Proteolysis of Prothrombin by Thrombin

Determination of kinetic parameters, and demonstration and characterization of an unusual inhibition by Ca²⁺ ions

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A discontinuous assay to measure the proteolysis of tritiated prothrombin to fragment 1 and prethrombin 1 by thrombin has been devised, based empirically on extracting the radiolabeled fragment 1 into 4% p-toluene sulfonic acid. The assay is shown to be valid for the measurement of the initial rate of reaction in simple mixtures and has been used to determine the Michaelis-Menten parameters. The \( K_m \) for prothrombin per ml, and the \( k_{cat} \), 6.9 s⁻¹, indicate that, in the absence of other controls, attack of thrombin on prothrombin would be overwhelming during coagulation in plasma and that the prothrombin concentration would be rate-limiting. However, Ca²⁺ ions at concentrations around 1 mM reduce the rate of proteolysis by at least 20-fold. This inhibition is rapidly reversible by the addition of chelating agents. Measurement of initial rates at Ca²⁺ concentrations up to 1.5 mM shows that the inhibition is multisite and perhaps involves cooperative binding of the metal ions. The effect of Ca²⁺ on the kinetic parameters has also been investigated. \( k_{cat} \) could not be measured because thrombin could not be saturated with prothrombin under conditions amenable to assay, but the \( K_m \) is increased, showing that the inhibition by Ca²⁺ is at least partially competitive. An independent qualitative demonstration that Ca²⁺ is inhibitory in the reaction of native prothrombin with thrombin has been obtained by measurement of intrinsic fluorescence.

Three peptide bonds in bovine prothrombin are hydrolyzed during its activation to thrombin by Factor Xa (1–3). Two of the cleavages, at Arg²⁷₄-Thr and Arg³²₃-Ile, are catalyzed by Factor Xa, and are both necessary and sufficient for thrombin formation (4). However, evidence suggests that the third, at Arg⁵₆-Ser, is extraneous to thrombin formation and, indeed, is catalyzed by thrombin itself rather than Factor Xa (5). Thus, it is prevented if an inhibitor of thrombin is included in the activation mixture (6, 7). Conversely, if prothrombin and thrombin are incubated together without Factor Xa, Arg⁵₆-Ser is the only peptide bond cleaved, at least over a period of several minutes (8, 9). The products are fragment 1, the NH₂-terminal segment, and prethrombin 1, the remaining 426 amino acid residues (1–12).

Fragment 1 contains the γ-carboxyglutamic acid residues which are primarily responsible for binding prothrombin to phospholipids via Ca²⁺ ions (13, 14). Prethrombin 1 comprises the thrombin sequence from residue 275 onwards (11, 12) together with a region which, in prothrombin, is required for interaction with Factor V, the protein cofactor of activation (15). Hydrolysis of Arg³⁷₆-Ser, therefore, removes that part of prothrombin necessary for its association with phospholipids. This probably explains why purified prethrombin 1 is a relatively poor substrate for Factor Xa, a phospholipid-requiring enzyme (16–18).

In a previous paper, we suggested that the cleavage of prothrombin by thrombin during activation is a manifestation of a product-mediated control (18). This was based on the observation that in slow activations, in which Factor X or Factor V is scarce, relatively more prethrombin 1 is formed than in fast activations, and less is further cleaved to thrombin by Factor Xa. It was postulated that this is not only because prethrombin 1 is a poor substrate per se, but also because of other constraints on the system such as the rapid inactivation of Factor V (18). Thus, the action of thrombin diverts activation of remaining prothrombin to an alternative, less efficient pathway.

Whether control involving proteolysis of prothrombin by thrombin occurs during coagulation is another matter. In studies of the fate of radiolabeled prothrombin in clotting human plasma or blood, investigators have been unable to detect any formation of fragment 1 (19). This suggests that there may be additional mechanisms which protect prothrombin from thrombin attack in this more complex system.

