Two Forms of Human Thrombin

ISOLATION AND CHARACTERIZATION*

GERARD F. LANCHANTIN, JACK A. FRIEDMANN, AND DONALD W. HART

From the Division of Laboratories and Medical Research Institute, Cedars-Sinai Medical Center, and the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles, California 90029

SUMMARY

When preparations of human prothrombin are rapidly activated to thrombin (EC 3.4.4.13) in the presence of a partially purified enzyme from Taipan snake venom (Oxyuranus scutellatus scutellatus), the thrombin activity isolated from the activation mixture by ion exchange chromatography is associated with a single molecule of molecular weight of approximately 39,000. The subunit structure of this blood-clotting enzyme was examined by sodium dodecyl sulfate electrophoresis and was found to consist of two polypeptide chains connected by disulfide bonds, of molecular weight of approximately 22,000 and 7,000. NH₂-terminal amino acid analysis by the 5-dimethylaminonaphthalene-1-sulfonyl procedure indicated that the larger chain contained NH₂-terminal isoleucine while the smaller chain contained NH₂-terminal threonine. With time, the enzyme self-digests itself without change in the clotting activity of the system to produce a smaller thrombin molecule of molecular weight of approximately 26,000, which consists of a 19,000 and a 7,000 molecular weight peptide with NH₂-terminal isoleucine and threonine, respectively. The other peptide, which is inactive against fibrinogen, is split from the larger chain of the parent molecule presumably because of autolysis. It was found to have a molecular weight of approximately 11,000 and to contain a single NH₂-terminal glycine. These structural changes were confirmed by isolation of the various thrombin peptides by gel filtration in 10% acetic acid. Slow and incomplete activation of human prothrombin in the presence of concentrated citrate solution or activation with a human Factor Xa preparation results in the isolation of a mixture of both thrombin molecules from the activation systems, including the inactive thrombin peptide. These molecules showed the same sequence of structural changes due to autolysis without a corresponding change in clotting activity as did the venom-derived enzyme.

Thrombin preparations containing a mixture of the larger and smaller molecules, particularly those in which the smaller molecule predominates, have a significantly higher clotting activity than preparations containing only the larger molecule. No evidence was obtained for the existence of a three-chain polypeptide human thrombin molecule as has been reported by others for the bovine enzyme. Since preliminary observations seemed to indicate that under our conditions of study bovine and horse thrombin preparations contain similar species of molecules as those described above for human thrombin, methods of preparation of prothrombin or activation (or both) employed by others may account for this apparent discrepancy. These studies suggest that the thrombin derived from human, bovine, and possibly horse protbin appear quite similar in size, NH₂-terminal amino acids, and subunit structure, their relative composition in a prothrombin activation mixture being dependent only upon the rate or yield (or both) of thrombin generated from its zymogen, rather than the source of activators.

During the coagulation of blood, the conversion of prothrombin to thrombin plays a central role common to the intrinsic and extrinsic pathways leading to the formation of the fibrin clot. Thus, information on the structural changes which take place during this zymogen to enzyme transformation is important to an understanding of the mechanism and role which other clotting factors may have on this stage of blood coagulation. Much of the earlier work dealing with the events which initiate prothrombin activation as well as the somewhat complex nature of the fragmentation of the zymogen as it is converted to thrombin has been reviewed recently (1, 2) and will not be discussed here. More recent studies dealing almost exclusively with bovine preparations have shown that upon activation, a single polypeptide chain prothrombin molecule is sequentially degraded to a series of fragments, some of which contain molecules of two to three polypeptide chains, and that more than one of these molecules is associated with fibrinogen-clotting activity (3-7). Thus the multiple molecular forms of bovine thrombin suspected some years ago (8-10) are now well known although the nature of these forms, particularly as they are derived from the zymogen under differing activation conditions, is not well established (6, 8, 11, 12). For example, Seegers et al. (8) were among the first to isolate two different clotting forms of bovine thrombin which had hydrodynamic molecular weights corresponding to 33,000 and 23,000. The smaller thrombin molecule had about twice the specific activity (i.e. thrombin

* This study was supported in part by Research Grants HE 09939, HE 07703, and FR 05468 from the National Institutes of Health, United States Public Health Service.
clotting units per mg of protein) as the larger molecule. Presumably these differences were due to the degradation of the larger thrombin molecule with the release of a small acidic peptide that was an inhibitor of its clotting activity. Following this, Rosenberg and Waugh (12) identified and partially isolated six thrombin heteromorphs from a commercial thrombin source, some of which displayed different specific activities. Only three of these forms could be identified in thrombin preparations derived from the activation of purified prothrombin, however. While the reduced and alkylated thrombin preparations containing six active components displayed a subunit structure of at least five fragments of molecular weights from 32,000 to 5,000, the preparations derived from the purified zymogen yielded only the 32,000 and 5,000 molecular weight peptide. This suggested a single molecular weight class of about 37,000 for their multiple thrombin preparations which were heterogeneous with respect to electrophoretic mobility. More recent studies (6) seem to indicate that bovine thrombin consists of three forms corresponding to molecular weights of 39,000 (two chains), 28,000 (two chains), and 28,000 (three chains) with relative specific activities based on clotting activity to be 2:1:0.5. The specific activity of these three forms based on esterase assay all appear to be identical, however. This work has been confirmed, particularly the identification of the two-chain and three-chain form of bovine thrombin, but with the finding that both these forms have essentially the same specific clotting activity (11). Amino acid sequence studies have been performed on a two-chain polypeptide thrombin molecule by Magnusson (2) which did not display heterogeneity under a wide variety of conditions.

