Interaction of Soybean Trypsin Inhibitor with Thrombin and Its Effect on Prothrombin Activation*

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SUMMARY

Because of its importance to an understanding of the mechanisms involved in the conversion of prothrombin to thrombin, the inhibitory properties of soybean trypsin inhibitor on this system were investigated.

Kinetic studies showed that the inhibitor participates in a bimolecular reversible reaction with one of the components of the prothrombin-thrombin conversion system and to have an association constant dependent upon the rate of prothrombin conversion. With the use of three different systems for the activation of prothrombin, soybean trypsin inhibitor was found to block the conversion of the zymogen at two stages in the activation sequence. The first stage occurs during the initial peptide bond-breaking step which produces a polymorphic prothrombin intermediate plus a peptide fragment. The second point of inhibition occurs when this intermediate is split into smaller molecules, one of which has thrombin activity. Indirect evidence suggests that the proteolytic activity of thrombin is responsible for these initial events and that some of the bonds normally broken at these two stages are similar to bonds which have been implicated with the reactive site of soybean trypsin inhibitor.

By the use of gel filtration columns equilibrated with buffers containing soybean trypsin inhibitor, it is possible to show that human or bovine thrombin (mol wt approximately 30,000 g) combines with soybean trypsin inhibitor (mol wt approximately 20,000 g) to form a mole for mole dissociable enzyme-inhibitor complex (mol wt approximately 50,000 g). This was confirmed by ultracentrifuge analysis. The complex thus formed is enzymatically active against fibrinogen (Arg-Gly bonds) and synthetic ester substrates but its formation is not affected when these catalytic properties are inhibited by sulfonation or alkylation of the residues at the active site of thrombin. Because of this, it is suggested that the mechanism of inhibition may be associated with alterations of secondary binding sites on the enzyme or with steric effects.

In spite of extensive studies of the interaction of the Kunitz soybean trypsin inhibitor with various proteolytic enzymes (1-7) the mechanism whereby soybean trypsin inhibitor delays the coagulation of blood, or more specifically, the conversion of prothrombin to thrombin (EC 3.4.4.13) remains obscure. In 1961, Glendening and Page (8) reviewed some of the early work concerning this problem and provided evidence that STI either combines with prothrombin to form a highly dissociable complex or, more probably, with an intermediate in the prothrombin-thrombin transition. Following this, Alkjaersig, Deutsch, and Seegers (9), with a different experimental approach, found that STI prevents the generation of thrombin activity when prothrombin is activated in spite of the fact that intermediates of the reaction are readily formed. Because of this and the stoichiometry of the system used, they did not feel that a dissociable complex between intermediate and STI seemed likely. This was supported to some extent by the very careful kinetic studies of Shulman and Heerson (10), who found that the rate of thrombin formation from the hypothetical intermediate to be dependent on alterations of secondary binding sites on the enzyme or with steric effects.

With other experiments and for reasons which are discussed, these results cannot be explained on the basis of Factor X contamination of the thrombin preparations used. Although these studies do not dispute the possible interaction of soybean trypsin inhibitor with preparations having Factor X activity, they do not support the view that the inhibitor combines with intermediates or derivatives of the prothrombin-thrombin transition. Rather, these studies indicate that soybean trypsin inhibitor interacts with thrombin to block its specific proteolytic capacity to initiate prothrombin activation.

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upon the rate of prothrombin activation and to compete with 
the rate of formation of an essentially irreversible intermediate-
STI complex. Recently, this STI-sensitive intermediate has been 
identified by Kipfer and Seegers (11) as autoprothrombin C, a 
derivative of the prothrombin molecule which is essential 
for the conversion of the zymogen to thrombin (12). However, 
others believe that autoprothrombin C is identical with Factor 
Xa (thrombokinase, activated Stuart Factor, etc.), a protein 
whose genetic, biological, and immunological properties are 
independent from that of prothrombin and whose blood-clotting 
activity is inhibited by complex formation with STI (13-17). 
Thus far, to our knowledge, no complex of STI with a prothrom-
bin intermediate (or derivative) or a blood-clotting factor has 
ever been isolated, and, apart from kinetic studies, no other 
evidence has been presented that such a complex exists.

From the above studies, there is also no evidence that STI 
inhibits the clotting (proteolytic) or esterolytic activity of 
thrombin. This seems to be unique because thrombin has a 
number of physical and kinetic properties similar to trypsin (18), 
chymotrypsin (7, 18), and coconase (4), whose activities are 
inhibited by the soybean protein (1, 4, 7) and because STI, al-
though capable of combining with functionally inactive enzymes 
(5), is not known to join in complex with zymogens or inter-
mediates between zymogens and active enzymes. In addition, 
thrombin, like trypsin, hydrolyzes trypsin-sensitive bonds (Arg-
Ile or Lys-Ile, or both), in trypsinogen and chymotrypsinogen 
A (19) and these bonds are also similar to those which presumably 
must be hydrolyzed for the conversion of prothrombin to throm-
bin (20) and which have been implicated with the reactive site of 
STI itself (2).

On the basis of these observations, we, therefore, decided to 
re-examine this problem which is of considerable importance to 
an understanding of the nature of the activation of prothrom-
bin.