To allow evaluation of the quantitative importance of thrombin feedback on prothrombin, an assay has been devised to measure its initial rate accurately. The method is analogous to one we recently used to measure the activation kinetics of Factor X, based on the initial rate of release of tritiated peptide from radiolabeled substrate (20). The present assay has been used to determine kinetic parameters for the attack of thrombin on prothrombin in mixtures of the two and is applicable to testing possible mechanisms of protection which would pertain in plasma, such as the presence of Ca²⁺ ions, phospholipids, and substrates which compete for thrombin. The results show that, as expected from previous qualitative observations, prothrombin is cleaved readily by thrombin; however, Ca²⁺ ions at concentrations in the order of 1 mM inhibit the reaction. The mechanism of inhibition is wholly or partly competitive in type, and multisite.

**Experimental Procedures**

**Materials**

Benazamidine hydrochloride, p-toluene sulfonic acid monohydrate,
acrylamide, and N,N'-methylenebisacrylamide were purchased from Aldrich. Sodium lauryl sulfate and 1.0 mM CaCl2 solution were products of British Drug House. 2-(N-Morpholino)ethanesulfonic acid was from Aldrich. Sodium lauryl sulfate and 1.0 mM CaCl2 solution were purchased from Amicon (PM 10 membrane), and stored at -20°C in 50% glycerol. Dimethyl sulfoxide and ethylene glycol were Eastman chemicals.

Methods

Protein Purifications—Prothrombin was prepared from a barium citrate eluate of bovine plasma and rechromatographed on DEAE-Sephadex (21, 22). The leading edge of the chromatographic peak was free of Factor VII and Factor IX activity, and the material gave a single band by electrophoresis on 10% polyacrylamide gels in sodium dodecyl sulfate using the stacking system of Laemmli (23). Prothrombin was stored at -80°C in 30 mM benzamidine-HCl, 1 mM EDTA, TBS, 0.1% BSA, and 0.05 M Tris, pH 7.5.

Bovine thrombin was made by the modification of the method of Fenton et al. (24). To 900 ml of bovine citrated plasma (Pel-Freez) was added 10 mM BZA1 and then 60 ml of 1.0 mM BaCl2. The mixture was stirred for 30 min, and then the barium citrate precipitate with prothrombin complex bound to it was centrifuged (Servall RC-3 centrifuge) at 2,500 g for 30 min at 20°C. The supernatant was brought to 65% saturation with solid ammonium sulfate and 400 ml of 5 mM BaCl2, 0.5 mM BZA, centrifuged and suspended in 100 ml of 35% saturated ammonium sulfate, 10 mM BZA. The pH was adjusted to 7.5 with 1.0 M NaOH. The mixture was stirred for 30 min at 4°C and then centrifuged for 15 min (Servall RC-2B, 16,000 x g). The supernatant was brought to 65% saturation with solid ammonium sulfate and then to pH 7.5 with NaOH. After stirring for 30 min at 4°C, the precipitated prothrombin complex was recovered by centrifugation (30 min at 16,000 x g) and then dissolved in 150 ml of TBS. Ammonium sulfate was removed by dialysis versus 2 X 10 liter of TBS. The prothrombin in the complex was activated at 37°C by adding 10 mM CaCl2 and 20 ml of crude bovine brain suspension (29). The appearance of thrombin was monitored by clotting assay. The reaction was stopped after 5 min by adding 20 mM EDTA and 10 mM BZA, and the brain was removed by centrifugation for 1 h (RC-2B, 27,000 x g). The pH was adjusted to 6.0 with acetic acid and the thrombin was purified by ion exchange chromatography on CM-Sephadex (2 X 30 cm column), eluting with a 1-Liter gradient of 150 ml of 50 mM NaCl, pH 6.0, 10 mM BZA. The thrombin was found by clotting assay. The product was a single band (unreduced) by gel electrophoresis. It was stored at -80°C in 0.7 M LiCl, 2.5% polyethylene glycol 6000, 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.0 (24). Thrombin coagulant activity remained constant under these conditions for more than a year.

