrophoresis. In comparison, these preparations migrate anomalously with a relative mobility less than prothrombin, and the 16-hour preparation indicates a reasonable association of clotting and CTN esterase activity with the protein zone. The 500-hour preparation is similar to the 16-hour preparation, but as anticipated, only esterase and no clotting activity was detected.

Inhibition Studies—Previous work by others has shown that PMSF and DFP inhibit thrombin activity in a manner similar to other estero-proteolytic enzymes (35, 36). It was therefore of interest to determine if a difference in the titration curve could be detected with the esterase fraction (\( Peak III \) of the 500-hour mixture) and the clotting plus esterase fraction (\( Peak III \) of the 16-hour mixture) with the use of these compounds.

Fig. 11A presents the results of an experiment which shows the marked time dependence for the inhibition of thrombin activity in sera of different ages stored at 0°C. The plots of clotting times (Fig. 11B) show that clotting activity in sera from donors of the same age is initially identical but later diverges, indicating a difference in thrombin resistance. The clotting times for sera of the same age, but stored at different temperatures had been stored at different temperatures, were essentially identical, indicating that the difference in thrombin resistance results from differences in the sera of the donors, rather than from differences in the conditions of storage.
In this connection it should be noted that from one experiment reported by Seegers et al. (35), we have calculated that a 346-fold molar excess of PMSF over that of highly purified bovine thrombin produced only 50% inhibition of clotting activity in 2 hours.

Because of the slowness with which the sulfonation reaction went to completion and the fact that the reaction rate is known to be dependent on the PMSF concentration (30), we turned our attention to the titration of thrombin activity with DFP. Fig. 11B presents data indicating that with this inhibitor there is also a time dependence on the inactivation of thrombin clotting and esterase activity. With the use of a similar system of measurements this effect was not noted by Miller and Van Vunakis (36), who used bovine thrombin preparations of less purity than those employed here. As shown in the 1-hour incubation experiments presented in Fig. 12B, this time dependence is considerably less than with PMSF but more than with chymotrypsin. Of particular note is the marked difference between the clotting and esterase activity in the presence of a 50-fold molar excess of PMSF, assuming a molecular weight of 30,000 g for human thrombin. This was a somewhat unexpected finding, since previous studies had shown that the reaction of PMSF with chymotrypsin is completed in about 1 hour under similar conditions (30).

Comparisons were made after 1-hour incubation of enzyme with the inhibitor because the instability of the thrombin preparations precluded following the degree of inhibition over longer periods of time. These experiments also included the titration of chymotrypsin esterase activity. In Fig. 12A it is apparent that the titration of chymotrypsin with PMSF follows a stoichiometric reaction, the experimental values being in close agreement with the inhibitor and enzyme and a calculated concentration of 1 × 10⁻⁴ M chymotrypsin (noted by Arrow a) based on a molecular weight of 27,400 g (30). The experimental values. Curve b (---) corresponds to the experimental data obtained for clotting activity (○) and esterase activity (●) for a thrombin preparation isolated from a 16-hour activation system (specific activity = 2,880 clotting units and 480 esterase units per mg). Based on a molecular weight of 30,000 g, the thrombin concentration was 7 × 10⁻⁴ M (noted by Arrow b). Curve c (-----) similar to Curve b except the thrombin preparation was isolated from a 6-hour activation system (specific activity = 880 TAMe esterase units per mg), and the enzyme concentration was calculated to be 6 × 10⁻⁴ M (noted by Arrow c). Experimental data (△) obtained for esterase activity only since clotting activity was undetected. Additional details are given in text.

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An incidental observation was the finding that if the inhibition system included imidazole buffer, 0.15 M, pH 7.0 (a common buffer used in blood clotting studies), the degree of inactivation of thrombin activity by PMSF was substantially reduced.
from that of the control (Fig. 13A) for the rate of loss of clotting activity. Since the activation of human prothrombin in citrate solution appears to be a proteolytic process (22), this experiment was little affected. Inclusion of STI (2 mg) to the system almost completely inhibited the activation of prothrombin under these conditions, with less than 4% of the clotting activity being developed after 2 days of activation.

In another experiment (not shown), 100 µg of a Peak III preparation isolated from a 500-hour activation system (specific activity, 889 esterase units per mg; no detectable clotting activity) were added to prothrombin under conditions identical with those given in Fig. 13. The curve obtained did not differ significantly from that of the control (Fig. 13A) for the rate of loss of clotting activity. Since the activation of human prothrombin in citrate solution appears to be a proteolytic process (22), this experiment would seem to provide additional evidence for the differences in enzymatic activity of the two activation products evolved.

**DISCUSSION**

In our previous studies a sequence of activity and protein changes following the citrate activation of human prothrombin was observed (20). The data obtained showed that prothrombin was reduced in size during activation to a fragment of about 30,000 to 35,000 molecular weight, which had clotting activity. It was also noted that other smaller molecules were present in the activation system, which seemed to increase in concentration as the clotting activity declined during prolonged exposure to citrate ion. On the basis of clotting assays, the highest specific activity of thrombin isolated from 16 hour citrate activation mixtures by gel filtration was only half that anticipated, assuming that thrombin has twice the specific activity of prothrombin. This implied that another activation fragment, of about the same size as the clotting enzyme, also existed in the activation system. Similar observations have recently been made by Aronson and Menach (34), who used a biologically activated prothrombin system.