EXPERIMENTAL PROCEDURE

Materials

Prothrombin was isolated from human plasma by a method 
previously described (21) and, in some cases, was further purified 
by DEAE-cellulose chromatography (22). The products thus 
obtained have high relative specific activities, are homogeneous,
and have constant amino acid composition. In addition, the 
physical and biochemical purity of these products are given 
in limits, by DEAE-cellulose column chromatography, disc 
and immunoelectrophoresis, and sedimentation velocity analysis, 
and have constant amino acid composition. In addition, the 
isolation procedure does not yield significant amounts of protein 
when applied to serum or the plasma of a patient congenitally 
deficient in prothrombin activity. Further details concerning 
the physical and biochemical purity of these products are given 
elsewhere (21-23, 27, 28). Human thrombin was prepared from 
prothrombin either by activation in 25% (w/v) sodium citrate 
solution (27) or by biological activators (i.e. two-stage method) 
(25-29) and the enzyme was isolated from these mixtures by ion 
exchange chromatography (28). A similar method was used to 
purify bovine thrombin from a commercial source (Parke Davis) 
(29). The human thrombin preparations thus obtained had 
negligible activity against casein (30) and lysine ethyl ester (29) 
substrates and other properties quite different from plasmin and 
trypsin (29).

A 3-mg preparation of Factor X which had been isolated from 
8 liters of human plasma by hydroxyapatite chromatography 
and preparative disc electrophoresis (31) was a gift from Dr. 
David L. Aronson of the National Institutes of Health. Prior 
to freeze-drying and shipment in Dry Ice, it had a Factor X 
specific activity (Denson method) of 1709 clotting units mg⁻¹ 
of protein (31) in which 100 units are arbitrarily defined as the 
clotting factor activity found in 1 ml of freshly collected normal 
plasma. This represents a purification of approximately 
1690 times. After receipt in this laboratory, the dry powder had 
a Factor X specific activity of 1680 units mg⁻¹, with the assay 
method of Hougie (32), and no prothrombin- or thrombin-clot-
ting activity could be detected in the preparation.

Part of the Factor X preparation was converted to its active 
form (Factor Xa) by dissolving it in 25% sodium citrate solution 
and allowing it to stand for 16 hours at 24°C in a manner similar 
to that used for the citrate activation of prothrombin (27). The 
Factor X preparation before activation had no detectable TAME 
esterase activity (less than 0.1 unit mg⁻¹) while following 
activation it had approximately 300 TAME esterase units mg⁻¹ 
and negligible but detectable thrombin-cotting activity (2 μg 
of clotted fibrinogen in 20 min). Additional details concerning the 
electrophoretic patterns and other properties of these prepara-
tions are presented in Fig. 7 and under "Results."

Soybean trypsin inhibitor (three times crystallized, Lot 5493 
and 5494) and trypsin (crystalline, salt-free, Lot N1210) were 
purchased from Worthington. Trypsin-modified STI and tryp-
sin-STI complex were prepared according to the procedure 
of Ozawa and Laskowski (2). This involved incubation of 2 to 5 
moles of trypsin/100 moles of inhibitor at pH 3.75 for 24 to 
72 hours, separation, and isolation of the modified inhibitor 
and the enzyme-inhibitor complex by gel filtration followed by 
dialysis and lyophilization.

Unless otherwise indicated, all other reagents were of analytical 
reagent grade.

Methods

Enzyme Measurements—Clotting measurements for prothrom-
bin activity by the two-stage method of Ware and Seegers (25)

4 ARONSON, D. L., AND MUSHINSKI, J. F., Abstracts Twelfth 
Congress of International Society of Hematology, New York, 1968, 
p. 171.

5 We are indebted to Dr. Samuel I. Rapaport in whose labora-
tory this clotting factor assay was performed.
and thrombin activity (National Institutes of Health method) were carried out as previously described with National Institutes of Health thrombin (Lot 3B) as reference (27). Thrombin esterase activity was determined at 28° by the hydrolysis of p-tosyl-L-arginine methyl ester (Calbiochem) or Nα-tosyl-L-lysine methyl ester (Cyclo Chemicals) with a recording pH-stat (28).  

Thrombin esterase activity was inhibited by titration with phenylmethyl sulfonyl fluoride (Sigma) and Nα-tosyl-L-lysinochloromethyl ketone (Calbiochem) by methods previously described (28). Benzethonium (Schwarz Bio-Research) and e-amino caproic acid (Lederle) at a concentration of 0.1 mM did not inhibit thrombin esterase activity against TAME. Trypsin and the inhibition of trypsin activity by STI were determined spectrophotometrically with benzoylarginine-p-nitroanilide as substrate (34). 

**Disc Electrophoresis**—Polyacrylamide disc electrophoresis was carried out with a model 12 Canaco apparatus and the protein-stained bands were analyzed by a model F Densitometer equipped with a recorder and integrator (model SR, E. H. Sargent and Company, Anaheim, California). Additional details are given elsewhere (24). 

**Ultracentrifugation**—Sedimentation velocity analyses were performed in a Beckman-Spinco model E analytical ultracentrifuge at 20° (27). 

**Spectrophotometric Measurements**—Protein was measured by absorption at 280 nm (Beckman DU or DB spectrophotometer) and corrected for Rayleigh light scattering at 320 nm (24). The optical factors for the protein concentration of human prothrombin, human thrombin, trypsin, and STI were computed from the published E1% values of 13.0 (23), 16.2 (18), 14.4 (7), and 8.5 (7), respectively. An E1% = 5.8 based on the weight of the dry powder was found for the human Factor X preparation. 

