**Kinetic Properties and Molecular Size of Thrombin-activated Factor V***

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**SUMMARY**

Native bovine factor V exists in three forms of differing molecular size, the oligomeric interconvertible forms A and C and a phospholipid-containing complex designated form L. Thrombin increases the activity of form A to a greater extent than form L and does not alter the activity of form C. Thrombin-activated form A and form C function as potent inhibitors of thrombin activation of form A but cannot inhibit the action of thrombin on other substrates as \(\alpha\)-N-tosyl-L-arginine methyl ester and fibrinogen.

No change in the molecular size of any of these three forms was detected after exposure to thrombin. Factor V derived from serum by endogenous thrombin action appeared to have a smaller Stokes radius than that of form A, the predominant form in plasma. However, the serum factor V associates to a form similar in size to form A after storage in 50% glycerol.

These results suggest the formation of a complex between these inhibitor forms of factor V and activated factor V (form A), suggesting that both substrate and product inhibition occur.

Factor V is a plasma protein which accelerates the conversion of prothrombin into thrombin by activated factor X in the presence of phospholipid and calcium. Despite the availability of highly purified preparations of bovine factor V, disagreement exists as to whether thrombin can increase its activity (2, 3). In this laboratory most preparations of purified factor V were found to undergo more than a 2-fold increase in activity after incubation with purified bovine thrombin (4). However, occasional preparations, particularly when frozen in the absence of glycerol or exposed to high concentrations of certain anions, were not activated by thrombin. More recent studies (5) have indicated that factor V exists in three different molecular forms; form L appears to be a phospholipid-protein complex, form C (mol wt 38,000) is homogeneous by polyacrylamide electrophoresis and form A is an oligomer of form C. The relative concentration of form A and C changes as a function of glycerol or anion concentration. The availability of these purified forms of factor V now make possible an assessment of their relative thrombin susceptibility.

The rate of thrombin activation of factor V was also found to be inversely related to factor V concentration (4), a finding consistent with substrate or product inhibition. In the process of thrombin action on factor V an altered form of factor V appears which loses activity at 2.5 times the rate of native factor V.

Knowledge of the effect of this product of thrombin action on the rate of activation might elucidate the nature of the inhibition.

Papahadjopoulos, Houge, and Hanahan (6) demonstrated that the molecular size is altered after incubation with thrombin. Other workers (2), with the use of a highly purified preparation of factor V, also observed a decrease in molecular size as determined by gel filtration following exposure to thrombin. Since native factor V is now known to occur in at least three different molecular forms, re-evaluation of the effect of thrombin on the molecular size of factor V seemed desirable.

This investigation will examine the effect of thrombin on each of the three forms of factor V in regard to its thrombin susceptibility, the effect of the reaction products on the activation reaction, and the molecular size of these products.

**EXPERIMENTAL PROCEDURE**

**Thrombin**—Bovine thrombin was purified by the method of Yin and Wessler (7). This preparation has a specific activity of about 1000 NIH units per mg of protein (1 NIH unit of thrombin clots a 1% solution of fibrinogen in 15 sec at 27°C) and as previously demonstrated (4) is not contaminated with measurable amounts of clotting factors I, II, V, VIII, Xa, XI, and plasminogen or plasmin. A more purified preparation of bovine thrombin, specific activity 2260 ± 250 NIH units per mg (8) gave similar results.

**Factor V Preparation, Assay, and Activation**—The method of purification, characterization, and assay of plasma factor V and
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Fig. 1. Thrombin activation of the different oligomeric forms of factor V. O-O, purified factor V prior to gel filtration; ▲ ▲ ▲, form A; △ △ △, form L, and ▼ ▼ ▼, form C, respectively, isolated by gel filtration on Sepharose 4B. The initial concentration of factor V was 0.30 unit per ml. The preparations were separately incubated with 1.0 NIH unit of purified thrombin, and factor V activity was determined at various times.

Fig. 2. Effect of thrombin-activated factor V on activation of various forms of Factor V. A, experiment in which the initial concentration of factor V was 4.00 units per ml. B, experiment in which 4.60 units of factor V were mixed with an equal volume of native factor V (4.40 units per ml). C, initial concentration of factor V was 0.90 unit per ml. D, experiment identical with B except that the concentration of factor V was 0.74 unit per ml and native factor V, 0.96 unit per ml.