The present studies support the above findings and provide evidence for conditions in which the esterase and clotting activities of thrombin are both dependent and independent of one another. During the initial citrate activation of prothrombin, the TAME esterase and fibrinogen-clotting activities generated by the system appear to have identical properties under the conditions studied. Both are derived from prothrombin at almost equal rates (Fig. 1), have similar chromatographic (Fig. 3), gel filtration (Fig. 6), and electrophoretic (Fig. 10C) properties, and the activity of each is about equally inhibited by PMSF (Fig. 12A) and DFP (Fig. 12B). From these observations, along with the sedimentation data (Figs. 8 and 9), it might seem quite reasonable to conclude that both the esterase and clotting activities of thrombin are associated with the same active center on a protein with a molecular weight of approximately 30,000 g. It is, however, only after maximum activation occurs and upon prolonged exposure to citrate that differences in the properties of these enzyme activities are detected. This is apparent from the increase in the specific activity of the esterase enzyme following chromatography (Figs. 4 and 5) and gel filtration (Fig. 6) of activation systems in which the clotting enzyme has virtually disappeared (Fig. 2). In addition, the relative inhibition of enzyme activity by PMSF (Fig. 12A) and DFP (Fig. 12B) is altered, and the difference in the catalytic properties of the two enzymes on prothrombin activation is striking (Fig. 13). These and our previous data (20), when coupled with the findings that human and bovine prothrombin have only one NH₂-terminal group (alanine), as opposed to at least two NH₂-terminal groups for thrombin (isoleucine and threonine (22)), might suggest the possibility that the majority of the esterase and clotting activities of thrombin are associated with different active sites or perhaps two different species of molecules.

This latter interpretation must be viewed with caution, however, since with much simpler estero-proteolytic enzyme systems (i.e. trypsin, chymotrypsin, etc.), small changes in structure can alter their kinetic properties. For example, it is possible that an initially homogeneous enzyme having a single active site could be modified so as to alter its relative activity toward different substrates as well as its chromatographic behavior. This might occur by loss of an amide or sulff acid residue or hydrolysis of a single peptide bond. Whether or not this happens during prolonged exposure of thrombin to citrate is not known and would require amino acid analysis and peptide mapping of the esterase and clotting protein peaks to determine if such a minor structural change did take place. This is not evident from a comparison of the electrophoretic (Fig. 10), sedimentation (Fig. 9), and gel filtration (Fig. 6) properties of such fractions isolated in this

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**Fig. 13.** Effect of clotting fraction on the citrate activation of prothrombin. A, prothrombin, 6 mg, dissolved in 2 ml of 25% (w/v) sodium citrate. B, prothrombin, 6 mg, dissolved in 2 ml of 25% (w/v) sodium citrate containing 43 µg of a Peak IV preparation (1055 clotting units per mg) isolated by chromatography of a 500-hour citrate activation mixture. Aliquots taken at times indicated and assayed for clotting and esterase activity. See text for further explanation.
study. In this regard it has been suggested that Peak IV (Fig. 5) might be molecularly different and arise from prothrombin independently of the main thrombin component (Fig. 3). Prolonged exposure to citrate might then alter the characteristics of all components in such a way that the main activation product loses clotting activity. This would allow Peak IV material to be separated because of its structural difference and to display altered kinetic properties.

Seegers has demonstrated that, upon acetylation of bovine thrombin or upon activation of acetylated bovine prothrombin, there is a marked increase in the esterase activity coincident with a disappearance of fibrinogen-clotting activity (9). Similar observations have been made with other estero-proteolytic enzymes, e.g., pepsin (37). As noted earlier, the modification in the relative esterase and clotting properties of acetylated thrombin has been interpreted in terms of alterations in secondary binding sites (4) or steric effects (2). Whether such an interpretation might serve in the present study is uncertain, since the conditions used and the results obtained are somewhat different. In our experiments with human thrombin, the esterase activity remains unchanged during the loss of clotting activity in citrate (Figs. 1 and 2), which results in an increase in the specific activity of the esterase enzyme following chromatography (compare Figs. 3B and 4B). This is due to a decrease in the protein content of the enzyme peak, rather than an increase in the activity per se, as occurs in the acetylation experiments.

