Multiple Active Forms of Thrombin

III. POLYPEPTIDE CHAIN LOCATION OF ACTIVE SITE SERINE AND CARBOHYDRATE*

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SUMMARY

The polypeptide chain location of active site serine and carbohydrate in the multiple forms of thrombin present in Parke-Davis thrombin have been investigated by means of [3H]diisopropylfluorophosphate incorporation and carbohydrate staining techniques. Both the sodium dodecyl sulfate electrophoretic technique and gel filtration in 6 M guanidinium chloride have been applied to the resolution of individual thrombin forms and their component chains.

The results of these studies indicate that the active site serine is contained in the 33,000-dalton chain of the large (39,000 dalton) thrombin, and in the 18,000- and 14,000-dalton chains of the smaller (28,000 dalton) thrombins. Carbohydrate analysis of the resolved thrombin components indicate that, although the large thrombin contains covalently attached carbohydrate, the smaller enzymes do not.

Multiple active forms of thrombin (IIa) have been observed in a number of laboratories (1–5). Two previous communications from this laboratory (6, 7) have clearly demonstrated that multiple forms of thrombin existed in both commercial "biothrombin" obtained from Parke-Davis and in preparations of the enzyme obtained by activation of the highly purified prothrombin in 25% sodium citrate-plasma. Three major forms of thrombin were observed which differed substantially in their relative activities toward fibrinogen, but which had identical specific activities toward the ester substrate Nα-tosyl-l-arginine methyl ester. Three IIa1 structures were proposed: (a) IIa-A,

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1 The abbreviations used are: IIa, thrombin; L-TAMe, Nα-tosyl-l-arginine methyl ester; DFP, diisopropylfluorophosphate; TLCK, Nα-tosyl-l-lysinechloromethyl ketone.

a 33,000-dalton chain disulfide linked to a 6,000-dalton chain; (b) IIa-B, an 18,000-dalton chain disulfide linked to a 10,000-dalton chain; (c) IIa-C, disulfide-linked chains with molecular weights of 14,000, 4,000, and 10,000. Further, activation studies conducted with biothrombin (6) and prothrombin (7) indicate that the small thrombins (IIa-B and IIa-C) are sequentially derived from IIa-A, which in turn was derived from a single chain 39,000-dalton precursor (7). Fig. 1 presents the tentative structures of the thrombins initially described in our laboratory (3) together with the designations we have previously applied to them (7). At a recent meeting (August 1972) of the committee on thrombin nomenclature of the Third Congress of the International Society on Thrombosis and Haemostasis, a nomenclature system, consistent with that applied to other serine proteases was devised. The new designations for the multiple thrombins and their respective polypeptide chains are presented in Fig. 1. These new designations are applied to specific thrombin structures and their chains in the remainder of this text.

Thrombin is a member of the family of proteolytic enzymes known as serine proteases (9) and possesses trypsin-like specificity (9, 10). Both the diisopropylfluorophosphate (4, 11) and Nα-tosyl-l-lysinechloromethyl ketone (TLCK) (12, 13) incorporation into the protein have been studied, and the peptides into which these labels are incorporated have been identified. Variability in the size of the molecular species into which both the diisopropylfluorophosphate (4) and TLCK (13) is incorporated has been noted, and this is most readily explained on the basis of a variety of thrombin structures present in the respective preparation used for these investigations.

The precursor of thrombin, prothrombin (II), is a glycoprotein (14), and thrombin also contains carbohydrate (14). Some preparations, however (4), have been shown to be devoid of carbohydrate. This variability may also be due to the relative composition of thrombin forms in any given preparation.

It was felt in this laboratory that the determination of the polypeptide chain locations of active site serine and carbohydrate in the multiple active forms of thrombin would provide useful structural information relevant to the origin of the polypeptide chains in thrombin, as well as a means of testing the structures for thrombin proposed in this laboratory (6) and others (8, 13).