Radiolabeled fragment 1 and prothrombin 1 were prepared by incubating 8 mg of tritiated prothrombin (190,000 cpm per mg) in 4 ml of 50 mM NaCl, 25 mM Tris-Cl, pH 7.5, with 2 units of bovine thrombin per ml for 30 min at 37°C. The reaction was stopped by adding 5 ml of 1% polyethylene glycol 400, 24% p-toluenesulfonic acid (concentration determined by refractive index) was added, and the samples were centrifuged (Beckman Microfuge 152, 4 min). The supernatants were aspirated into clean tubes, and triplicate samples were counted in 1 ml of toluene-based scintillation mixture (Baker “Scintrex” containing 5% water to clarify the scintillant) using a Beckman LS-3155T counter.

Fluorescence Measurements—These were made in an Aminco SPF 500 ratio spectrophotofluorimeter with xenon lamp. The proteins were diluted in 1% polyethylene glycol 4000/TBS, which was also used as the reference.

RESULTS

Development of the Assay—In agreement with published findings, the only products obtained upon incubation of prothrombin with thrombin that were detected by electrophoresis were fragment 1 and prethrombin 1. The rate of fragment 1 generation should, therefore, reflect the proteolytic activity of thrombin in this reaction.

Since prothrombin carries about 65% of its sialic acid complement on the fragment 1 region (Asn residues 77 and 101, Refs. 11, 27), a feasible method of measuring initial rate was to radiolabel the substrate with tritium (see “Method”), extract the radiolabeled fragment from samples taken during the reaction and measure the rate of appearance of tritium in the extracts by liquid scintillation counting. To find a suitable

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1 The abbreviations used were: BZA, benzamidine hydrochloride; EGTA, ethylene glycol bis(β-aminoethylether)N,N’-tetraacetic acid; SDS, sodium dodecyl sulfate.

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extractant, tritiated fragment 1 and prothrombin, purified chromatographically, were compared with tritiated prothrombin with respect to their solubility in a number of complexing acids. It was found that 4% p-toluenesulfonic acid was optimal for recovering fragment 1; fragment 1 was 40% soluble, whereas prothrombin and prothrombin 1 were only 2.5% soluble. The recovery of prothrombin and fragment 1 was proportional to the total final concentration up to 400 μg and 3 μg/ml, respectively, i.e. the proteins partition between supernatant and precipitate at concentrations to be used in kinetic studies. To check whether fragment 1 could be extracted from mixtures containing excess prothrombin, such as would be encountered during initial rate measurements, purified fragment 1 at several concentrations up to 0.5 and 2 μg/ml, respectively, was added to prothrombin at two concentrations, 11 and 57 μg/ml. The recovery of radioactivity in the 4% p-toluenesulfonic acid supernatant was proportional to the amount of fragment 1 added. These experiments show that 4% p-toluenesulfonic acid (pH approximately 1.0) is a suitable medium for making initial rate measurements. As little as 0.1 pmol of fragment 1 can be detected and measured in the presence of 10 pmol of prothrombin in a single sample, with 0.02% albumin as carrier protein, 5 mM BZA, and 10 mM EDTA also present.

When tritiated prothrombin at a concentration of 100 μg per ml was incubated with 2 units of thrombin per ml, the prothrombin was completely cleaved in 2 to 3 min, as judged by changes in staining pattern on SDS-polyacrylamide gel electrophoresis. The amount of radioactivity extracted into the 4% p-toluenesulfonic acid supernatant after complete cleavage was consistently 43 ± 2% of the total for all batches of tritiated prothrombin. This figure was used to calculate the molarity of substrate converted in unit time during initial rate measurements, assuming a negligible decrease in the concentration of soluble tritiated prothrombin.