**Gel Filtration Analysis**—In some experiments, gel filtration was carried out on columns, 1 × 60 cm, of beaded Sephadex G-75, G-100, or G-200, equilibrated at 24° with either 0.2 M NaCl-Tris buffer, pH 7.4, or 0.5 M NaCl (27). In most experiments, the gel columns were equilibrated and eluted with buffers containing 1.0 mg of STI ml−1 ( Worthington, one crystallized, Lot 7 CB and 7 H4), further details of which are given under “Results and Discussion.”

**RESULTS AND DISCUSSION**

**Effect of STI on Biological (Two-Stage) Conversion of Prothrombin to Thrombin**—Since we were using prothrombin preparations of human origin, we repeated some of the work of Shulman and Hearon (10) who studied the effects of STI on bovine prothrombin conversion. The results that we obtained were similar to theirs (Fig. 1), but differ in interpretation. When STI is added to the two-stage system which measures prothrombin as a function of the total amount of thrombin activity generated (25), increasing amounts of inhibitor result in a progressive decrease in thrombin units (Fig. 1A). Unlike the inhibition of trypsin activity by STI (7) this is not a linear (stoichiometric) function of inhibitor concentration, but closely approximates a bimolecular reversible reaction similar to the STI-chymotrypsin (1) and STI-coconase (4) systems. The experimental values calculated from the data of Shulman and Hearon (10) for a bovine prothrombin preparation (specific activity = 1 977 NIH units mg−1) converted to thrombin at optimum concentrations of two-stage reagents. B ( ), calculated from the data of Shulman and Hearon (10) for a bovine prothrombin preparation (specific activity ~1488 Iowa units mg−1) at a concentration of 5 to 25 μg ml−1 of activation mixture. C, the experimental points for two prothrombin preparations converted to thrombin at one-third the optimum concentration of two-stage reagents shown in A. One preparation ( ) was the same as that used in A and the other ( ) had a specific activity of 1150 NIH units mg−1. (See text for details.)
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Fig. 2. Thrombin activity measurements and polyacrylamide disc electrophoresis patterns following the activation of human prothrombin by biological (two-stage) reagents in the presence and absence of soybean trypsin inhibitor. In this experiment, 1.1 mg of prothrombin were added to a test tube containing 1.6 ml of two-stage reagent (25). With the exception of acaenin, this included NaCl, CaCl₂, a bovine tissue thromboplastin suspension, buffer, and absorbed bovine plasma in the concentrations and proportions given in the original paper (25). A second test tube similar to this contained, in addition, 2 mg of STI. Both tubes were incubated at 28° and, at various times, 0.1-ml aliquots were taken for the measurement of thrombin activity, the results of which are shown in A. In addition, 0.1-ml aliquots were also taken at the same time, diluted with 0.1 ml of 5% sodium citrate to stop the reaction, and quick frozen at -50°. These were then limited to very dilute solutions of the zymogen. If very large amounts of the activators, the rate and final yield of the thrombin activity generated are markedly diminished (10). From what has been discussed above, such a system would not only be expected to be more sensitive to inhibition by STI, but would also present the advantage of measuring the protein changes which take place at concentrations that can be detected by physical methods. One of several such experiments is presented in Fig. 2. In the system used, slightly more than 50% of the prothrombin added to the two-stage reagents was converted to thrombin in 6 hours and the addition of STI to a duplicate system completely blocked the generation of thrombin activity (Fig. 2A).

Disc electrophoresis patterns (Fig. 2B) of aliquots taken from the time course study in the absence of STI show the formation of a prothrombin derivative in a stoichiometric manner much the same as STI combines with trypsin. That this is not the case is clearly evident from the kinetic measurements shown in Fig. 1 and comparisons made between the k_{ass} of this system and others discussed earlier. As shown below, there are several rate-limiting reactions during the conversion of prothrombin to thrombin, two of which are inhibited by STI.

The two-stage system for thrombin analysis is generally limited to very dilute solutions of the zymogen. If very large amounts of prothrombin are added to the test tube containing 1.6 ml of two-stage reagent (25). With the exception of acaenin, this included NaCl, CaCl₂, a bovine tissue thromboplastin suspension, buffer, and absorbed bovine plasma in the concentrations and proportions given in the original paper (25). A second test tube similar to this contained, in addition, 2 mg of STI. Both tubes were incubated at 28° and, at various times, 0.1-ml aliquots were taken for the measurement of thrombin activity, the results of which are shown in A. In addition, 0.1-ml aliquots were also taken at the same time, diluted with 0.1 ml of 5% sodium citrate to stop the reaction, and quick frozen at -50°. These were then limited to very dilute solutions of the zymogen. If very large amounts of the activators, the rate and final yield of the thrombin activity generated are markedly diminished (10). From what has been discussed above, such a system would not only be expected to be more sensitive to inhibition by STI, but would also present the advantage of measuring the protein changes which take place at concentrations that can be detected by physical methods. One of several such experiments is presented in Fig. 2. In the system used, slightly more than 50% of the prothrombin added to the two-stage reagents was converted to thrombin in 6 hours and the addition of STI to a duplicate system completely blocked the generation of thrombin activity (Fig. 2A).
Fig. 3. Thrombin-clotting activity and electrophoretic changes accompanying the citrate activation of human prothrombin in the presence and absence of soybean trypsin inhibitor. In this experiment, 40 mg of prothrombin were dissolved in 4.0 ml of 25% (w/v) trisodium citrate at 24°C. At intervals, two 0.2 ml aliquots of the mixture were taken. To one of these, 0.2 ml of 25% sodium citrate was added, diluted with H2O to T/2 = 0.15, and then immediately assayed for thrombin activity. A portion of this dilution was also quick frozen for subsequent disc electrophoresis. The thrombin values obtained are shown by the solid circles connected by the solid lines (●—●) in A. The disc electrophoresis patterns of these samples are shown in B. To the second aliquot, 0.2 ml of 25% sodium citrate containing STI (1 mg per ml) was added and the mixture was allowed to stand for the remaining 24-hour period at which time they were all assayed for thrombin activity. The thrombin values obtained are shown by the open circles and dashed lines (○-○-○) in A. The disc electrophoresis patterns of these samples are shown in C. For purposes of orientation, all of the disc patterns in C should be compared with that of the 24-hour sample in B.