EXPERIMENTAL PROCEDURES

Bovine "biothrombin" was obtained from Parke-Davis. Fibrinogen (85% clottable) was from the Sigma Chemical Com-
Thrombin was purified by the method of Lundblad (15) and assayed for clotting activity by using the procedure described previously (6). The preparations used in these studies contained on the average 2200 NIH units per mg. [3H]Diisopropylfluorophosphat (3.3 Ci per mmole) was obtained as a 1.5 mCi solution in ethylene glycol from Amersham-Searle, Arlington Heights, Ill. Sodium dodecyl sulfate was obtained from the Schwarz-Mann Chemical Company, St. Louis, Mo. [3H]Diisopropylfluorophosphate (3.3 Ci per mmole) was diluted with cold (5.44 M) diisopropylfluorophosphate to provide a stock solution (0.1 M) with a specific activity of 50 mCi per mmole. For most studies, the batch eluted enzyme from the sulfoethyl-Sepharose column (in 0.25 M sodium phosphate, pH 6.5) was pooled, and sufficient stock [3H]diisopropylfluorophosphate was added to bring the solution to $5 \times 10^{-4}$ m in this reagent. The solution was then adjusted to pH 8.1 with 1 M NaOH, and a second addition of [3H]diisopropylfluorophosphate was made to bring the final concentration to $10^{-4}$ m in diisopropylfluorophosphate. This treatment eliminated all enzymatic activity. The labeled protein was then removed from excess reagent by either precipitation in trichloroacetic acid or by dialysis versus 0.2 M acetic acid. These reagents were removed by methanol followed by ether extraction and by lyophilization, respectively.

Separation of Thrombins and Component Chains—The multiple thrombin components were separated with disulfide bonds intact by means of both sodium dodecyl sulfate electrophoresis (16) and by gel filtration on 6% agarose in 6 M guanidinium chloride (17). Both methods were also applied to the resolution of constituent thrombin polypeptide chains following reduction of disulfide bonds with 0.1 M 2-mercaptopropanol and, in the case of the agarose gel filtration procedure, S-carboxymethylation. Sodium dodecyl sulfate gels were stained with Coomassie blue; after destaining, they were scanned with a Gilford 2410 spectrophotometer equipped with a model 2410 gel scanner and an integrating Barber Coleman recorder. Depending on protein loading of the gel, scans were performed at either 600 or 650 nm. Guanidinium chloride columns were monitored spectrophotometrically by using an Isco UA-4 monitor equipped with a heat recorder.

Fluorescent modification of the protein for analysis on the guanidinium chloride column was performed with either fluorescein isothiocyanate or rhodamine B by the methods of Mann and Fish (18) and Mann et al. (19). Relative fluorescence was measured for column fractions by using an Aminco fluorometer equipped with the appropriate filters.

Detection of Radioactive Components—Radiolabeled protein

\[
\begin{align*}
\text{OLD DESIGNATION}^{(a)} & & \text{PROPOSED STRUCTURE} & & \text{NEW DESIGNATION}^{(b)} \\
\ IIa-A & & \begin{array}{c}
\text{NH}_2 \\
3,000 \text{ daltons}
\end{array} & & \alpha \text{ Thrombin}^{(c)} \\
\ IIa-B & & \begin{array}{c}
\text{B}_1 \\
10,000 \text{ daltons}
\end{array} & & \beta \text{ Thrombin}^{(d)} \\
\ IIa-C & & \begin{array}{c}
\text{B}_3 \\
4,000 \text{ daltons}
\end{array} & & \gamma \text{ Thrombin}^{(e)}
\end{align*}
\]

Fig. 1. Tentative structures for the major thrombin species. (a) The designations IIa-A, IIa-B, IIa-C were those used previously to describe the thrombins isolated in this laboratory (see Reference 7). (b) The new designations, $\alpha$, $\beta$, $\gamma$, are those agreed to by the Committee on Nomenclature of the Third Congress of the International Society on Thrombosis and Haemostasis. (c) The structure designated $\alpha$ Thrombin is equivalent to that reported by Magnusson (see Reference 14). (d) The new designations $\alpha$, $\beta$, $\gamma$, are those agreed to by Magnusson (see Reference 14). (e) The structure designated $\alpha$ Thrombin is probably similar to the variant reported by Glover and Shaw (see Reference 13). The chain identifications are a reflection of the work presented in this article and that of Glover and Shaw. (e) It is apparent from our previous studies and those reported here that the chains designated $B_1$ and $B_2$ are derived from the $B_1$ chain of $\beta$-thrombin. However, it is not at present known which of these chains is linked to the $B_1$ chain, or whether $B_3$ or $B_4$ is the NH$_2$-terminal fragment of the $B_3$ chain.