With respect to human thrombin, the evidence that this enzyme exists in more than one form is less clear (13-17) even from the results obtained in this laboratory. Our earlier studies (14, 15) suggested that a chromatographically homogeneous enzyme preparation of molecular weight of approximately 30,000 to 35,000 could be isolated from citrate-activated preparations of purified human prothrombin. Following this, polyacrylamide disc electrophoresis demonstrated that more than two protein-stainable bands could be detected in these preparations (16), and while later work also detected two electroforetic components (17) they were not the same two as in the former studies. Others have isolated a single electrophoretic form of human thrombin of molecular weight of approximately 31,000 whose amino acid composition was found to be similar to that of bovine thrombin (18). The present work was undertaken to re-examine this problem in the light of what is known about bovine thrombin. We were particularly interested in learning whether or not human thrombin existed in more than one functionally active form, and if it did, could these forms result from the degradation of a parent thrombin molecule due to autolysis or from different activation pathways from prothrombin. The results obtained appear to suggest that the human thrombin system and possibly that of the horse as well are similar to that of the bovine in some respects such as size, subunit structure, and NH₂-terminal amino acids. However, the two forms of human, bovine, and horse thrombin identified in this work appear to differ in some respects with those of others in terms of specific activity of the two forms and the existence of a three-chain thrombin molecule. The results also suggest that the relative composition of thrombin heteromorphs in a prothrombin activation system is dependent upon the rate or yield (or both) of thrombin generated by prothrombin activation rather than the source of activators which convert the zymogen to the enzyme.

EXPERIMENTAL PROCEDURE

Materials—Human prothrombin was isolated from freshly collected acid-citrate, dextrose-anticoagulated plasma by methods previously described (16-18). The products thus obtained have high specific activities, are homogeneous, within limits, by DEAE-cellulose column chromatography, disc and immunoelectrophoresis, and sedimentation velocity analysis, and have constant amino acid composition, as summarized elsewhere (19). As discussed elsewhere, these preparations contain small amounts of other blood-clotting factor activity which, on a weight basis, comprise less than 1% of the weight of the preparations (19). In one experiment prothrombin was isolated from acid-citrate, dextrose-anticoagulated horse plasma by the same methods. In a few experiments this isolation procedure was also applied to bovine plasma with the use of the BaSO₄-adsorbed and eluted plasma fraction supplied by Sigma Chemical Company as starting material.

Thrombin was prepared after prothrombin activation in (a) 25% w/v sodium citrate solution, and (b) the presence of a prothrombin-converting enzyme partially purified from Taipan snake venom, and (c) the presence of a human Factor Xa preparation. The thrombin activity was isolated from these activation mixtures by gradient elution chromatography from columns of Bio-Rex 70 (Bio-Rad Laboratories) by a method previously described (15). By means of this method, thrombin clotting and esterase activity are eluted from these columns with the salt gradient, while the snake venom enzyme preparation and Factor Xa are not adsorbed to the column and are eluted in the column hold-up volume along with other nonthrombin activation fragments of prothrombin (2, 15, 17, 21).

Australian Taipan snake venom (Oxyuranus scutellatus scutellatus) was obtained from Sigma Chemical Company. Preliminary experiments indicated that the raw venom probably contained other proteolytic enzymes which nonspecifically digested prothrombin in addition to the enzyme which converted the zymogen to thrombin (22, 23). Consequently the enzyme was partially purified by equilibrating 50 mg of the raw venom by dialysis against 0.02 m Tris buffer, pH 7.4, and placing 5 ml of this solution on a column (1 × 10 cm) of DEAE-cellulose (Whatman DE52) in the same buffer. A linear salt gradient of increasing ionic strength with a linear gradient of 0.02 m Tris-1.0 m NaCl was then used to elute the prothrombin-converting enzyme, which was found to be associated with the last of four protein peaks emerging from the column at about 0.3 m NaCl. Based on the ability of aliquots of the column eluent fractions to convert purified prothrombin in an assay system similar to that of Aronson and Menache (24) and using a value at 280 nm of E₅₅₀ = 15 for the raw venom, a purification of over 40-fold was achieved by this method. By disc electrophoresis the preparation displayed one major and two minor protein-stainable bands. The venom enzyme preparation had to be used almost immediately in experiments to be described under “Results,” since it lost 50 to 90% of its prothrombin-activating capacity even when stored at −20°C for 2 weeks or longer.

1 The designation, Factor X, conforms to the recommendations of the International Committee for the Standardization of the Nomenclature of Blood-clotting Factors (20). By convention, the subscript refers to the activated form of the blood-clotting factor (i.e., Factor Xa).
Preparations of human Factor X (24, 25) were kindly supplied by Dr. David L. Aronson of the Division of Biologic Standards, National Institutes of Health. Two of these preparations displayed a single protein-stainable band by polycrylamide disc electrophoresis and assayed between 3000 to 4000 Factor X activity units per mg of protein (24, 25). In these assays, the Factor X activity of 1 ml of freshly collected human plasma is taken as being equivalent to 100 units. Factor X was converted to Factor Xa by activation in citrate (21) or by activation with a partially purified enzyme preparation (26) isolated from Russell’s viper venom (Vipera russelli) purchased from Sigma Chemical Company. After activation the Factor X preparations displayed a multicomponent protein system by polycrylamide disc electrophoresis. Following transverse sectioning of unstained electrophoretic disc gels of these preparations, followed by elution of the protein in 0.15 M Tris buffer, pH 7.4, only one of these components was found to be associated with Factor Xa activity when measured by its ability to convert purified prothrombin to thrombin in a system similar to that described by Aronson and Menache (24). The Factor Xa activity was found to be equivalent to 465 thrombin units generated from prothrombin per mg of Factor X per hour in a system containing Factor Xa and prothrombin (1 mg) in 1.0 ml of 0.15 M Tris-0.04 M CaCl₂ buffer. The Kₐ (apparent) for this reaction was found to be 1.6 × 10⁻⁴ M prothrombin (assuming 70,000 for the molecular weight of prothrombin) with Vₘₐₓ = 1000 units per ml per hour. These values are similar to those found by Aronson and Menache (24), and consequently these preparations were used in all subsequent experiments to be described under “Results.” Unless otherwise stated all other materials were of analytical reagent grade or better.