It was then determined whether the initial rate of release of radioactivity into the p-toluenesulfonic acid supernatant, v₀, was a function of the enzyme concentration at lower levels of thrombin. Tritiated prothrombin at 28 μg (0.39 nmol) per ml was incubated with four concentrations of thrombin between 0.01 and 0.08 unit (0.1 to 0.8 pmol) per ml. The rate curves are shown in Fig. 1a. The secondary curve, Fig. 1b, shows that the slope is proportional to thrombin concentration. The technique, therefore, gives a valid measurement of initial rate.

For the determination of kinetic parameters, v₀ values were measured at 12 prothrombin concentrations between 0.06 and 4 nmol (4 to 288 μg) per ml at a thrombin concentration of 0.02 unit (0.2 pmol) per ml. Rates were measured over 3-min time courses, five determinations at each substrate concentration. The rate curves were linear. The values of v₀ were determined by linear regression analysis and expressed as the mean of the five determinations ± SE. The direct plot of v₀/E versus (prothrombin) was fitted to a rectangular hyperbola (Michaelis-Menten equation) by a weighted nonlinear least squares program (Fig. 2). The Kₘ is 1.77 ± 0.22 nmol per ml (127 μg per ml), and the kₑₐₜ is 413 ± 24 min⁻¹.

This value of Kₘ differs from the one of 10 μg per ml given in a preliminary report (28). The disagreement appears to be due to a variable degree of alteration in the structure of prothrombin produced by the tritiation procedure. Overall, greater losses of activity by one-stage clotting assay were reflected in lower Kₘ values. Thus, more denatured prothrombin is a more effective substrate for thrombin, as would be expected of proteolytic reactions in general. The radiolabeled prothrombin used in the present determinations was at least 80% active by clotting assay. The extent of the reaction, measured in terms of the proportion of radiolabel released by thrombin under initial rate conditions, was unaffected by adding unlabeled fully active prothrombin up to a mole fraction of 0.9. This suggests that the present substrate is as close as possible to being kinetically normal and that the corresponding high value for Kₘ is correct.

It was also possible that there could be variation in the kinetic constants according to the batch of thrombin used. The thrombin throughout was more than 95% in the α-form by gel electrophoresis (see "Methods"), and several batches at a given concentration (determined by coagulation assay) were found to cleave 50 μg of prothrombin per ml at the same rate to within 5%, suggesting that cleavage was governed by similar kinetic parameters. Whether β-thrombin also catalyzes the reaction, and with different parameters, was not investigated.

Effect of Ca²⁺ on Proteolysis of Prothrombin by Thrombin—It was noted early on that when 1 mM EDTA was present, the rate of proteolysis of prothrombin by thrombin was about 4-fold higher than when EDTA was absent. This suggested that there was inhibition either by Ca²⁺ ions which had remained bound to prothrombin throughout its purification or by trace heavy metal ions, or both. To distinguish between these possibilities, EGTA, a specific chelator of Ca²⁺ ions, was substituted for EDTA. The rate was intermediate between that with EDTA and that without any chelator,
implying that both Ca\(^{2+}\) and other metals were present. However, the rate of proteolysis decreased upon addition of more EGTA, suggesting that EGTA might itself be inhibitory. The experiments described here were, therefore, performed in the presence of 1 mM EDTA, to which Ca\(^{2+}\) was added where necessary at concentrations exceeding 1 mM; any effects of stray metal ions were thus prevented.

When rates of cleavage of prothrombin by thrombin were measured in the presence of Ca\(^{2+}\) ions and phospholipids, inhibition by Ca\(^{2+}\) was specifically found. The rate of fragment 1 release was between 10- and 30-fold slower in the presence of 1 mM CaCl\(_2\), according to the batch of prothrombin used. Purified phospholipid (equimolar dioleoylphosphatidylserine and dioleoylphosphatidylcholine) micelles, prepared by sonication and chromatography (13), did not affect the reaction rate in the absence of Ca\(^{2+}\); and had no additional effect in the presence of Ca\(^{2+}\), and so were subsequently omitted.