Methods

Purification and [3H]Diisopropylfluorophosphate Labeling—Thrombin was purified by the method of Lundblad (15) and assayed for clotting activity by using the procedure described previously (6). The preparations used in these studies contained on the average 2200 NIH units per mg. [3H]Diisopropylfluorophosphat (3.3 Ci per mmole) was diluted with cold (5.44 M) diisopropylfluorophosphate to provide a stock solution (0.1 M) with a specific activity of 50 mCi per mmole. For most studies, the batch eluted enzyme from the sulfoethyl-Sepharose C-50 was from Bio-Rad Laboratories. All other reagents were of the best quality available commercially.

\[
\begin{align*}
\text{OLD DESIGNATION}^{(a)} & & \text{PROPOSED STRUCTURE} & & \text{NEW DESIGNATION}^{(b)} \\
\ IIa-A & & \begin{array}{c}
\text{NH}_2 \\
6,000 \text{ daltons}
\end{array} & & \alpha \text{ Thrombin}^{(c)} \\
& & \begin{array}{c}
\text{B}_2 \\
33,000 \text{ daltons}
\end{array} & & \\
\ IIa-B & & \begin{array}{c}
\text{NH}_2 \\
10,000 \text{ daltons}
\end{array} & & \beta \text{ Thrombin}^{(d)} \\
& & \begin{array}{c}
\text{B}_2 \\
18,000 \text{ daltons}
\end{array} & & \\
\ IIa-C & & \begin{array}{c}
\text{NH}_2 \\
10,000 \text{ daltons}
\end{array} & & \gamma \text{ Thrombin}^{(e)} \\
& & \begin{array}{c}
\text{B}_3 \\
4,000 \text{ daltons}
\end{array} & & \\
& & \begin{array}{c}
\text{B}_4 \\
14,000 \text{ daltons}
\end{array} & & 
\end{align*}
\]
in Coomassie blue-stained sodium dodecyl sulfate-acrylamide gels were determined as follows. After scanning, the gels were frozen on a block of Dry Ice covered with aluminum foil. The frozen gels (~10 cm in length) were then sectioned into about 100 lateral slices by using a homemade device composed of stainless steel razor blades. The slices were then teased from between the razor blades and deposited into glass scintillation vials (two to four slices per vial). To each vial was then added 1 ml of 6 mM guanidinium chloride, and the vials were capped and incubated for 12 hours at 90°C. This procedure solubilized the stained protein in the gel slice providing an equilibrium distribution of soluble protein between the aqueous phase and the free volume of the gel slice. Since the ratio of total aqueous volume to total gel slice volume was approximately 9 to 1, greater than 90% of the protein was obtained in a soluble phase in this fashion. At this point, 15 ml of a scintillation mixture consisting of 1,4-bis[2-(5-phenyloxazoyllbenzene (POPOP) (0.1 g), 2,5-diphenyl oxazole (PPO) (5.0 g), toluene (667 ml), and Triton X-100 (333 ml) were added. The vials were then counted in either a Packard or Nuclear-Chicago scintillation counter. This procedure provides approximately 12% counting efficiency as judged by internal 3H standardization. The efficiency is not affected by the stain.

Aliquots (0.1 ml) of the fractions collected from the gel filtration experiments were counted in the same scintillation mixture used for the sodium dodecyl sulfate electrophoresis gel.

Carbohydrate Location Identification—The locations of carbohydrate in the thrombins and their component chains were determined in sodium dodecyl sulfate gels using the Schiff-periodate method described by Fairbanks et al. (20). Approximately a 5-fold (~50 μg) increase in applied protein was required for carbohydrate staining. The Schiff-periodate-stained gels were scanned at 560 nm and then counterstained with Coomassie blue. Owing to the extinction of the stained protein, scanning of the counterstained gels was conducted at 650 nm.