FIG. 2. Effect of thrombin-activated factor V on activation of various forms of factor V—In a previous study (5), the effect of thrombin upon the rate and degree of activation of purified factor V was hypothesized to depend upon the relative concentrations of forms A and C. When form C predominated, activation by thrombin apparently did not occur. Each of the three forms of factor V isolated by gel filtration of Sepharose 4B (5) was incubated with thrombin, and a characteristic rate and degree of activation is observed for each form (Fig. 1). Form A; reaches in 1 min a maximum activation of 266% of the original form A activity and form L, 150% of the starting activity in 2 min. Form C did not significantly differ from the activation of factor V by thrombin are described by Colman (4).

Serum factor V was purified by the method (5) previously used for plasma. Freshly collected bovine blood without anticoagulant was incubated for 4 hours at 25° in glass containers, and the serum was collected by centrifugation at 2300 × g for 15 min at 4°.

Thrombin-activated factor V (factor V₃) was prepared by incubation of the cellulose phosphate eluate preparation of factor V with purified bovine thrombin (1 NIH unit per ml) at 17° until maximal activation was achieved (4). The resulting preparation was maintained at 2° and either used immediately in kinetic experiments or subjected to further purification by gel filtration through Sephadex G-200 or Sepharose 4B (5). In some experiments, gel filtration of factor V was performed first, and the isolated forms L, A, and C were similarly incubated with thrombin. After exposure to thrombin, the resulting products were designated forms Lₛ, Aₛ, and Cₛ, respectively.

Fibrinogen Clotting Time—Bovine fibrinogen (95% clotting ability) (Gallard Schlesinger Chemical Manufacturing Corporation) was used. The concentration was determined from the value $E_{\text{1}}^{50} = 15.1$. Fibrinogen (9 mg per ml) was dissolved at 37° in 0.02 M sodium Veronal HCl buffer (pH 7.4) containing 0.15 M NaCl. This fibrinogen solution (0.1 ml) was mixed with 0.1 ml of the same buffer. In some cases various preparaions of factor V diluted in this buffer were substituted for the buffer. Thrombin, 0.1 ml at concentrations specified in the text, was added, and the time required for clot formation was measured with an automatic clot timer (Fibrometer, Baltimore Biological Laboratories, Baltimore, Maryland).

Esterase Activity of Thrombin—The micromoles of methanol released from $N$-tosyl-$L$-arginine methyl ester (Cyclo Chemical Company) were quantified by a colorimetric method (9). The buffer used was 0.1 M sodium phosphate, pH 7.65, in 0.15 M sodium chloride.

Sephadex G-200 and Sepharose 4B Gel Filtration—These were performed as previously described (5) and Kᵥ values calculated by the method of Ackers (10). Appropriate column fractions were pooled for forms L, A, and C, respectively, and concentrated with an Amicon ultrafiltration apparatus with the use of a UM-50 membrane (Amicon Corporation, Cambridge, Massachusetts).

RESULTS

Effect of Thrombin on Activation of Various Forms of Factor V—In a previous study (5), the effect of thrombin upon the rate and degree of activation of purified factor V was hypothesized to depend upon the relative concentrations of forms A and C. When form C predominated, activation by thrombin apparently did not occur. Each of the three forms of factor V isolated by gel filtration of Sepharose 4B (5) was incubated with thrombin, and a characteristic rate and degree of activation is observed for each form (Fig. 1). Form A; reaches in 1 min a maximum activation of 266% of the original form A activity and form L, 150% of the starting activity in 2 min. Form C did not significantly differ from the activation of factor V by thrombin are described by Colman (4).

The term activation as used in this paper represents only an increase in measured activity and does not connote a change from a precursor to an active entity.

All forms of factor V after exposure to thrombin are designated as factor V₃.
Fig. 3. Effect of form C and thrombin-activated factor V on thrombin activation of factor V (form A). In these experiments various concentrations of factor V, form A (.20 to .50 unit per ml) were mixed with various concentrations (0 to .80 unit per ml) of form C (뤄) or form A (●). The mixtures were activated with 1 NIH unit of thrombin, and increase in factor V activity was followed until a maximum level was reached. The ratio of the maximum activity to the initial activity is plotted as a function of the ratio of inhibitor activity (concentration of form C or form A) to the concentration of form A in the incubation mixtures.

in specific activity from form C. As might be expected, an intermediate value is observed for the original cellulose phosphate eluate which contains a mixture of all three forms.