Methods—Clotting measurements for prothrombin activity (two-stage method) and thrombin activity (NIH method) were carried out as previously described (14) with NIH thrombin (Lot 3B) as reference. Thrombin esterase activity was determined by the hydrolysis of p-tosyl-L-arginine methyl ester (Calbiochem Corp.) with the use of a thermostatic recording pH-stat as previously described (15). Active site titrations of thrombin solutions were performed with p-nitrophenyl p-guanidinobenzoate (Cycle Chemical Company) as described by Waugh and Chase (27) with the use of the expanded scale (0 to 0.1 absorbance) of a Gilford model 2000 spectrophotometer. Protein in solution and in column effluents was measured by absorption at 280 nm (Beckman DU-2 spectrophotometer) and calculated from the optical factors summarized elsewhere for prothrombin, thrombin, and Factor X (17). In some experiments protein was monitored in column effluents following alkaline hydrolysis (28) and ninhydrin color development in dimethylsulfoxide (29).

Analytical polycrylamide disc electrophoresis was performed by either the method of Davis (30) with minor modification (16) or by the Churmbough method as detailed by Rosenberg and Waugh (12) using the Canalco model 1200 system. The former method was used previously by us for the identification of prothrombin activation fragments (16), while the latter method, although giving the same results, displays a better separation of the slower migrating protein bands, especially those associated with thrombin activity. Electrophoresis in 7.5% polycrylamide gels was also performed in the SDS* (unreduced) and SDS-containing mercaptoethanol (reduced) systems of Weber and Osborn (31). While electrophoresis of unreduced proteins does not necessarily give a good correlation between the logarithm of their molecular weights with electrophoretic mobility because of a variety of reasons (see “Discussion” in Ref. 92), this procedure was important in this work as well as in others (5, 6) for the preliminary identification of single chain peptides derived from activated prothrombin which then could be re-examined by a second electrophoretic analysis in the reducing solvent system. In some experiments the acrylamide gels were polymerized with twice the normal amount of cross-linker (31) or in the presence of 6 M urea solution in order to obtain better resolution of the faster migrating (smaller molecular weight) protein bands. The following proteins were used as molecular weight markers, the values of which are given elsewhere (31) for the unreduced and reduced forms: phosphorylase A and DNase (Worthington), ovalbumin and chymotrypsinogen A (Pharmacia), cytochrome c (Schwarz-Mann), and insulin (Novo). Protein bands were visualized by staining with Amido Schwarz (30) or Coomassie blue (31), and glycoprotein bands were stained with a periodate-Schiff base reagent (33). The distribution of protein bands separated by the above electrophoretic methods were, in some experiments, scanned by the use of a Canalco model F densitometer in conjunction with a model R Sargent-Welch recorder as previously described (16).

Thrombin was concentrated in solution by reverse dialysis against 40% (w/v) sucrose solution while other proteins were concentrated by ultrafiltration by means of a UM-10 Amicon membrane and ultrafiltration apparatus. For the separation of the peptides associated with thrombin activity, the thrombin preparations, purified by Bio-Rex 70 chromatography, were precipitated in the presence of 10% (w/v) trichloroacetic acid, washed twice with double their volume of cold acetone, and redissolved in 20% (v/v) acetic acid. They were then placed on a column (1 × 60 cm) of Sephadex G-100 (Pharmacia), equilibrated, and eluted with 10% acetic acid. Effluent fractions corresponding to each of the separated peptide peaks were then pooled and the solvent removed by vacuum in a desiccator. The dried peptides were then redissolved in an appropriate buffer and examined by electrophoresis and for NH₂-terminal amino acids.

End group analysis for NH₂-terminal amino acids was carried out by the method of Gros and Labouesse (34) with 3-dimethylaminonaphthalene-1-sulfonil chloride (Pierce Chemical Company). Approximately 10 to 100 μmoles of each protein, peptide, or mixture of these were derivatized, hydrolyzed in 6 N HCl (Aristar), dried, and dissolved in solvent. In earlier work, the thin layer chromatography procedure of Woods and Wang (35) was used with polyamide layer sheets (Cheng-Chin Company) and their two-dimensional solvent system. However, most of the work to be reported here was performed with Kodak silica gel sheets according to the method described by Zanetta et al. (36), which appears superior, particularly in the separation of dansylalanine from dansylmethionine and at volumes of 1 μl of sample. A variety of solvent systems were used to separate and identify the dansyl amino acid derivatives with toluene-pyridine-acetic acid (150:50:3.5, v/v/v) in one dimension and toluene-2-chloroethanol-ammonia (100:80:6.7, v/v/v) in the second dimension being the most effective. The identity of each spot was rechecked from its Rₙ value in at least two solvent systems against authentic derivatives (Schwarz-Mann Company and Pierce Chemical Company). In some cases the fluorescent spots were eluted from the silica gel sheets and their

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*Further details concerning these experiments will be the subject of a future communication.

¹The abbreviations used are: SDS, sodium dodecyl sulfate; dansyl, 3-dimethylaminonaphthalene-1-sulfonil.
fluorescence determined in a G. K. Turner model III fluorometer equipped with a high sensitivity attachment. In a few instances, the area of the fluorescent spots was determined and compared with various concentrations of a standard of the same dansylated derivative using the linear relationship between the logarithm of the weight per volume and the square root of the area of the spot (37).