RESULTS

[3H]Diisopropylfluorophosphate Incorporation

Electrophoretic Studies—Most of the studies reported in this communication were conducted with thrombin preparations which contained little or none of the 14,000 molecular weight chain. Data is therefore rather limited with respect to the proposed γ-thrombin. Our most extensive studies have dealt with thrombin samples which contained those polypeptide chains proposed to be associated with α- and β-thrombin.

Fig. 2 presents a photograph of the sodium dodecyl gel electrophoretograms of the thrombin preparations used for the majority of the work reported in this study. The primary difference between the gel patterns depicted in Fig. 2 and those previously reported (6) is the absence in the reduced gels of the 14,000-dalton chain. The major components in the nonreduced gels correspond to the 39,000- and 28,000-dalton thrombin components. Analysis of the reduced gels reveals components with molecular weights of 33,000 ± 3,000, 18,000 ± 2,000, and 10,000 ± 1,000.

The gels depicted in Fig. 2 were scanned and sliced, and the slices were counted for 3H (see "Methods"). The results of this experiment in terms of 3H counts per min and protein-bound dye absorbance is seen in Fig. 3, A and B.

The observed apparent molecular weights are 39,000 and 28,000. The apparent discrepancy is due to the presence of disulfide bonds. (See Reference 5.)

Fig. 3A demonstrates that both thrombin component bands are labeled. In addition, observation of Fig. 3A reveals that in addition to the two major protein bands (α and β), which correspond to the 39,000- and 28,000-dalton thrombins, respectively, a minor band (designated X) is also present. This component is present to a varying degree in all of our preparations; in previous work it was regarded as an impurity, since it accounts for, at best, about 4% of the total stained material in the gel. The significance of this component is discussed in the section dealing with carbohydrate location.

Fig. 3B presents the dye staining and 3H incorporation data for the same sample depicted in Fig. 3A, but with disulfide bonds reduced. Previous studies (6) have shown that the component

![Fig. 2. Sodium dodecyl sulfate gel electrophoretograms of the thrombin preparations used for the bulk of the studies reported in this article. The electrophoretograms labeled 2 and 4 represent the samples with disulfides intact, whereas those labeled 1 and 3 represent the identical samples with disulfides reduced.](image-url)
designated B arises from the larger, \( \alpha \)-thrombin (30,000 dalton), whereas those labeled \( B_1 \) and \( B_2 \) arise from the 28,000-dalton thrombin (\( \beta \)-thrombin). These results clearly show that the site of diisopropylfluorophosphate sensitivity in \( \alpha \)-thrombin is the 33,000-dalton chain, whereas in \( \beta \)-thrombin the active site serine is located in the 18,000-dalton chain (\( \delta \)).

The integrated areas of the stained protein components in Fig. 3, A and B were compared as follows. Since dye staining intensity is a fairly constant concentration-dependent function for any given component, the relative concentration of each component in a given gel can be compared. The relative areas in the unreduced sample of Peak \( \alpha \) to Peak \( \beta \) in the scan depicted in Fig. 3A is 59 to 41\%. Similarly, for the reduced sample (Fig. 3B), the area of Peak B corrected for the loss of the 6,000-dalton peptide (A chain) which is not fixed by the stain, relative to the sum of the components \( B_1 \) and \( B_2 \) is 58 to 42\%. Similarly, the ratios of \( ^3 \text{H} \) activity in the reduced and unreduced gels can be compared. The ratio of total radioactivity in the peak designated \( \alpha \) in Fig. 3B to that of Peak B in Fig. 3B is 2.3, whereas the ratio for Peak \( \beta \) (Fig. 3A) to Peak \( B_2 \) (Fig. 3B) is 2.1. These data strongly suggest, relatively speaking, the following. (a) Peaks \( B_1 \) and \( B_2 \) of Fig. 3B are derived from Peak \( \beta \) of Fig. 3A. (b) Peak B of Fig. 3B is derived from Peak \( \alpha \) of Fig. 3A. (c) The radioactive label incorporated in Peaks \( \alpha \) and \( \beta \) of Fig. 3A is totally contained in the component chains \( B \) and \( B_2 \), respectively, after reduction of the disulfide bridges. This relative method of comparison has been chosen since it does not rely at all on consistency of sample handling.