Ca\(^{2+}\) inhibition was rapidly reversible by manipulating the relative concentrations of Ca\(^{2+}\) and EDTA present (Fig. 3). When 54 pg (0.75 nmol) of prothrombin, 0.05% albumin, 54 pg (0.75 nmol) of prothrombin, 0.2 unit of thrombin per ml were incubated with 1 mM EDTA, rapid cleavage occurred, releasing 30% of the total tritium in 6 min (curve d). Addition of 5 mM CaCl\(_2\) at 2.5 min to an identical mixture stopped proteolysis (curve b). When 5 mM CaCl\(_2\) was present initially, no reaction could be measured (curve d). Chelation of this Ca\(^{2+}\) with 10 mM EDTA at 2.5 min allowed the reaction to commence (curve c), with no detectable lag.

**Characterization of the Inhibition by Ca\(^{2+}\)**—Two approaches were used to investigate the inhibition of proteolysis by Ca\(^{2+}\). First, 0.69 nmol of prothrombin and 0.1 pmol of thrombin per ml were incubated for 30 min in the presence of increasing concentrations of free Ca\(^{2+}\) up to 1.5 mM. Reciprocal rates (each the mean of two measurements) were plotted versus Ca\(^{2+}\) concentration (Dixon plot, Fig. 4a). The plot is curved, showing that binding of Ca\(^{2+}\) to at least two sites takes part in inhibition of the reaction. The direct plot of the same data (Fig. 4b) suggests that the inhibitory binding may be positively cooperative, because it is somewhat sigmoid in shape at the lowest Ca\(^{2+}\) concentrations. This varied in degree with different batches of tritiated prothrombin; however, the reliability of the rate measurements was not sufficient to warrant a firm conclusion that the inhibition is cooperative.

Second, the effect of Ca\(^{2+}\) on the Michaelis-Menten parameters was investigated. \(v_0\) was measured at a range of (prothrombin) from 0.076 to 2.8 nmol (5 to 200 pg) per ml in the presence of 1 mM CaCl\(_2\). The curve was fitted to a rectangular hyperbola as before. The values of \(K_m\) and \(k_{cat}\) have little meaning because the reaction is close to being first order with respect to prothrombin concentration over this range. Clearly Ca\(^{2+}\) causes an increase in \(K_m\), but any change in \(k_{cat}\) cannot be discerned. It may, therefore, be concluded that the inhibition is of a classical competitive or mixed type.

**Confirmation of Effect of Ca\(^{2+}\) by Fluorescence Measurements**—There was a chance that the inhibitory effect of Ca\(^{2+}\) was peculiar to those prothrombin molecules which had been chemically modified by tritiation. This was unlikely, because dilution of the labeled prothrombin with unlabeled, fully active protein did not alter the percentage of label released into 4% p-toluenesulfonic acid in the presence of Ca\(^{2+}\). Also, the level of tritiation of the substrate was about 1 g atom per mol, suggesting that the bulk of the prothrombin molecules bore the radiolabel. Nevertheless, it was desirable to confirm the Ca\(^{2+}\) effect, at least qualitatively, by an independent method using native prothrombin. For this, intrinsic fluorescence measurements were used.

Prothrombin shows intrinsic fluorescence, maximum at 330 nm, when excited at 280 nm (Fig. 6a, broken line). Upon addition of Ca\(^{2+}\) (5 mM), fluorescence intensity dropped slightly, but the shape of the emission peak remained largely unchanged (Fig. 6b, broken line). This agrees with the observations of Prendergast and Mann (29). When a catalytic amount of thrombin was added to prothrombin in the absence of Ca\(^{2+}\), there was a red shift of about 6 nm in the emission
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Fig. 4. Dependence of inhibition of fragment 1 formation upon Ca²⁺ concentration, at fixed prothrombin and thrombin concentrations. Incubations contained 0.05% albumin, 1 mM EDTA, 0.05 nM of 3H-II, and 0.2 pmol (0.02 unit/mL) of thrombin per ml. XO, total Ca²⁺ available in the presence of 1 mM EDTA, determined calorimetrically with murexide. a, reciprocal specific rate ((E)/υ₀) versus (Ca²⁺). Each point is the mean of two separate determinations (30-min time courses). The curve was fitted to a parabola using an unweighted, nonlinear least squares program, and is described by the equation, y = 0.1462x² - 0.0268x + 0.0062. b, Direct plot of specific rate (υ₀/(E)) versus (Ca²⁺); same data as in (a). The curve was drawn by inspection. The sigmoid shape of the curve accounts for the negative inflexion of Fig. 4a and the negative exponent in the equation describing the curve.