**RESULTS**

**Thrombin Derived by Citrate Activation of Human Prothrombin**—Fig. 1A presents the results of a typical experiment in which a prothrombin activation mixture was fractioned on a Bio-Rex 70 column following activation of the zymogen in 25% (w/v) sodium citrate solution for 24 hours. As in previous experiments (15), more than half of the protein of the activation mixture passes through at the hold-up volume of the column and has no detectable clotting activity associated with it (Peak I of Fig. 1A). After the initiation of a salt gradient, the thrombin activity is eluted from the column in essentially a single protein peak (Peak II of Fig. 1A). Measurements of the specific activity of this peak, however, indicate that the ascending limb contains other proteins which are inert or only partially active while activity measurements of the middle of the peak through its descending limb approach an almost constant specific activity of about 2700 units per absorbance unit. Disc electrophoresis was performed on selected fractions of Peak II noted in Fig. 1A, after concentration of the fractions to almost equivalent absorbance values by dialysis against 40% (w/v) sucrose solution. These results are shown in Fig. 1B, which demonstrates that the ascending portion of the absorbance peak (Fraction 78) contained about equal amounts of two protein bands (designated S-1 and S-2) with a small amount (10%) of another electrophoretic band (designated S-3), and the descending portion of the Peak II material (Fraction 95) contained principally S-3 (80%) with a smaller amount of the S-2 band (20%). These results thus indicated that in spite of the apparent constancy of thrombin specific activity from the middle of Peak II throughout its descending limb, this protein fraction was heterogeneous with respect to electrophoretic composition and suggested that both the S-2 and S-3 bands were associated with thrombin activity.

At this point it was not known whether this heterogeneity of electrophoretic components occurred in the activation mixture itself or whether it was due to the handling and manipulation of the mixture prior and subsequent to its chromatography on Bio-Rex 70. Consequently, this experiment was repeated except that following the citrate activation of prothrombin the mixture was diluted, immediately placed on the Bio-Rex 70 column equilibrated at 4°, rather than at 23°, and the effluent was analyzed as soon as it emerged. Under these conditions (not shown) the specific activity of the eluted thrombin peak (Peak II) was practically constant with fraction number throughout the entire protein peak at 1666 units per absorbance unit, and disc electrophoresis of the entire pooled Peak II material showed on densitometry only 2% of the S-1 band and 26% and 72% of the S-2 and S-3 bands (Fig. 2, left, 0° days stored). This preparation was then allowed to remain at room temperature in 0.3 M NaCl-0.1 M sodium acetate, pH 7.0, and aliquots were taken over a period of 7 days for clotting assays and electrophoretic analysis. The clotting measurements indicated that the preparation (approximately 0.61 absorbance unit per ml) had a stable activity for at least 7 days without any apparent loss of specific activity.

Fig. 1. A, ion exchange chromatography of human prothrombin following activation for 24 hours in 25% (w/v) sodium citrate solution, pH 7.4, at 23°. After activation of 20 mg of prothrombin in 2 ml of citrate solution, the mixture was diluted 10-fold with water and placed on a column (1.5 x 40 cm) of Bio-Rex 70 equilibrated with 0.1 M sodium acetate at 23°. The column was then washed with solvent to elute the first protein peak (Peak I), and then the thrombin activity (Peak II) was eluted with a linear salt gradient with a limit of 1.0 M NaCl-0.1 M sodium acetate. Fractions (1 ml) were collected which were analyzed for absorbance at 280 nm, thrombin specific activity (i.e., NIH thrombin-clotting units per absorbance unit), and chloride ion, as indicated. Arabic numerals refer to those peak tubes that were concentrated and analyzed by disc electrophoresis, the gel patterns of which are shown in B. Horizontal lines with corresponding Roman numerals refer to effluent fractions which were subsequently pooled. Recovery based on absorbance was 100%, with 66% in Peak I and 34% in Peak II. Recovery based on clotting activity was 92%. In B migration is from top to bottom.
Fig. 2, Alterations in the disc electrophoresis and SDS electrophoresis patterns with time of a human thrombin preparation. In this experiment a thrombin preparation was isolated from a prothrombin activation mixture by Bio-Rex 70 chromatography in a manner similar to that shown in Fig. 1A, except that the column was run at 4°. The Peak II fractions were pooled and allowed to stand in a sealed plastic test tube for 7 days at room temperature. During this time, aliquots were taken for disc electrophoretic analysis as shown on the left and also for clotting assays. Throughout this period of study the thrombin activity was virtually constant at 1498 ± 45 NIH clotting units per ml. On the left the S bands are designated, while on the right the approximate apparent molecular weights of the reduced peptides are presented. Migration is from top to bottom.

Activity of 1498 ± 45 units during this period, but there were marked alterations in the composition of its three electrophoretic components (Fig. 2, left). Densitometry of the disc gel patterns (Fig. 2, left) showed that with time there was almost a linear increase in the S-1 and S-2 bands and a linear decrease in the S-3 band, so that at 7 days the mixture consisted of 34% S-1, 56% S-2, and only 10% of the S-3 band. SDS electrophoresis of these preparations was also performed, the patterns of which are shown on the right of Fig. 2. These patterns show that the S-1, S-2, and S-3 bands seen on disc electrophoresis (Fig. 2, left) also consist of three major bands in unreduced SDS gels and 4 bands on reduced SDS gels. Comparing these bands and their corresponding apparent molecular weights (Fig. 2, right) with the disc gel patterns (Fig. 2, left) it is seen that it is the molecular weight component of 32,000 which diminishes during the time course that the thrombin preparation is allowed to remain at room temperature while the 19,000 and 11,000 molecular weight components increase with time. This suggested that the apparent conversion of the S-3 band to the S-2 and S-1 bands might be a proteolytic process, and that more than one of the bands is associated with thrombin-clotting activity and is composed of more than one peptide chain. Of particular note was the fact that the chromatographic fraction containing 90% of the S-2 band and 10% of the S-3 band had a specific activity of 2700 units per absorbance unit (Fig. 1A, A76), while the pooled fraction from the column run at 4° (see above) was composed of 26% S-2 and 72% S-3 (Fig. 2, left, zero time) and had a specific activity of 1666 units per absorbance unit.