appeared from the system, although slightly less than 50% of the prothrombin had been converted to thrombin at this time. This appears to be due to proteolytic digestion of the zymogen at a rate exceeding its conversion to thrombin (18).

With respect to the two-stage system in which the potential thrombin activity was completely blocked by ST1 (Fig. 2A), the disc patterns (Fig. 2B) show that this blockage principally occurs after the formation of the P-2 intermediate and that no further fragmentation into thrombin components occurs. This pattern is evident after 15 min of reaction time and undergoes no further change even after 240-min exposure to the two-stage reagents.

Effect of STI on Conversion of Prothrombin by Citrate—In order to study the effects of STI on the initial activation stage of prothrombin conversion, the 25% citrate activation system was next examined. This is a technically more convenient system to study since: (a) thrombin generation takes place autocatalytically over many hours rather than in minutes, (b) the reaction can be terminated and its composition stabilized at any time by dilution with water (to about 2.5% citrate), (c) the system is not contaminated with other protein reagents, and (d) the products formed appear comparable to those obtained in the two-stage system (22, 27, 28).

Fig. 3 presents an experiment in which STI was added at various times to a prothrombin preparation undergoing activation in 25% sodium citrate solution. In Fig. 3A, the solid curve represents the thrombin activity generated in the control system (no STI added) which reaches a maximum in about 16 hours and then diminishes slightly as a result of autolysis of the enzyme (28) by 24 hours. The disc electrophoresis patterns of samples of the reaction mixture taken during this time are shown in Fig. 3B and show a sequence of protein changes identical with that discussed above for the two-stage system and shown in Fig. 2B. Fig. 3A (dashed line) also shows the effect of STI when added at various times to the citrate activation mixture and indicates that it promptly terminates the generation of thrombin by the system. Fig. 3C presents the disc electrophoresis patterns of the STI-inhibited system in comparison to the uninhibited (control) system. This comparison indicates that, when the inhibitor is added at the same time (zero time) that activation is initiated by dissolving the zymogen in citrate solution and the mixture then incubated for 24 hours, the P-2 intermediate and F-1 fragment are only partially formed. If the inhibitor is added 30 min after activation is started and the mixture is allowed to incubate for 24 hours, no thrombin is generated and the system consists of the P-2 intermediate and F-1 fragment. Subsequent additions of ST1 over the remainder of the 24-hour period completely inhibit any further thrombin generation or change in the disc pattern of the protein mixture. From these experiments, therefore, it appears that STI blocks the activation of prothrombin at two stages, namely, the conversion of zymogen (P-1) to the intermediate (P-2) and the conversion of the intermediate
The effect of STI on the inactivation and proteolysis of prothrombin by thrombin.

**A. ACTIVITY MEASUREMENTS**

Prothrombin + STI

<table>
<thead>
<tr>
<th>PRoThROMBIN UNITS/ML</th>
<th>MG. STI/ML</th>
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<tbody>
<tr>
<td>1000</td>
<td>4</td>
</tr>
<tr>
<td>500</td>
<td>2</td>
</tr>
<tr>
<td>1000</td>
<td>1</td>
</tr>
<tr>
<td>500</td>
<td>0.5</td>
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<tr>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
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**B. ELECTROPHORETIC PATTERN**

Prothrombin + STI + Thrombin

**FIG. 4.** The effect of STI on the inactivation and proteolysis of prothrombin by thrombin. A, a human prothrombin preparation (approximately 0.5 mg per ml) was incubated for 3 hours at 28°, 0.15 M Tris buffer, pH 7.4, with various concentrations of STI in the absence (■) and presence (○) of human thrombin (0.56 unit per ml, approximately 0.1 µg ml⁻¹) and then measured for two-stage prothrombin activity. B, polyacrylamide disc electrophoresis patterns of the various mixtures of prothrombin, thrombin (P-2) to the thrombin components (S-bands). It is also important to note that in the disc patterns presented in Figs. 2B, 3B and C, and 4B, there is no evidence that an irreversible complex is formed between an intermediate and STI as suggested by the kinetic studies of Schulman and Hearon (10) and Alkajaersig et al. (9).