Effect of Factor Vt on Activation of Factor V by Thrombin—Because higher concentrations of factor V lower the rate of activation by thrombin, substrate or product inhibition has been suspected (4). In preliminary experiments, once factor V was exposed to thrombin further activation could not be achieved despite the addition of more thrombin. Accordingly, form A was mixed with plasma factor Vt together with additional thrombin. Factor Vt substantially inhibited the degree of activation of form A (Fig. 2). Without factor Vt, thrombin increased the activity of form A 2-fold (Fig. 2A); with Vt, the maximum activation was only 1.3-fold (Fig. 2B). Similar results were noted even when different concentrations of factor V and Vt were employed (Fig. 2, C and D). Similar studies were performed with the use of serum-derived factor V or form C, neither of which could be activated by thrombin. Both markedly inhibited the thrombin activation of form A.

To ascertain the quantitative relationships between these inhibitory forms of factor V and thrombin activation of form A, experiments were performed using various concentrations of form C or form A, and fixed concentrations of form A. The extent of activation was calculated as a ratio of the maximum activity obtained after thrombin exposure divided by the initial activity of form A. When this ratio was plotted against the relative concentrations of the inhibitory forms a linear relationship was obtained (Fig. 3). The degree of inhibition was identical for form C and form A, and is directly proportional to the factor V activity of the inhibitors. Moreover, when the ratio of inhibitor to form A exceeds 1.5, complete inhibition of thrombin activation is manifest. When the above experiments were performed with form C previously incubated at 37° until devoid of factor V activity, no inhibition of thrombin activation was observed.

Effect of Thrombin-treated Factor V on Hydrolysis of Fibrinogen and Tosyl-AME—Factor Vt inhibits thrombin activation of factor V, and it seemed pertinent to explore whether this altered form also inhibited the action of thrombin on other substrates. Accordingly, fibrinogen and tosyl-AME were each incubated with thrombin with and without factor Vt. The kinetic constants (Vₘ and Kₘ) of thrombin proteolysis of fibrinogen (Fig. 4, top) were unaffected. Similarly, the kinetic constants of thrombin esterolytic action on tosyl-AME did not appear to be grossly altered (Fig. 4, bottom). In further experiments, a constant concentration of factor Vt was tested against various concentrations of thrombin. No effect was noted on the velocity of the clotting of fibrinogen (Fig. 5).

Purification and Gel Filtration of Various Forms of Factor V Following Exposure to Thrombin—The effect of thrombin on the molecular size of the various forms of purified factor V was studied. After exposure to thrombin, form L underwent gel filtration through Sepharose 4B. The only activity peak detected was eluted in the void volume of the column and was unchanged from form L. No change in elution volume of form C₄

4 The abbreviation used is: tosyl-AME, a-N-toluenesulfonyl-L-arginine methyl ester.
Fig. 5. Effect of thrombin-activated factor V on the proteolysis of fibrinogen by various concentrations of thrombin. The velocity of fibrin formation is determined in the absence ($\times$) and presence (○) of 2.4 units per ml of factor V as a function of thrombin concentration.

**Table I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$K_D$ (Sephrose 4B)</th>
<th>$K_D$ (Sephadex G-200)</th>
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</thead>
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<td></td>
</tr>
<tr>
<td>Forms L, A, C</td>
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<td>0.65c</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Form A</td>
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<td>0</td>
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<td>0.57&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Form C</td>
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<td>1.00&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Form C&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.054&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum V (50% glycerol)</td>
<td>1.00</td>
<td>0.054&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> $K_D$ values determined and calculated as described previously (5). Each value is the mean of at least duplicate determinations.

<sup>b</sup> Data from Philip, Moran, and Colman (5).

<sup>c</sup> This value corresponds to a Stokes radius of 8.5 ± 0.1 nm and an estimated molecular weight of 170,000 (5).

<sup>d</sup> This value corresponds to a Stokes radius of 5.2 ± 0.2 nm and a molecular weight of 38,000 (5).

Discussion

In this study, marked differences were observed in both the rate and extent of activation when the various purified forms of factor V were exposed to thrombin. Loss of ability to activate factor V preparations has been observed in preparations frozen in the absence of glycerol or exposed to high concentrations of anions, a condition known to favor existence of form C. Apparently contradictory results employing similar preparations of factor V were obtained by Esnouf and Jobin (3), who found no activity increase with thrombin, and Barton and Hanahan (2), who found increased specific activity after thrombin exposure. Those experiments can be reconciled by assuming that their preparations contained varying amounts of forms A and C. The dispute regarding thrombin activation between its proponents (11, 12) and opponents (13, 14) may be similarly understood.