Fig. 3A presents an experiment in which the Peak II material, isolated from a prothrombin activation mixture by ion exchange chromatography (similar to that depicted in Fig. 1A) was separated into its component peptides by gel filtration on a column of Sephadex G-100 equilibrated in 10% (v/v) acetic acid. Based on absorbance at 280 nm, four peptide fractions were evident which corresponded to elution volumes (Vf) of 65, 73, 97, and 150 ml, respectively. Ninhydrin analysis of these fractions gave essentially the same pattern except that no ninhydrin-positive material was detected at Vf = 150 ml, nor were there any electrophoretic protein bands or NH2-terminal amino acids detected in this fraction. Since the recovery of starting material based on absorbance at 280 nm was greater than 200% in this experiment, it was concluded that the peak at Vf = 150 ml was an artifact probably due to the leaching of a small molecular weight material with a high extinction coefficient from the plastic tubing and test tubes used to handle the thrombin preparation. This phenomenon occurred in some but not all of these experiments and has been observed by others.

The SDS electrophoresis patterns of the unreduced and reduced peptides isolated by gel filtration in Fig. 3A are shown in Fig. 3B along with the gel patterns of the Peak II mixture before gel filtration (m in Fig. 3B). The peptide peak with Vf = 65 corresponds to the larger of the peptides of the mixture (mol wt 38,000 to 42,000 unreduced) and consisted principally (85%) of a peptide chain with a molecular weight of 32,000 and a smaller peptide (approximately 15%) of molecular weight of 7,000. The next smaller polypeptide, corresponding to Vf = 73, was of molecular weight approximately 24,000 to 28,000 in the nonreducing SDS system and was also found to contain at least two chains, which in the reducing SDS electrophoresis systems gave molecular weights corresponding to 19,000 and 7,000. These correspond to 78% and 22% of the stainable material in the gel. The last peak, at Vf = 97, showed essentially a single peptide chain in both the nonreducing and reducing systems, which in the latter corresponded to an apparent molecular weight of 11,000. All of the gel patterns presented in this paper are presented in a manner similar to that depicted in Fig. 1A.

The rationale for monitoring peptide peaks in the column effluents by ninhydrin analysis as well as by absorbance at 280 nm was based on the fact that the "A" chain of bovine thrombin contains only one tryosine residue and no tryptophan (2). D. F. Waugh, personal communication.

1 These calculations have been corrected for the small amount of overlapping peptide from adjacent peaks. Assuming the amount of dye staining is proportional to the amount of protein distributed in these gels, a peptide of molecular weight 7,000 would be expected to represent approximately 18% of a 37,000 molecular weight peptide and 27% of a 29,000 molecular weight peptide. The values obtained by densitometry of the gels in
Fig. 3. A, gel filtration separation in acetic acid of the thrombin peptides isolated by ion exchange chromatography of a 24-hour citrate-activated human prothrombin preparation. In this experiment, a thrombin preparation was isolated from a 24-hour citrate activation mixture of prothrombin by chromatography on a Bio-Rex 70 column. The thrombin eluted with the salt gradient (Peak II material in Fig. 1A) was pooled, precipitated with 10% (w/v) trichloroacetic acid, washed twice with double the volume of cold acetone, and dissolved in 3 ml of 20% (v/v) acetic acid. It was then placed on a column (1 x 60 cm) of Sephadex G-100 (fine), equilibrated, and eluted with the same solvent at a flow rate of 30 ml per hour. Fractions (3 ml) were collected. The Arabic numerals refer to the peak tubes that were analyzed by SDS electrophoresis (Fig. 3B), while the horizontal lines represent those tubes which were pooled for NH$_2$-terminal amino acid analysis. The peptide with a peak corresponding to a $V_e = 150$ is an artifact. See text for details.

Fig. 3B stained faintly and atypically for carbohydrate with the periodate-Schiff base reagent (compared to other glycoprotein, i.e. prothrombin in the same system). By disc electrophoresis the pooled peak at $V_e = 65$ had the mobility of the S-3 thrombin band, while the peaks at $V_e = 73$ and $V_e = 97$ corresponded to the S-2 and S-1 bands, respectively.

this experiment were sufficiently close to these theoretical values as to lead us to conclude that no more than one peptide with a maximum molecular weight of 7,000 was split from the parent peptide by mercaptoethanol reduction of one or more disulfide bonds.

NH$_2$-terminal amino acid analysis of the Peak II material gave nearly equal molar amounts of isoleucine, threonine, and lesser amounts of glycine. Following gel filtration, the pooled fractions noted by the horizontal lines in Fig. 3A gave isoleucine and threonine for the peak at $V_e = 65$ and $V_e = 73$ and only glycine for the peak at $V_e = 97$. No other NH$_2$-terminal amino acids were encountered in these experiments.