**Effect of STI on Proteolysis of Prothrombin by Thrombin**—Previous studies have shown that thrombin itself attacks the prothrombin molecule and produces alterations very similar to those which occur initially during biological or citrate activation of the zymogen (22). In order to examine whether or not STI could block these changes, experiments were performed in which small amounts of thrombin were added to prothrombin in the presence of increasing concentrations of STI. One example of such an experiment is presented in Fig. 4. Fig. 4A shows that, in the presence of a very small amount of thrombin, about 40% of the prothrombin activity disappears in 3 hours of incubation (i.e., from 790 to 460 units ml⁻¹ at zero STI concentration). With the addition of increasing amounts of STI to this system, relatively less prothrombin activity is lost and, at a concentration of 1.25 mg of STI ml⁻¹ of reaction mixture (Fig. 4A), the activity of prothrombin is almost identical with that anticipated from the effect of STI alone on its two-stage activity (i.e., 640 and 630 units ml⁻¹, respectively). This experiment is interpreted to mean that STI blocks the proteolytic attack of thrombin on the prothrombin molecule. Further support for this is shown in Fig. 4B in which the reaction mixtures of Fig. 4A were analyzed by disc electrophoresis. From a comparison of the upper and lower patterns of the figure, it is evident that thrombin in the absence of STI converts the prothrombin band (P-1, upper far left gel pattern) to the P-2 intermediate and F-1 fragment (lower far left gel pattern). Increasing concentrations of STI, when added to this system (gel patterns from left to right), show that the inhibitor blocks this transition.

**Isolation and Properties of STI-Thrombin Complex**—From the disc electrophoresis patterns shown in Figs. 2 to 4, there is no evidence by this method that STI forms an irreversible complex with prothrombin, its activation fragments, or thrombin. From gel filtration studies of prothrombin activated by citrate-containing STI, similar conclusions were reached (28). This would seem to be quite unusual since such methods have been found to be quite sensitive in detecting irreversible protein inhibitor-enzyme complexes (2, 3, 5). However, considering the kinetics of interaction of STI with the prothrombin activation systems described previously (Fig. 1), this is not too surprising for a system that undergoes rapid reversible dissociation. Transport methods which separate two reactants in a medium devoid of either reactant would tend to dissociate a complex of the two if such a complex were formed. Therefore, only systems in which a protein boundary moves through a solution of the other so as to maintain each of the reactants saturated with the other would be expected to be sensitive to complex formation provided, of course, that the complex formed had properties significantly different from either of the reactants. In the system under study, two such methods based on this reasoning were used, namely, filtration through Sephadex gels equilibrated with STI and analytical ultracentrifugation.

Fig. 5A shows the protein elution pattern of a Sephadex G-75 column equilibrated with STI following which a thrombin solution is placed at the top of the column and allowed to permeate through the gel. The thrombin emerges from the column as an absorbance peak superimposed on the STI base line absorbance.
with a distribution coefficient \((K) = 1.17\). Following this peak, the absorbance of the effluent falls below the STI base line absorbance, and then returns to its equilibrated value followed by a second absorbance peak at \(K = 2.54\) (this peak is due to small peptide autolysis products of thrombin (28)). The protein elution pattern thus obtained (Fig. 5A) is typical of what would be expected if one reactant, in this case thrombin, interacted with a second reactant, i.e., STI, which was uniformly distributed throughout the gel matrix. As the thrombin boundary moves down the gel column, it progressively forms complexes with STI until a saturation peak of both proteins is reached. This peak would then migrate through the remaining gel column length at a rate greater than that of either the uncombined thrombin or STI. This would then result in an absorbance peak \((K = 1.17, \text{Fig. } 5A)\) representing the thrombin-STI complex followed by a “negative” absorbance peak at a \(K\) value at which the STI that joined in complex with thrombin should have emerged. In order to confirm this, a second experiment was performed similar to the first, except that the thrombin was first saturated with STI before it was placed on the STI-equilibrated column, the results of which are presented in Fig. 5B. Under these conditions, the thrombin-STI complex emerges at approximately the same absorbance peak \((K = 1.22)\) as in the first experiment \((K = 1.17)\) and the unreacted STI emerges at an absorbance peak \((K = 1.51)\) approximately the same as the “negative” peak \((K = 1.32)\) also. These latter peaks are comparable to a control experiment (Fig. 5C) in which STI alone was passed through the column \((K = 1.54)\). Fig. 5D shows a theoretical protein elution pattern for a thrombin peak \((K = 1.42)\) which was calculated to occur if no complex between thrombin and STI was formed. This is based on the calibration of the Sephadex columns with a series of globular proteins of known molecular weights (27) including human thrombin \((30,000 \text{ g})\) (28) and STI \((21,000 \text{ g})\) (7). By this technique, it was also possible to calculate from the distribution coefficient of the thrombin-STI complex a molecular weight of \(50,000 \pm 5,000 \text{ g}\) for the complex. This would be equivalent to a mole to mole ratio of thrombin to STI in the formed complex. Additional confirmation of the higher molecular weight of the complex relative to that of STI and thrombin was obtained from the ultracentrifuge experiment shown in Fig. 6, in which an \(s_{20,w}^{	ext{m}}\) of 4.4 for the thrombin-STI complex was obtained.7

In other experiments with the STI-equilibrated gel column method, it was possible to show that thrombin derived from biologically (two-stage) activated human and bovine prothrombin and citrate activated human prothrombin (see “Materials”) gave elution patterns identical with that shown in Fig. 5A. However, it was not possible to show on Sephadex G-200 columns any interaction between STI and prothrombin (P-1), mixtures of prothrombin and its intermediate (P-2) and activation frag-

7 This value is probably slightly overestimated as a result of the skewing of the boundary.
ments (F-1), or the protein preparations used to calibrate the STI equilibrated gel columns (i.e. albumin and cytochrome c). This indicates rather conclusively that the inhibitor does not form complexes with any intermediates of the prothrombin conversion system as previously proposed by other workers (8-11).