From the standpoint of the enzymatic measurements, it is possible, however, that the kinetic properties of thrombin may have been altered sufficiently so that there was an apparent separation of the two activities. This could have occurred during prolonged citrate exposure by changing the Michaelis constants ($K_m$), pH optimum, and salt dependence or sensitivity of product inhibition of either the esterase or clotting activity or both. For example, if the $K_m$ for the fibrinogen-thrombin reaction (which is unknown) increased because of the citrate alteration of thrombin, the clotting time measurements would be delayed without necessarily being reflected by the TAME or C1IN esterase measurements, in which case the substrates are used in large excess. In such a situation, one would expect to obtain either a single protein peak having only esterase activity and no detectable clotting activity, or (assuming changes in chromatographic behavior) an esterase peak and another protein peak having both activities. However, from Fig. 5, Peak III satisfies this criterion but Peak IV, which admittedly represents less than 10% of the original clotting activity and only one-third of the specific activity, has no detectable TAME esterase activity above that due to the spontaneous hydrolysis of the substrate (i.e., less than 0.05 $\mu$M H$^+$ min$^{-1}$). This would suggest that the kinetic properties of the TAME esterase activity (if associated with the clotting peak) have also changed, so that it is not evident under the single rate assay procedures employed. Possibly the selection of an ester substrate somewhat more flexible, in terms of kinetic conditions, than the TAME procedure used in the majority of this study, as well as measurements of the rate of fibrinopeptide release rather than the less satisfactory gel point of the fibrinogen-thrombin reaction, would provide a more definitive answer to this problem.

These experimental findings are not in accord with the hypothesis that the estero-proteolytic activities of thrombin are dependent upon a monomer-dimer association (6, 13). The evidence for this has not been documented and seems to be based largely on the studies of Cohly and Scheraga (38) and Schrier, Broomfield, and Scheraga (39) and, to some extent, Harmison et al. (33) whose data demonstrated a marked dependence of sedimentation coefficient and Archibald molecular weight on the protein concentration of bovine thrombin. Our studies give some indication that such a relationship exists with human prothrombin preparations (Fig 9A), and this also appears to be the case with bovine preparations (9, 32). However, there is no evidence that human thrombin products behaved similarly. Regardless of the relative esterase and clotting activities of the thrombin fractions isolated in our experiments, all the preparations behaved as undissoeating systems having no dependence of sedimentation coefficient on concentration or ionic strength.

In addition the $V_c/V_n$ ratio on gel filtration column was not concentration dependent.

Consideration of the role which other clotting factors, particularly Factor X, might have on the citrate activation of prothrombin is also of importance in the interpretation of these results. Since the prothrombin preparations used were impure, it is quite reasonable to suspect that they contained a significant amount of Factor X. Unactivated Factor X has been reported to be a "contaminant" of most prothrombin preparations, including those of Seegers, and to be activated in citrate solution and biologically in a manner similar to prothrombin (see references in Lehner and Deutsch (16)). Consequently, very potent Factor X preparations can be isolated from serum. Like thrombin, the activated enzyme is of small molecular size and has appreciable TAME esterase activity (15). Very small and practically unmeasurable amounts of thrombin (less than 0.05 NIH clotting unit per ml) have high Factor X activity (23). Certain other similarities have been noted (9). However, unlike thrombin, most Factor X preparations have negligible fibrinogen-clotting activity and have an esterase activity which is inhibited by STI (15) but not by PMSF or DFP (33).

We have no evidence to indicate that Factor X played a significant role in the results presented. Soybean trypsin inhibitor, in a ratio of 500:1 (w/w), has been shown to have no effect on the TAME esterase activity of the preparations employed (10). In addition, activated Factor X activity is usually not adsorbed to the type of exchange column used in these studies and appears in the initial hold up volume (9, 22). Whether the resistance of our esterase preparations to PMSF and DFP inhibition might be indicative of Factor X is difficult to document since other workers did not consider the relative molar ratio of inhibitor to enzyme. This aspect, as well as the possibility that the esterase preparations isolated might have other clotting factor activities (IX, XI, XII, etc.), remains to be examined.

In conclusion, this study indicates that thrombin is similar to prothrombin in its conversion to thrombin in citrate solution. Attempts to remove this material from our prothrombin products by a variety of chromatographic procedures (16, 21, 22, 34) have repeatedly resulted in products of the same specific activity as the unchromatographed materials. However, the chromatographed preparations consistently showed delayed citrate activation curve. In fact, one product (34) gave maximum activation (90% of the potential two-stage prothrombin activity) of clotting and TAME esterase activity only after exposure to 25% sodium citrate for 150 hours.

4 We are indebted to the reviewers of this paper for bringing these possibilities to our attention.
certain other estero-proteolytic enzyme systems in which an active center on the enzyme molecule can be modified so as to alter its relative activity toward different substrates. Whether such modifications are the result of structural or kinetic alterations, or both, in thrombin molecules having a single active center, or whether they are due to different active centers possibly associated with different molecules is, from the present study, not clear. Regardless of these interpretations, the data underline the possibility that under certain conditions, the TAMe esterase and fibrinogen-clotting activities are not necessarily related to one another. This is believed to be of importance in considering whether or not certain clotting factors which also possess esterase activity and prothrombin-activating properties are, in fact, distinct protein molecules in themselves (14) or represent products of prothrombin (9).

Acknowledgment—We would like to thank Professor John W. Mehl for reading the manuscript and making several helpful suggestions.

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