Fig. 5. Rate of fragment 1 formation as a function of prothrombin concentration in the presence of 1 mM Ca²⁺. Incubations contained 1 mM EDTA, 2 mM CaCl₂, 0.05% albumin, TBS, and 2 pmol (0.2 unit/mL) of thrombin per ml. Other details as in legend to Fig. 2, including curve fitting. The same substrate concentrations (abscissa) were covered as in Fig. 2, but the ordinate is expanded to accommodate lower values of specific rate.

peak (Fig. 6a, solid line), conveniently measured as an increase in intensity with time at 370 nm. The rate of increase was proportional to the thrombin concentration (not shown). Almost no shift occurred upon addition of thrombin up to 1 unit/mL in the presence of Ca²⁺ (Fig. 6b, solid line). The emission spectrum of thrombin alone (not shown) was the same in the presence and absence of 5 mM Ca²⁺. These results confirm that Ca²⁺ inhibits the reaction of prothrombin with thrombin studied in more detail using tritiated prothrombin and (tentatively) suggest that the main effect of Ca²⁺ is directed toward the substrate, to which Ca²⁺ is already known to bind at a number of sites. Any binding of Ca²⁺ to thrombin is not evident from fluorescence measurements.

Fig. 6. Fluorescence ratio emission spectra for prothrombin and its cleavage products formed in the absence (a) and presence (b) of Ca²⁺. Excitation wavelength, 280 nm. Solutions contained 16 μg of unlabeled prothrombin per ml in 1% polyethylene glycol 4000/TBS 1 mM EDTA. a, spectrum in the absence of Ca²⁺ before (-----) and after (----) incubation with thrombin (1 unit per ml) at ambient temperature for 10 min. b, spectrum in presence of 5 mM CaCl₂ before (-----) and after incubation with thrombin as in (a). Note quenching due to Ca²⁺, indicated by lower relative intensities compared to (a).
DISCUSSION

The discontinuous assay of the formation of radiolabeled fragment 1 permitted the kinetics of the cleavage of bovine prothrombin by bovine thrombin to be studied in a simple system, and Michaelis-Menten parameters were determined. A comparison of these parameters with those obtained for bovine thrombin in its action on fibrinogen (30, 31) is given in Table I. The parameters obtained in this laboratory for the proteolysis of Factor X by a- VII/tissue factor are also included (29). The $k_{cat}$ for bovine prothrombin on thrombin is rather less than that for thrombin on fibrinogen, but the $K_m$ is also lower; consequently, the coefficient of proteolytic efficiency, $k_{cat}/K_m$, is very similar for the two reactions catalyzed by thrombin. The coefficient for Factor X activation is at least an order of magnitude larger, mainly because the reaction has a lower $K_m$. This is interesting, since the concentration of Factor X is also considerably less than the concentration of fibrinogen in plasma; hence, both fibrinogen and Factor X are at rate-limiting levels in the physiologic situation. Prothrombin is also rate-limiting in its reaction with thrombin, but this information is of less physiologic importance because the $K_m$ is altered in the presence of Ca$^{2+}$ ions (see below).