Similar studies were conducted in a semiquantitative fashion with a sample of thrombin which contained substantial quantities of the 14,000-dalton chain. Fig. 4 presents the data for a reduced sodium dodecyl sulfate electrophoretogram of this sample. These results clearly establish that, when present in a preparation, the 14,000-dalton chain contains the site of diisopropylfluorophosphate incorporation, indicating that \( \gamma \)-thrombin contains the active site serine in the 14,000-dalton chain. Unfortunately, these studies were conducted at a time when a great deal of effort was not expended in order to obtain quantitative relationships between label incorporation and protein concentration.

Gel Filtration Studies—Quantitative studies of label incorporation were made by means of gel filtration in 6 M guanidinium chloride of both the reduced and unreduced thrombin preparations. Gel filtration studies of cross-linked random coils (disulfide bonds intact) provide a means of resolution of thrombin components (6) but does not permit valid molecular weight evaluation (17, 18).

Gel filtration studies of reduced proteins in 6 M guanidinium chloride has been shown (17-19) to provide valid molecular weight estimates for polypeptide chains. In this solvent, reduced polypeptide chains behave as linear random coils (21) possessing no residual noncovalent structure. The molecular radius of a linear random coil is a function of the number of...
Pseudomolar specific activities were computed by using the equation

\[ SA' = \frac{cpm}{A_{280}} \times M \]

where \( SA' \) is the pseudomolar specific activity, \( cpm \) is the integral of the radioactivity curve, \( A_{280} \) is the integral of the protein absorbance at 280 nm, and \( M \) is the respective molecular weight of the component. Previous studies (3, 6) have provided molecular weights of 39,000 and 28,000 for these components.

By using this approach, values of \( 2.29 \times 10^4 \) cpm per “mole” \(^2\) for Component 1 and \( 2.03 \times 10^4 \) cpm per “mole” \(^2\) for Component 2 were obtained. Thus on a relative basis, given the assumption of equivalent extinction coefficients, Components 1 (α-thrombin) and 2 (β-thrombin) have roughly the same extent of \([3H]diisopropyfluorophosphate incorporation on a pseudomolar basis.

In a similar experiment to that represented in Fig. 5 conducted with unlabeled protein, it was observed that the fractions represented by Peaks α and β were active in the fibrinogen clotting assay following their renaturation in 0.01 M imidazole, 0.1 M NaCl (pH 7.4), containing 1% CaCl\(_2\). In these experiments, approximately 30% of the original specific activity of the thrombin was regained.

Fig. 6 presents the elution profile obtained on gel filtration of the identical sample after reduction and S-carboxymethylation of the protein. The assignment of the peak identity correlation is based on our previous work (3, 6) and the sodium dodecyl sulfate data presented earlier. The determined molecular weights in this experiment are as follows: \( B_1, 30,000; B_3, 20,000; B_4, 9,500; A, 5,700; Y, 4,500. \) Only two components contain the \([3H]diisopropyfluorophosphate label, B_1 and B_3; a result predicted by the sodium dodecyl sulfate studies presented in an earlier section of this report. For the purposes of calculation of pseudomolar specific activities, the assumption of equivalent extinction coefficients for all species was again applied to Component B. The value obtained, assuming that the extinction coefficient of the 33,000-dalton chain is about the same as that for α-thrombin and β-thrombin, is \( 2.24 \times 10^4 \) cpm per “mole.” Although this assumption is reasonable for Component B, inspection of Fig. 6 indicates that the assumption of equivalent extinction coefficients is not valid for Components \( B_1 \) and \( B_2. \)

\(^2\) The term “mole” is used since these comparative calculations use an extinction coefficient of unity for all components.
However, if one assumes that 1 mole of \( \beta \)-thrombin will yield 1 mole each of \( \beta_1 \) and \( \beta_2 \), the ratio of the areas of \( \beta_1 \) to the sum of \( \beta_1 + \beta_2 \) should yield the appropriate extinction coefficient correction. The molar extinction coefficient ratio of \( \beta \)-thrombin to \( \beta_2 \) obtained in this fashion is 1.26. By using this correction factor and Equation 1, the pseudomolar extinction coefficient for \( \beta_2 \) becomes 2.25 \( \times 10^4 \) cm per “mole.”