Based on the data thus far presented in Figs. 1 to 3, it appeared that the thrombin S-3 electrophoretic band was a single component of molecular weight approximately 39,000 which contained two peptide chains of molecular weight 32,000 and
7,000, with NH₂-terminal isoleucine and threonine. With time, this S-3 component appears to be degraded, presumably due to autolysis, to yield the S-2 electrophoretic band which is also a two-chain molecule of molecular weight about 26,000 having a 19,000 molecular weight chain and a 7,000 molecular weight chain which also terminate in isoleucine and threonine. The peptide fragment split from the parent S-3 thrombin molecule, presumably toward its COOH-terminal end, is the S-1 electrophoretic band, a single chain of molecular weight approximately 11,000, which has an NH₂-terminal glycine. This transformation of the S-3 thrombin to the S-2 thrombin plus the S-1 thrombin fragment occurred without any change in the fibrinogen-clotting activity of the system. Since the specific activity measurements in Fig. 1A indicate that the S-1 thrombin band is not associated with appreciable thrombin activity, it was concluded that the S-3 and S-2 components retained the active site of the enzyme during autolysis so that their activity against fibrinogen substrates remained in spite of a reduction of almost one-third of the apparent molecular weight. Attempts to resolve these electrophoretic bands by rechromatography of the Peak II material on Bio-Rex 70 or according to the method of Lundblad (38) on columns of SP-Sephadex C-50 were unsuccessful.

**Thrombin Derived by Activation of Human Prothrombin by Taipan Snake Venom Enzyme**—Citrate activation of human prothrombin for 16 to 24 hours generally results in only about 40 to 70% of the potential enzyme activity of the zymogen being converted to thrombin (15). In order to determine if the S-3 and S-2 thrombin molecules were derived separately from prothrombin or sequentially, as the data in Fig. 2 seemed to suggest, it was necessary to use an activation system which was more rapid and quantitative than the citrate system. In addition, it was important to determine if only a single thrombin molecule could be detected and to determine its specific activity. Since previous studies had shown that an enzyme in Taipan snake venom was capable of rapidly and quantitatively converting prothrombin to thrombin in the initial absence of other clotting factors (22, 23) this system was utilized for further experimentation. The enzyme was purified from the raw venom as described under “Materials” and 0.7 mg of this preparation was added to 15 mg of prothrombin in 15 ml of 0.02 M Tris buffer, pH 7.0, and incubated at 28°C. In spite of the fact that this system contained no calcium, which others maintain is necessary for prothrombin-converting activity (22, 23), over 90% of the prothrombin two-stage units were converted to thrombin units in less than 20 min, and the thrombin activity generated by the system was stable for over 24 hours in the presence of the venom enzyme. Fig. 4A presents the Bio-Rex 70 elution profile of this mixture after 30 min activation with the snake venom enzyme. In contrast to the citrate activation experiment shown in Fig. 1A, the total Peak I (hold-up volume) absorbance and the Peak II absorbance measurements are nearly identical, and the absorbance measurements of Peak II demonstrate a much sharper elution profile than was evident in Fig. 1A. Specific activity measurements (not shown) of the Peak II protein were virtually constant with fraction number throughout the fractions noted by the horizontal line and Roman numeral II (Fig. 4A) at 1,565 ± 45 units per absorbance unit. The pooled Peak II fractions showed an essentially single protein-stainable band on conventional disc electrophoresis (labeled CD in Fig. 4B) with the

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* Titration of human thrombin solutions with p-nitrophenyl-p-guanidinothiocarbamate indicated a concentration of 8.2 to 9.4 X 10⁻¹⁴ M active sites per 1,000 units of thrombin clotting activity per liter; human prothrombin has a specific activity of 1,500 clotting units per mg of protein and a hydrodynamic molecular weight of 70,000 (19). Assuming that thrombin has a molecular weight of 35,000 and about twice the specific activity as its zymogen, a solution of 1,000 thrombin clotting units per ml would give a concentration of 9.4 X 10⁻¹⁴ M thrombin, which is in good agreement with the active site titration data.
mobility of the S-3 band. By SDS electrophoresis the unreduced protein also exhibited a single protein-stainable band (labeled UR in Fig. 4B) of molecular weight about 40,000 which, when reduced with mercaptoethanol (R in Fig. 4B), suggested the presence of two peptides of 32,000 and 7,000 in apparent molecular weight. Fig. 5, Gel d, shows the SDS gel pattern of this preparation after it was stored for 7 days at -20° and then reduced and analyzed. While there was little change in the preparation stored in the frozen state (compare Fig. 4A, Gel R, with Fig. 5, Gel d), the preparation that was allowed to remain at room temperature demonstrated the same subunit changes that occurred in citrate-derived thrombin mixtures (Fig. 2). In addition, the pooled Peak I material in Fig. 4A yielded only NH₂-terminal isoleucine and threonine, as did the frozen preparation of Fig. 5, Gel d, while after autolysis for 7 days the preparation contained both of these NH₂-terminals as well as glycine. Gel filtration in 10% acetic acid of the Peak 11 material shown in Fig. 4A, as described in Fig. 3A, also demonstrated a single peptide peak at Vₑ = 65 (and a small artifactual peak at Vₑ = 150).

Fig. 5 also shows the results of SDS electrophoresis of mercaptoethanol-reduced prothrombin before activation (Gel a), prothrombin after 30 min of activation with the venom enzyme and before chromatography (Gel b), and the inactive Peak I material (Fig. 4A) which appeared in the column hold-up volume. From these gel patterns it is evident that upon activation, human prothrombin of molecular weight of approximately 72,000 (Fig. 5, Gel a) is degraded to a series of activation fragments of lower apparent molecular weight ranging from 58,000 to 15,000, and possibly less. Quantitatively, the most conspicuous protein in the activation mixture is a protein of molecular weight of approximately 32,000 (Fig. 5, Gel b) which is totally removed from the system by chromatography of the mixture on Bio-Rex 70 (Fig. 5, Gel c) and is eluted from the column by the salt gradient (Fig. 5, Gel d). Thus, no other reduced peptides corresponding to S-2 thrombin mol wt 19,000 plus 7,000) or the S-1 thrombin fragment (mol wt 11,000) can be detected in the activation mixture itself (Fig. 5, Gel b) or in the Peak 1 material (Fig. 5, Gel c).