Kinetics of factor V activation by thrombin performed on fractions, it seemed important to analyze the effect of endogenous thrombin on native factor V as it occurs in clotting blood. Factor V purified from bovine serum was filtered on Sepharose 4B (Fig. 6). Unlike plasma factor V which emerges as three separate peaks (5), serum factor V emerges in a single peak. The $K_D$ of this peak is 1.00 (Fig. 6, Table I) identical with the $K_D$ of form C. The specific activity (tube 43) was 50 units per mg. Since forms A and C are known to be interconvertible (5), it was established whether the smaller molecular size represented dissociation of form A<sub>1</sub> during the purification, rather than any effect of thrombin. Since form C undergoes association to form A in 50% glycerol (5), the serum factor V was tested under similar conditions. Tubes 40 to 48 were pooled, concentrated to the original concentrations of the cellulose phosphate eluate (25 units per ml), adjusted to contain 50% glycerol (v/v), and stored at -20°C for 7 days. This preparation was then subjected to gel filtration on Sephadex G-200. The calculated $K_D$ of serum factor V now was similar to form A<sub>1</sub> but not form C<sub>1</sub> (Table I).
preparations containing forms L, A, and C were thought to be compatible with substrate and product inhibition (or both) (4). In the light of the existence of oligomeric forms of factor V and the properties of factor Vt, this reaction product was further investigated. When form A1 was mixed with native form A, it markedly inhibited the increase of factor V activity ordinarily produced by thrombin on native form A alone. Serum factor V, presumably also a thrombin-activated form, was markedly inhibitory. This product inhibition was independent of the molecular size of the thrombin-treated factor V since serum-derived V with K2 corresponding in size to form A1 or C1 was equally effective. That the inhibitor is probably an altered form of factor V is suggested by the linear relationship of inhibitory activity to factor V activity of the thrombin.

Activation was not possible with form C as a substrate; it markedly inhibited thrombin activation of form A. The inhibitory activity was proportional to the factor V activity of form C. Since form C is apparently homogeneous (5), it must be directly responsible for the apparent substrate inhibition observed in preparations containing both forms A and C (4). Forms A and C are interconvertible and probably represent oligomeric forms of factor V. The susceptibility of form A to thrombin activation may be due to conformational changes produced by association. Form C is insusceptible to thrombin activation and functions as an inhibitor as well. One might be tempted to deduce that any form of factor V insusceptible to thrombin would function as an inhibitor; thus, form C, serum-derived factor V, and factor Vt are all inhibitors and cannot be activated by thrombin. However, papain and Russell viper venom, two proteolytic enzymes which activate factor V (15) also produce an altered factor V which cannot be further activated by thrombin. However, papain and Russell viper venom, two proteolytic enzymes which activate factor V (15) also produce an altered factor V which cannot be further activated by thrombin. Neither the papain- nor the Russell viper venom-activated factor V inhibits the activation of factor V by thrombin. Moreover, the digest of factor V with tryptic and plasmin, two enzymes which destroy factor V activity, fail to interfere with thrombin activation of factor V. Thus, form C, factor Vt, and serum V appear to function uniquely as inhibitors of factor V activation by thrombin.

Fibrinogen, another substrate of thrombin, exhibits similar behavior to factor V. Certain genetically abnormal forms of fibrinogen (16), in one case the result of single amino acid substitution (17), fail to clot or clot slowly when exposed to thrombin. Abnormal fibrinogens also inhibit the thrombin-initiated polymerization of fibrinogen to fibrin (18). A naturally occurring form of fibrinogen, a product of in vivo proteolysis by plasmin, polymerizes slowly (19), and later derivatives inhibit thrombin-initiated polymerization (20).

One possible mechanism by which factor Vt or form C inhibits thrombin activation of form A involves the binding of thrombin to altered factor V. These altered forms would then reduce the effective thrombin concentration, which determines the degree and extent of the activation. If this were true, factor Vt should inhibit any of the actions of thrombin. This inhibition did not occur as evidenced by the normal thrombin hydrolysis of another protein substrate, fibrinogen, as well as a synthetic ester substrate, tosyl-AME, in the presence of factor Vt. The failure to observe inhibition reflects either the lack of a thrombin-factor Vt complex or a preferential bonding to fibrinogen and tosyl-AME by thrombin. Although the active site of thrombin for factor V may be different from that for fibrinogen and tosyl-AME, the latter mechanism seems unlikely since the polypeptide hirudin is a potent inhibitor of the action of thrombin on both fibrinogen and factor V (4).