**Mechanism of Interaction of Thrombin and STI**—It was of interest next to determine the nature of the binding sites on both the thrombin and STI molecules themselves. In the experiments presented in Fig. 5, A and B, the effluent fractions containing the absorbance peaks were analyzed for clotting and TAME esterase activity (not shown in the figure). The peak tubes of both these activity measurements corresponded to the protein absorption peaks of the thrombin-STI complex (i.e. $K = 1.17$ in A and $K = 1.22$ in B of Fig. 5), with recoveries exceeding 86% of the enzyme activity placed on the column. These experiments suggested that the binding site on thrombin must be different from the catalytic site for these activities and must be located at a site of the enzyme molecule with a different proteolytic activity. In order to test this assumption, a solution of thrombin ($-0.5$ mg ml$^{-1}$) having an activity of 350 TAME units and 1925 clotting units ml$^{-1}$ was incubated with phenylmethyl sulfonyl fluoride (final concentration $2.5 \times 10^{-4}$ M) for 1.5 hours (28), at the end of which time 6 TAME units and 33 clotting units ml$^{-1}$ remained (98% inhibition). This mixture was then placed on the STI-equilibrated column and an elution pattern identical with that shown in Fig. 5A was obtained. In another experiment, TLCK (final concentration $1.5 \times 10^{-4}$ M) was incubated with thrombin (2 hours at 24°C) which reduced its TAME esterase activity >97%. This mixture also gave a Sephadex pattern identical with that shown in Fig. 5A. These experiments indicate that the serine and histidine residues at the active site of thrombin are apparently not implicated with complex formation, which may suggest an allosteric type of binding. In comparison, diisopropylphosphoryl derivatives of trypsin and chymotrypsin do not interact with several protein inhibitors, although their TPCK and TLCK derivatives do so (5).

With regard to the binding site on the STI molecule which reacts with thrombin, experiments were performed to determine the inhibitory effects of trypsin-modified STI and the trypsin-STI complex preparations (see "Materials") on the two-stage assay procedure and in the same concentrations as those presented in Fig. 1. All preparations of the trypsin-modified STI were found to be effective inhibitors of the two-stage system (76 to 100% of virgin STI) and were equally inhibitory toward tryptic activity. We, therefore, performed experiments with preparations of human Factor X and X$_2$ (see "Materials") in order to explore this possibility and these are briefly summarized in Fig. 7 and the discussion which follows.

Fig. 7A shows the disc electrophoresis pattern of a human Factor X preparation which consisted of two major protein bands of about equal amount and some minor (<1%) bands as well. The two major bands migrated as serum albumin (Fig. 7B) by themselves as well as when added to serum (Fig. 7C), and this relative mobility was independent of concentration (Fig. 7G). Bovine Factor X preparations also display two bands of about the same relative mobility by disc electrophoresis (26), but a greater mobility than albumin by moving boundary electrophoresis (17). Human prothrombin, on the other hand, migrates as the G$_2$ (group-specific) serum protein band on disc columns (Fig. 1D) by itself and when added to serum (22).

Although the above data do not support the contention that STI interacts with a prothrombin intermediate, they do not rule out the role which Factor X and Factor X$_2$ might have on the interpretation of these results. The Factor X-X$_2$ system has many physical and chemical properties, some of which are similar and others dissimilar to the prothrombin-thrombin system (17). This has led to the controversy as to whether they are distinct enzyme systems (17) or "derivatives" of one another (12). Factor X$_2$ is considered to be an enzyme activated from its zymogen, Factor X, after a complex series of interactions of other blood clotting factors. Although there is some disagreement (13), Factor X$_2$ is also believed to be an activator which directly converts prothrombin to thrombin and whose blood clotting and esterolytic activities are inhibited by STI (13-16). Consequently, it might be logical to suppose that the prothrombin and thrombin preparations used in these studies and those of others might be "contaminated" with Factor X and Factor X$_2$, respectively. We, therefore, performed experiments with preparations of human Factor X and X$_2$ (see "Materials") in order to explore this possibility and these are briefly summarized in Fig. 7 and the discussion which follows.

Ozawa and Laskowski (2) have provided evidence that the reactive site of STI, and possibly other protein inhibitors as well, contains either an Arg-Ile or Lys-Ile bond sensitive to tryptic hydrolysis. Whether the hydrolysis of such bonds is sine qua non for STI activity has been questioned (5), although there is agreement that Arg and Lys residues are required for trypsin inhibitors (6). Thrombin presumably attacks these same bonds when it activates trypsinogen (Lys-Ile) and chymotrypsinogen (Arg-Ile) (19). Prothrombin has one NH$_2$-terminal Ala and, thus far, no COOH-terminal amino acid has been found (20). On the other hand, thrombin has principally NH$_2$-terminal Ile and COOH-terminal Arg. When the zymogen is converted to the enzyme, COOH-terminal Arg and Lys and NH$_2$-terminal Tyr and Ala are isolated from the peptide fragments of the activation mixture (20). This suggests that Arg-Ile and Lys-Ile bonds are also hydrolyzed during activation of the zymogen.