At the present time, we are not certain what becomes of the absorbance at 280 nm of the component designated \( X \) (Fig. 5) following its reduction and S-carboxymethylation. Prior to reduction and S-carboxymethylation, \( X \) elutes at a position consistent with an apparent molecular weight of 8500 (Fig. 5). Following reduction and S-carboxymethylation of the total sample (Fig. 6), only a single symmetrical peak \( (B_2) \) is observed in the region near that where \( X \) was eluted. Were \( X \) (in terms of absorbance at 280 nm) still present at its original position, it would most certainly have been detected. Preliminary studies have been conducted in order to determine what becomes of \( X \) following reduction and S-carboxymethylation. The fractions containing \( X \) (Fig. 5) were pooled, labeled with fluorescein isothiocyanate, reduced, S-carboxymethylated, and rechromatographed. Fluorescent components with apparent molecular weights of 8500 and 5000 were detected.

The data of Figs. 5 and 6 were also compared based on the assumption that the sums of the absorbances of component chains (with disulfides reduced) should be equivalent to that of the parent, disulfide bridged component. The ratio of the sum of reduced component chain absorbance at 280 nm (Fig. 6) to that of the parent component (Fig. 5) should be equivalent to the ratio of the quantity of sample applied to the column operated with disulfides reduced (Fig. 6) to that of the column operated with disulfides intact (Fig. 5). The ratio of the sum of \( B \) and \( A \) (Fig. 6) to \( \alpha \) (Fig. 5) is 1.21, whereas the ratio of the sum of \( B_1 \) and \( B_2 \) (Fig. 6) to \( \beta \) (Fig. 5) is 1.20. A similar comparison of the protein-bound \( \Phi \) for the reduced system (Fig. 6) to that for the unreduced system (Fig. 5) gives a ratio value of 1.25. These ratios indicate that, on the average, 22% more protein was applied to the column operated with the reduced protein. If the integrated area for Component \( X \) observed in the unreduced sample is multiplied by 1.22, the area value determined is essentially equivalent to that for Component \( Y \) observed in the column profile for the reduced sample. These data, when coupled with that observed for the fluorescein-labeled, reduced carboxymethylated \( X \), suggest that the portion of Component \( X \) responsible for its absorbance at 280 nm is equivalent to Component \( Y \) in the reduced column. This analysis, however, is speculative in nature, and a complete identification of the nature of Component \( X \) must await the isolation of more substantial quantities of this material.

Although previous results have indicated that the components labeled \( B_1 \) and \( B_2 \), and \( A \) and \( \beta \), in the elution profile presented in Fig. 6, are derived from \( \beta \)-thrombin and \( \alpha \)-thrombin, respectively, it was decided to test this hypothesis directly within the experimental system described here. The fractions from the column operated with the unreduced protein (Fig. 5) were pooled so as to obtain enriched levels of the partially resolved Components \( \alpha \) (Fractions 56 to 62) and \( \beta \) (Fractions 67 to 73). The fractions containing Component \( \alpha \) were labeled with fluorescein isothiocyanate, whereas those containing the Component \( \beta \) pool were labeled with rhodamine \( B \). Following removal of most of the excess fluorescent reagent by dialysis, the pools were combined, reduced, S-carboxymethylated, and reappplied to the 6% agarose column. The result of this experiment is presented in Fig. 7. These results clearly indicate that Components \( B_1 \) and \( B_2 \) have been correctly assigned to Peak \( \beta \) and that Components \( A \) and \( B \) (Fig. 6) are derived from Component \( \alpha \) of Fig. 5.