Thrombin Derived by Activation of Human Prothrombin by Human Factor Xa Preparation—It is generally agreed that Factor Xa is the enzyme responsible for the physiological activation of prothrombin and that the enzyme activity of Factor Xa is markedly augmented kinetically by Factor V, phospholipid and calcium ion (1). By itself, Factor Xa converts prothrombin to thrombin but at a very slow rate and in poor yield (24-25). Since we were principally interested in the thrombin(s) derived from prothrombin by Factor Xa, we incubated 50 μg of this enzyme isolated as described under “Materials” with 10 mg of human prothrombin in 10 ml of 0.1 M NaCl-0.02 M Tris buffer at 28°. Clotting measurements on aliquots were made at intervals over a period of 3 hours, by which time only 30% of the potential thrombin activity of the zymogen was converted to thrombin. The mixture was then chromatographed on Bio-Rex 70 in a manner similar to that shown in Fig. 1A, and the fractions corresponding to the Peak II material were pooled and analyzed by SDS electrophoresis, the results of which are shown in Fig. 6, Gel A. Here again, the reduced proteins gave a pattern identical with that seen for citrate-derived thrombin (Fig. 2) as well as the thrombin which was generated from prothrombin by the snake venom preparation and then allowed to autolyze (Fig. 5, Gel d). The peak fraction taken from the gel pattern shown in Fig. 6, Gel A, had a relative specific activity of 1849 units per absorbance unit and consisted principally of the S-3 thrombin component with lesser amounts of the S-2 thrombin and S-1 peptide.

In other experiments it was of interest (to be discussed later) to determine if thrombins derived from purified prothrombin preparations of other species were similar to that found for human preparations. We therefore selected the cow and the horse because of previous work done on bovine materials (2-12) and the apparent physicochemical similarities between bovine and horse prothrombin of molecular weight 70,000 associated with the major intermediate of this preparation. From these gel patterns it is evident that upon activation, human prothrombin of molecular weight of 70,000 is composed of a single chain with NH₂-terminal alanine. Upon activation, NH₂-terminal serine, glutamate, threonine, isoleucine, and glycine appear (in order) in the system along with a number of fragments and intermediates, as well as thrombin. One of the major intermediates is a protein of molecular weight 20,000 to 60,000 seen in Fig. 5, Gels b and c, as well as other fragments. Because of the high anionic charge of some of these glycopeptides, they may be bound by SDS in an atypical manner (32), which can make interpretation of mobility and hence apparent molecular weight hazardous by this electrophoretic method.

In a subsequent manuscript (in preparation) we will report on the sequence of structural changes which takes place during the conversion of human prothrombin to thrombin by the various activation systems described in this paper. This sequence is similar, but not identical with that described by Mann et al. (5, 6) and by Stenn and Blout (7). Evidence will be presented to show that human prothrombin of molecular weight of 70,000 is composed of a single chain with NH₂-terminal alanine. Upon activation, NH₂-terminal serine, glutamate, threonine, isoleucine, and glycine appear (in order) in the system along with a number of fragments and intermediates, as well as thrombin. One of the major intermediates is a protein of molecular weight of 20,000 to 60,000 seen in Fig. 5, Gels b and c, as well as other fragments. Because of the high anionic charge of some of these glycopeptides, they may be bound by SDS in an atypical manner (32), which can make interpretation of mobility and hence apparent molecular weight hazardous by this electrophoretic method.
human prothrombins (19). Horse thrombin was selected because (a) its zymogen is about twice the molecular weight of both the human and bovine (39), (b) the equine enzyme has been reported to be a single molecule of molecular weight of approximately 35,000 (40), and (c) earlier work indicated a rather substantial difference among the specific activities of the prothrombins isolated from all three species (41). Fig. 6, Gel B, presents the SDS electrophoresis pattern of a typical bovine thrombin preparation isolated after citrate activation of bovine prothrombin by Bio-Rex 70 column chromatography (i.e. Peak II). Fig. 6, Gel C, is one experiment in which a horse thrombin preparation was isolated in a similar fashion. Comparison of all three gels shown in Fig. 6 (Gel A, human; Gel B, bovine; and Gel C, horse) indicate a considerable degree of similarity in the reduced thrombin peptides among all three species although there are substantial differences in their specific activities. It would thus appear that in these three mammals the mechanism of prothrombin activation and the subsequent generation of thrombin proceeds via proteolytic pathways which are very similar. The specific activities of these thrombin preparations, which are presented in Fig. 6, are relative to the bovine fibrinogen substrate used and are based on absorbance measurements. The extinction coefficients at 280 nm have been reported as 16.2 for human (42), 19.5 for bovine (43), and 15.2 for equine (40). In all probability these values were obtained on mixtures of multiple thrombin components.