From these findings, therefore, it might be logical to conclude that STI would be an effective competitive inhibitor of thrombin because of the similarities in reactive site with these protein substrates, but would not necessarily affect the catalytic activity of thrombin toward other bonds such as on fibrinogen (Arg-Gly bonds) or synthetic ester substrates.

**Experiments with Factor X and Factor X$_2$**—Although the above data do not support the contention that STI interacts with a prothrombin intermediate, they do not rule out the role which Factor X and Factor X$_2$ might have on the interpretation of these results. The Factor X-X$_2$ system has many physical and chemical properties, some of which are similar and others dissimilar to the prothrombin-thrombin system (17). This has led to the controversy as to whether they are distinct enzyme systems (17) or "derivatives" of one another (12). Factor X$_2$ is considered to be an enzyme activated from its zymogen, Factor X, after a complex series of interactions of other blood clotting factors. Although there is some disagreement (13), Factor X$_2$ is also believed to be an activator which directly converts prothrombin to thrombin and whose blood clotting and esterolytic activities are inhibited by STI (13-16). Consequently, it might be logical to suppose that the prothrombin and thrombin preparations used in these studies and those of others might be "contaminated" with Factor X and Factor X$_2$, respectively. We, therefore, performed experiments with preparations of human Factor X and X$_2$ (see "Materials") in order to explore this possibility and these are briefly summarized in Fig. 7 and the discussion which follows.

Fig. 7A shows the disc electrophoresis pattern of a human Factor X preparation which consisted of two major protein bands of about equal amount and some minor (<1%) bands as well. The two major bands migrated as serum albumin (Fig. 7B) by themselves as well as when added to serum (Fig. 7C), and this relative mobility was independent of concentration (Fig. 7G). Bovine Factor X preparations also display two bands of about the same relative mobility by disc electrophoresis (26), but a greater mobility than albumin by moving boundary electrophoresis (17). Human prothrombin, on the other hand, migrates as the G$_2$ (group-specific) serum protein band on disc columns (Fig. 1D) by itself and when added to serum (22).

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* Other experiments (to be published) also indicate that TPCK-treated trypsin, but not chymotrypsin or plasmin, initially attacks the prothrombin molecule and cleaves protein fragments similar to that obtained with thrombin. Since there are between 61 and 62 arginine and lysine residues in the human prothrombin molecule (20), proteolytic activation of the zymogen seems to be confined to only a few selective peptide bonds containing these residues.

* By immunoelectrophoresis, the Factor X preparation does not show any precipitin lines against anti-human serum (personal communication from Dr. D. L. Aronson), nor do the prothrombin preparations against anti-human serum or anti (G$_2$) group specific component (unpublished observations).
FIG. 7. A comparison of polyacrylamide disc electrophoresis patterns of human preparations of prothrombin, thrombin, Factor X, activated Factor X, and serum. The materials analyzed and the approximate weight of protein applied to the gels were as follows. A, Factor X preparation (78 µg); B, human serum (300 µg); C, Factor X added to serum (378 µg); D, prothrombin (75 µg), specific activity 1470 two-stage NIH units mg⁻¹, without an albumin marker; E, Factor X (84 µg) after “activation” in 25% sodium citrate solution for 24 hours (see “Materials”), 300 TAME esterase units and 1.0 clotting unit mg⁻¹; F, partially activated prothrombin (12 µg) with an albumin marker (1 µg)—preparation similar to that in Fig. 4, but activated with less thrombin; G, Factor X (16 µg), same as in A above; H, partially activated prothrombin (12 µg) added to Factor X (16 µg) and immediately placed on the disc column; I, citrate-activated thrombin (15 µg), same preparation as used in Fig. 4, but with an albumin marker (see Footnote 10); J, Factor X (16 µg) added to thrombin (15 µg) and incubated for 3 hours at 24°C. Proteins were dissolved or diluted in 0.1 M NaCl-0.05 M Tris buffer. Further details are described in text.

From these comparisons, there appears to be no evidence by disc electrophoresis of Factor X contamination in the prothrombin preparations used. Recently, Tishkoff, Williams, and Brown (26) presented data which show that when as little as 3% of the protein of prothrombin preparations contain Factor X this is readily apparent on the disc electrophoresis patterns. In fact, their prothrombin preparations which contained significant amounts of other clotting factors (i.e. Factors VII, IX, and X) were obviously heterogeneous by disc electrophoresis and displayed five to seven protein bands which, upon chromatographic isolation, corresponded to each of the clotting factor activities.