The results for the elution profiles presented in Figs. 5 to 7 are summarized in Table I. The values for molecular weight are in good agreement with those reported earlier by using the same technique (3). Also, the sum of the molecular weights of Components \( B_1 \) and \( B_2 \) is equivalent, within experimental error, to the previously reported sedimentation equilibrium value for the molecular weight of the \( \beta \)-thrombin with disulfide bonds intact in the same solvent (3). Similarly, the sum of the molecular weights of \( A \) and \( \beta \) is equivalent, within experimental error, to that determined for \( \alpha \)-thrombin by sedimentation equilibrium in 6 M guanidinium chloride (4, 6).

**Carbohydrate Location**—The location of the carbohydrate on the various species of thrombin and their component chains was determined by Schiff-periodate staining of the resolved components in sodium dodecyl sulfate gels. It was during these studies that the nature of the component designated \( X \) in Figs. 3 and 5 became of interest.

Fig. 8, \( A \) and \( B \) presents the absorbance scans for the unreduced and reduced thrombin preparation in terms of absorbance at 280 nm of bound Schiff reagent and absorbance at 650 nm for Coomassie blue (see “Methods”).

Examination of Fig. 8A reveals three interesting features.
Fig. 8A, absorbance scans of an electrophoretogram prepared with nonreduced thrombin (Gel 2 of Fig. 2). Absorbance at 650 nm (Coomassie blue, ) and 560 nm (Schiff reagent, ) are plotted as a function of position (cm). B, absorbance scans of an electrophoretogram prepared with reduced thrombin (Gel 1 of Fig. 2). Absorbance at 650 nm (Coomassie blue, ) and 560 nm (Schiff reagent, ) are plotted as a function of position (cm).

(a) α-Thrombin contains covalently bound carbohydrate; (b) β-thrombin possesses no covalently bound carbohydrate; (c) Component X bears nearly an equivalent amount of carbohydrate relative to Component β (β-thrombin), as there is carbohydrate in Component α (α-thrombin). The values for the ratios of carbohydrate stain to Coomassie blue for Component α is 1.59, whereas that of carbohydrate in X to Coomassie blue in β is 1.10. Similar examinations of Fig. 8B (disulfide bonds reduced) indicates that the carbohydrate of α-thrombin is covalently linked to the large chain, whereas the mobility of the carbohydrate-containing component (X) is unchanged by reducing conditions. The apparent molecular weight of Component X in sodium dodecyl sulfate gels is 14,000. The coidentity of X in Fig. 8A and Component X in Fig. 5 has been established by sodium dodecyl sulfate electrophoresis of the component isolated by gel filtration. Gel filtration studies of Component X following reduction and rechromatography on 10% agarose gives components with apparent molecular weights of 8,500 and 5,000. Neither technique, of course, provides a valid molecular weight estimate since examination of Fig. 8B suggests X is primarily composed of carbohydrate. If a comparative analysis is conducted, the ratio of carbohydrate stain to Coomassie blue in X (Fig. 8A) is 13.3. The α-thrombin band in the same gel (a) has a ratio of carbohydrate to Coomassie blue of about 1.59. Since thrombin is thought to be about 5% carbohydrate by weight, the ratio of staining for X would suggest that this component is about 42% carbohydrate by weight.

The identification of this material (X) is most interesting since it appears to represent the glycopeptide portion of α-thrombin which is split off in the formation of β-thrombin. Although this conclusion is somewhat speculative, it is supported by three observations. (a) The ratio of carbohydrate in X (Schiff stain) to protein (Coomassie blue) in β (Fig. 8A) approaches the value of this ratio for Component α. (b) The conversion of α to β occurs with total loss of covalently bound carbohydrate. (c) The relative amount of Component X in this preparation is somewhat greater than usually observed, and this factor appears to coincide with the absence of the 14,000-dalton chain associated with γ-thrombin. Since γ-thrombin is a degradation product of β-thrombin, this factor suggests that the enzyme preparation studied is rather fortuitous in that it represents an early stage of degradation of the enzyme. Such a system should possess a relatively greater amount of noncovalently associated cleavage fragment.