**DISCUSSION**

From the results presented a model of the human thrombin molecule, consistent with the data obtained, can be constructed showing the relationship of the two thrombin heteromorphs, their apparent molecular weights, subunit structure, and NH\_2-terminal amino acids. This is presented schematically in Fig. 7. In some respects, this model is similar to that of Seegers et al. (8) who isolated by chromatography two active thrombin molecules from their bovine thrombin preparations. These had hydrodynamic molecular weights of 33,000 and 23,000, and both had NH\_2-terminal isoleucine and threonine. The smaller molecule, which was derived from the larger one due to the splitting off of a theoretical peptide of molecular weight about 10,000, had a thrombin specific activity about twice that of the parent enzyme. This doubling of specific activity of thrombin in spite of the fact that the molecule was reduced in molecular weight to only about one-third was believed to be due to the removal of the peptide which inhibits the clotting activity of the larger molecule (44). Both of the thrombin molecules, and presumably mixtures of them, have quite stable clotting activities in solution even at room temperature (45). Except for the obvious differences in molecular weights of the thrombins, our human preparations demonstrate similar behavior, although we did not detect a 2-fold difference in specific activity between the S-3 and S-2 forms of human thrombin. Our S-3 form of human thrombin was found to have specific activities of about 1565 units per absorbance unit, which is almost 98% of the theoretical value expected if prothrombin (mol wt 70,000, 1,154 units per absorbance unit, \( E_{100}^{\text{ex}} = 127.7 \)) were completely converted to a single thrombin molecule (mol wt 39,000, 1,154 units per absorbance unit, \( E_{100}^{\text{ex}} = 162.4 \)). Our S-2 form of human thrombin has a specific activity of about 2700 units per absorbance unit, or about 109% of the theoretical value calculated if the zymogen were completely converted to the small thrombin molecule (mol wt 26,000, 2469 units per absorbance unit, \( E_{100}^{\text{ex}} = 16.2 \)). Thus,
in contrast to the work reported for bovine thrombins by Seegers et al. (8), our results indicate only one-third increase in specific activity as the larger thrombin molecule is degraded to the smaller one.

The model presented in Fig. 7 is also similar to that proposed by Mann et al. (5, 6) for their bovine thrombin preparations except (a) they isolated a single chain (inactive) molecule which had the same molecular weight as their most active two-peptide chain thrombin molecule; (b) as their thrombin molecules autoxjyzed, the subunit alterations which occurred were accompanied by a substantial loss of thrombin-clotting activity but not es-
erase activity; and (c) they found an active three-peptide chain thrombin molecule. As noted earlier, Glover and Shaw (11) also isolated a three-peptide chain thrombin molecule from a commercial thrombin source but could not confirm the difference in fibrinogen-clotting activity between this and the two-chain molecule. Earlier work by Rosenberg and Waugh (12) showed that two and sometimes three electrophoretic forms of bovine thrombin could be derived from single animal plasmas with remarkable consistency of composition, equivalent specific activity, and clotting stability (46).

With respect to the current work, we could not identify an inactive thrombin precursor of molecular weight of about 40,000 in our venom enzyme activation mixtures (Fig. 5, Gel b and c). This is probably due to the rapidity of prothrombin conversion and the almost quantitative yield of thrombin in this system. This precursor, first observed by Seegers et al. (47), may exist in slowly converting citrate activation mixtures of prothrombin, however. Our principal concern in this work was that this intermediate was not part of our isolated thrombin preparations.

In contrast to the bovine thrombin work of Mann et al. (5, 6) and Glover and Shaw (11), we were not able to isolate or identify a three-chain polypeptide thrombin molecule that had active fibrinogen activity. Even after human thrombin preparations were allowed to remain on a laboratory bench for 7 days at room temperature (during which time the solution pH was unchanged), no evidence was obtained for this molecule by chromatography or electrophoresis of the system, and no thrombin activity was lost from the preparation. The reasons for this are not clear, since we also examined bovine and horse thrombin preparations (Fig. 6) and could find no subunit structure corresponding to a three-chain peptide. Most of the studies of other workers cited above used a commercial source of thrombin (Parke-Davis Thrombin, Topical) as starting material for their isolation procedure, although some of their work was done with purified bovine preparations which were activated to thrombin. It is possible that these products may have contained small amounts of other proteolytic enzymes, such as plasmin (EC 3.4.23.14) (see “Discussion” in Ref. 48). Our previous studies (17) and those of Magnusson (49) who has performed sequence studies with bovine thrombin and finds it to consist of only two peptide chains (2) has shown that plasmin does not exist in these preparations. It would thus seem to be of some importance to ex-
clude the presence of other proteolytic enzymes in bovine throm-
bin preparations which could produce alterations in the enzyme similar to those of the chymotrypsin-trypsin system (50). As another alternative, it should also be noted that bovine and human citrate-activated prothrombin will generate thrombin up to a maximum, after which the thrombin activity declines from the system (14, 15). It is possible that in concentrated citrate solution (which we have not as yet studied in detail) the three-peptide chain thrombin molecule appears during the loss of enzyme activity from the system. Although this is probably an artificial situation, it is of interest to pursue if only to ac-
count for the existence of this heteroform. Perhaps the re-
cent success of affinity chromatography of bovine thrombin by
Thompson and Davie (51) will settle this issue. Others have
claimed to have found a three-chain polypeptide thrombin mol-
ecule, but from the data presented (52) it is difficult to decide if
this molecule is active or represents a nonthrombin activation
fragment.

These results do not as yet shed light on the physiological
activation of human prothrombin or determine if the apparent
autolysis of isolated thrombin also occurs in human blood. Some
years ago we demonstrated that human thrombin was bound
to plasma α-2 macroglobulin (trypsin-binding macroglobulin)
(53), and this was later shown by Shapiro to be one of the im-
portant physiological mechanisms for the inactivation of throm-
bin by the serum antithrombins (54). The thrombin products
used at that time were probably similar to those of different
mixtures of the two enzyme forms identified in this study, both
of which seem to have been bound to the macroglobulin.

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Gerard F. Lanchantin, Jack A. Friedmann and Donald W. Hart

J. Biol. Chem. 1973, 248:5956-5966.

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