Fig. 7E shows the disc pattern obtained when Factor X is converted to Factor Xₐ by citrate activation (see “Materials”). In this experiment, there is a slight increase in mobility of the more anodic of the two bands of the original Factor X while the other band virtually disappears and is partially accounted for by a new band appearing at the origin. While, at present, it is not known which of these protein-stainable bands has the Factor Xₐ activity, it is interesting to note that chromatography of Factor Xₐ has been reported to yield two peaks, both of which have Factor Xₐ-clotting activity (31). Fig. 7, F to H, shows that there is no similarity in migration rates between Factor X and the activation fragments of partially converted prothrombin. These comparisons indicate that within the limitations of this technique there is no physical evidence that Factor X is an intermediate of the prothrombin-thrombin system, nor is it an appreciable contaminant of such a system. Finally, Fig. 7, I and J, shows that when Factor X is added to thrombin the Factor X bands are converted to those characteristic of Factor Xₐ (Fig. 7E) obtained by citrate activation of the Factor X. In spite of the fact that the thrombin preparations used contain two protein-stainable bands (Fig. 7I),¹¹ none of the bands in either system have the same relative mobility.

At the present time, we are not aware of any reports dealing with the proteolytic activity of thrombin against Factor X. It is possible that some Factor X preparations (14, 17, 31), including those used in this study, are activated in citrate whereas others are not (15), as a result of the contamination of the former materials with small amounts of thrombin.

¹¹ The thrombin preparations used in these experiments consist of two protein-stainable bands (S-2 and S-3) by disc electrophoresis (Fig. 7). The S-3 band represents an inactive protein (no esterase or clotting activity), which is often difficult to separate from the thrombin peak (S-2 band) by ion exchange chromatography (28), and is similar in some respects to the “inactive” trypsin found in most trypsin preparations. There is no evidence that human thrombin is molecularly heterogeneous because of reversible dissociation to smaller subunits or charge differences (32) as has been reported for bovine preparations.
From the results of the experiments shown in Fig. 7, and the discussion above, it does not appear that the Factor Xa-cotting proteins could have physically contaminated the preparations used in this study to any appreciable extent. Previous analyses of these prothrombin preparations for specific blood clotting factors have shown them to have negligible activities in this regard (21), the significance of which is difficult to interpret because of the assay systems themselves (see discussion and references in References 22, 27, 28). Certainly, we cannot exclude the possibility of a few percent which would be beyond our methods of detection. However, such contamination, if it existed, could hardly account for the experiments detailed in Fig. 5 and described in the relevant text.

Further evidence for the insignificant contamination of our thrombin preparations with Factor Xa, based on a comparison of their biochemical properties, is somewhat more difficult. This is due primarily to conflicting and, at times, inconsistent reports on the properties of Factor Xa, many of which are not fully documented and which were carried out with preparations containing small but significant amounts of thrombin (13, 14, 16, 31). However, some comparisons are possible and these are as follows.

1. The clotting and esterase activities of the thrombin preparations used in these experiments are readily inactivated by phenethylmethyl sulfonyl fluoride and TLCK and previously we have shown complete inactivation by careful titration with DFP (29). These active site specific inhibitors also prevent the proteolytic attack of thrombin on prothrombin. On the other hand, the esterase and clotting activities of Factor X, including preparations used in these experiments, have been reported to be resistant to DFP inhibition by most workers (13, 31, and p. 32 of Reference 12), but not all (17).

2. The fibrinogen clotting activity and the esterase activity of thrombin are unaffected by STI even at a 500:1 weight ratio (29). The Factor Xa esterase activity is about 40 to 70% inhibited by STI (17, 31).

3. As noted elsewhere, our thrombin preparations catalyze the hydrolysis of TLMa at a rate 2.1 times greater than TLMa while purified Factor Xa has been reported to catalyze the hydrolysis of TLMa at a rate 6 times greater than TLMe (33).

4. Thrombin initially attacks the prothrombin molecule (P-1), producing the activation fragments F-1 and P-2 (Fig. 4) and then the F-2 and S-4 fragments (22). These reactions apparently terminate at this time since no significant thrombin activity is generated under these conditions and no protein band (S-2 band in Fig. 7f) is seen on disc electrophoresis of the incubation mixture (22). These studies suggest that thrombin may only initiate prothrombin activation while a variety of other agents such as blood clotting factors (two-stage reagents), polycations (citrate salts, polylysine), etc. (see discussion in Reference 31) are responsible for the terminal stages of the conversion process. The fact that Factor Xa preparations, some of which contain significant amounts of thrombin and probably other factors as well (16, 31), convert prothrombin to thrombin in an almost quantitative and linear fashion with incubation time is consistent with this view. However, whatever interpretation of these experiments may prove to be correct, the reported interaction of Factor Xa with prothrombin appears to be quite dissimilar with that of thrombin.

In conclusion, these studies show that STI forms a complex with thrombin which blocks the initial activation and subsequent conversion of prothrombin to thrombin. Although the enzyme-inhibitor complex is fully active against other thrombin substrates such as fibrinogen and synthetic esters, this is not a completely unexpected finding since several other proteins may alter the esteroproteolytic activities of thrombin in different ways (see discussion and references in Reference 28). Perhaps, more importantly, the results also indicate that some of the events which occur during the conversion of prothrombin to thrombin may be attributable to proteolysis of the zymogen by thrombin, which, in many respects, does not differ markedly from other well known zymogen enzyme systems (i.e. trypsinogen, pepsinogen, etc.). They do not answer completely, however, the historical question as to the events which must initiate the first formation of thrombin molecules and thus trigger the activation system, or the role which other blood clotting factors may have in this respect.

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