The renaturation studies conducted with the equivalent of Component β from gel filtration in 6 M guanidinium chloride (Fig. 5) indicates that Component X is not required for the activity of β-thrombin.

DISCUSSION

The primary conclusions which can be drawn from our data are depicted graphically in Fig. 9. The results presented here provide support for the hypothetical thrombin structures previously suggested by our laboratory. The [3H]diisopropylfluorophosphate labeling studies clearly show that the active site serine is located in the 33,000-dalton chain of the largest throm-
bin (α-thrombin), a result fully consistent with the tentative sequence for this molecule proposed by Hartley (8). In the partially degraded β-thrombin, the active site serine is located in the 18,000-dalton chain (B2), whereas in γ-thrombin the 14,000-dalton chain (B3) bears the serine 195 equivalent of chymotrypsin. Since previous studies (6) have shown that the smaller thrombin components are derived from α-thrombin and in view of the homology to chymotrypsin, it is apparent that the 18,000-dalton (B2) and 14,000-dalton (B3) chains must be derived from the COOH-terminal portions of the large chain of α-thrombin.

The carbohydrate data demonstrate that as expected (8) the large chain of α-thrombin contains a covalently bound carbohydrate moiety. These data further show that in the transition from α-thrombin to the 28,000-dalton β-thrombin, the glycopeptide bearing the carbohydrate is excised from the large (B) chain of α-thrombin. Although somewhat speculative, the available evidence suggests that this glycopeptide fragment remains noncovalently associated to some extent to β-thrombin. Renaturation studies of the resolved covalently associated components of β-thrombin indicate that the glycopeptide is not essential to the clotting activity of β-thrombin.

The covalent structure of β-thrombin indicates that it is composed of two disulfide-linked chains. If one assumes a mean amino acid residue weight of 110 g per residue, there would be about 91 ± 9 residues in the smaller chain (B1) (10,000 ± 1,000 daltons) and about 164 ± 16 residues in the active site serine containing large chain (B2) (18,000 ± 2,000 daltons). This would predict that since the equivalent of the chymotrypsin serine 195 is in the large chain (B2), the equivalent of the chymotrypsin histidine 57 would be located in the smaller chain (B1). Glover and Shaw (13) have in fact observed in their studies of TLCK incorporation into the active site histidine of thrombin a small chain present in various amounts did not incorporate the label. Their composition studies suggested that this small chain is composed of residues 1 to 73 (or 76) from the NH2 terminus of the large (B) chain of α-thrombin. This chain was observed to be disulfide bridged to a larger chain. The chain length for the active site histidine containing chain reported by Glover and Shaw and its disulfide attachment to a larger chain suggest that the component they described is equivalent to our β-thrombin, 10,000-dalton chain (B1); a possibility already suggested by these authors.

The tentative sequence proposed by Hartley for thrombin (8) corresponds very closely to the structure we designate α-thrombin. It is apparent that the β-thrombin chains are derived from the large (B) chain of α-thrombin (the B chain of Magnusson and Hartley). It is further apparent from our results and those of Glover and Shaw (13) that the site of cleavage may be near residue 80, and that the resulting fragment chains (B1 and B2) of β-thrombin are disulfide bridged. Further, the transition of α-thrombin to β-thrombin results in the loss of both the covalently bound carbohydrate and the small (A) chain of α-thrombin (the A chain of Magnusson and Hartley). These results are inconsistent with several of the features of the proposed sequence for the following reasons. (a) The sequence proposed by Magnusson indicates about 260 residues in the large (B) chain (14), with attachment of the carbohydrate somewhere between residues 58 and 69, whereas the site of the disulfide bridge between the large and small chains is somewhere between residues 111 and 135 (8). Excision of either the glycopeptide, or the bridging cysteine from the large chain would result in fragments much smaller than those observed in β-thrombin. Excision of both components would result in three chains, all of which would be smaller than those observed for β-thrombin. (b) A second difficulty lies in the placement of the intrachain disulfide bonds in the Hartley (8) model. No intrachain disulfide bond exists in the region of residues 80 to 112 which would account for the disulfide link observed between the 10,000- and 18,000-dalton chains of β-thrombin.

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