The results of this and earlier studies (4, 15) suggest that the inhibition by factor Vt of thrombin activation of form A may be due to the interaction of factor Vt and factor V. Analogously, fibrinogen and fibrin monomer complexes (products of fibrinogen proteolysis by thrombin) have been shown to exist by Shainoff and Page (21). Forms A1 and C1 not only undergo no further changes of activity upon incubation with thrombin, but their inhibitory action depends on their factor V activity. It seems reasonable that these forms may combine with activated factor V, just as form C associates to form A. The conformational changes which result might render the normally susceptible bonds resistant to thrombin.

The increase of activity of factor V by thrombin constituting a positive feedback in blood coagulation is limited by the instability of factor Vt (4). The relative amounts of the various oligomers of factor V and the formation of any factor V product which inhibits the reaction between thrombin and factor V would serve as further regulatory mechanisms which determine the rate and extent of thrombin formation.

The changes in activity of each form of factor V following thrombin activation were not correlated with changes in molecular size. Form A does not alter its Stokes radius on either Sephadex G-200 or Sepharose 4B but does undergo almost a 3-fold increase in specific activity. Form C does not undergo a change in elution volume on Sephadex G-200, and no activation is observed. Form L undergoes a considerable increase in specific activity but does not change in molecular size after exposure to thrombin. It remains in the excluded volume of Sepharose 4B, apparently associated with phospholipid in a large micellar form. The specific activity of form L, intermediate between form A1 and form C1, may be due to a mixture of forms A and C associated with the phospholipid. On the other hand, form A may be present in a complex with phospholipid which alters its ability to be activated by thrombin.

Factor V purified from serum represents the effect of endogenously derived thrombin occurring during the coagulation process. No further activation by thrombin of serum factor V could be demonstrated; in fact, it served as an inhibitor of thrombin activation of native factor V. The serum factor V, after storage in low glycerol concentration (10%), emerged as a single component on Sepharose 4B and corresponded to form O of plasma factor V. These results agree with those by Papahadjopulos et al. (6), who found the serum-derived factor V chromatographed with a greater K2 than did plasma factor V. However, serum-derived factor V after storage in 50% glycerol reverted to a size corresponding to form A. It is possible that the presence of 10% glycerol during gel filtration may have favored the reassociation of thrombin-activated factor Vt, thus accounting for the fact that form A1 has the same molecular size as form A. Even this explanation is still consistent with the proposition that thrombin action may not affect the sites critical for association in factor V.

The existence of several forms of factor V has been thought to be due to its interaction with thrombin. Ware, Murphy, and Seegers (22) distinguished between proaccelerin derived from plasma and acelerin derived from serum. Papahadjopulos et al. (6) found that crude factor V incubated with crude thrombin underwent a change in behavior on Sephadex G-200; the distribution coefficient (K2) for factor V on Sephadex G-200
prior to thrombin treatment was 0.050 and after thrombin, 0.18. These figures agree with our values for form A (0.050) and form C (0.22). The molecular size changes observed by those investigators after exposure to thrombin occurred under conditions (high chloride ion, no glycerol) in which form A to C transformations might be expected in the absence of thrombin (4). Alternatively, since the thrombin used in those studies contained other proteolytic enzymes (7) and the factor V was of low specific activity, the results of that study are not directly comparable to the present investigation. From the experiments in this study, thrombin activation of factor V is not necessarily accompanied by changes in molecular size.

The lack of alteration of molecular size by thrombin does not mean it has no proteolytic action on factor V. Thrombin may split off peptide(s) too small to detectably alter the molecular weight as in the case of its proteolysis of fibrinogen (23). Since the error of the estimated Stokes radius is at least 4%, such small changes would not be appreciated. Alternatively, thrombin may cleave bonds without liberating a peptide as in the plasminogen interaction with streptokinase (24). Factor V activated by thrombin exists in at least two different oligomeric forms similar to factor V in plasma. However, serum factor V, unlike plasma factor V, does not undergo activation with thrombin even when it exists in an oligomeric form corresponding to form A. Again suggesting that some alteration in properties other than molecular size has taken place.

Other molecular differences between native factor V and factor V_t have been demonstrated. The latter appears to have a higher isoelectric point and emerges at higher ionic strength from cation exchangers (25, 26). Preliminary experiments suggest that thrombin activation of form A is accompanied by a change in net charge.6 Experiments to locate peptide fragments and document end group changes are in progress.

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6 R. W. Colman, unpublished results.

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