The finding that Ca$^{2+}$ ions at concentrations in the physiologic range inhibit proteolysis of prothrombin by thrombin was unexpected. We are accustomed to think of Ca$^{2+}$ as a help rather than a hindrance in coagulation (32). This may mean that the phenomenon is unique to this reaction through the characteristics of the substrate. Prothrombin has been shown by physical techniques such as circular dichroism (33), fluorescence (29, 34), and light scattering (35) to undergo a conformation change upon binding of Ca$^{2+}$. This is borne out by the fluorescence data reported here. It is probably binding to the fragment 1 region of prothrombin which has the most profound structural effect, since it is known that binding to fragment 1 also causes a conformation change, is relatively tight, and is positively cooperative (29, 36-38); binding to prothrombin 1 is looser and not cooperative (29, 38). In this regard, it is interesting that there is some indication of cooperativity in the response to Ca$^{2+}$ of the rate of fragment 1 formation; moreover, the concentration of Ca$^{2+}$ at which the reaction rate is reduced by 50% agrees well with the concentration at which prothrombin is half-saturated, 0.6 mM (35, and references cited therein). One might, therefore, suppose that a Ca$^{2+}$-induced conformation change in prothrombin buries the Arg$^{156}$-Ser bond, or otherwise blocks it, making it inaccessible to thrombin.

Alternatively, inhibition of proteolysis could be afforded by the binding of prothrombin molecules to one another by Ca$^{2+}$ ions. Prothrombin dimerization was studied by Agarwal and co-workers (39) but was seen at higher protein concentrations the binding of prothrombin molecules to one another by Ca$^{2+}$ ions. According to their findings, prothrombin at 50 μg per ml or below would be mainly monomeric unless Ca$^{2+}$ ions alter the equilibrium between prothrombin molecules in favor of dimerization. There are no data published on this at present, although aggregation could account for the discrepancies in the numbers of Ca$^{2+}$-binding sites obtained for prothrombin in different laboratories.

A direct effect of Ca$^{2+}$ on the enzymic activity of thrombin cannot be entirely ruled out. The increase in $K_m$ reported here could be explained by simple competition of Ca$^{2+}$ at the active site, although the lack of a reliable value of $k_{cat}$ means that this assumption is not justified yet. It may be significant that Ca$^{2+}$ stabilizes two other substrates of thrombin, Factors V and VIII, during their purification and storage. This has been assumed to be structural stabilization (31, 40) but could in addition be because Ca$^{2+}$ ions protect them from attack by traces of thrombin.

The implications of the kinetic parameters for coagulation are as follows. In this discussion I make free use of the assumption that the activation of prothrombin is quantitative as well as qualitatively similar in the bovine and human systems. The argument of qualitative similarity appears to be valid. Fragment 1 formation is catalyzed wholly by thrombin during activation of purified human (7) and bovine (6) prothrombin. Also, unpublished preliminary results using the assay of release of radiolabeled fragment 1 suggest that negligible amounts are formed during in vitro coagulation of bovine plasma, in agreement with the findings of Aronson and co-workers using human plasma and blood (19). Whether similar kinetic constants govern the cleavage of human as of bovine prothrombin by thrombin remains an open question at present, as does the extent of inhibition of cleavage by Ca$^{2+}$ ions.

In the hypothetical case of coagulation without Ca$^{2+}$ ions, the $K_m$ (the concentration of prothrombin at which the rate of cleavage is 0.5 $V_{max}$) is similar to the plasma concentration. One would therefore expect, in the absence of inhibition, cleavage of about 200 molecules of prothrombin per molecule of thrombin produced. Two-stage assays show that the amount of thrombin produced during coagulation in vitro of human blood (determined by integration over the thrombin generation test curve, for example, Ref. 41) is 30 unit min per ml, or 0.3 nmol min per ml. This amount of free thrombin, generated in spite of the presence of irreversible thrombin inhibitors, would lead to cleavage of 60 nmol of prothrombin per ml, considerably more than the 1.4 nmol per ml present in plasma at the outset. In other words, in this hypothetical case, no unactivated prothrombin would survive the coagulation process but would all be converted to fragment 1 and prothrombin 1.

The fact that fragment 1 cannot be found in clotting blood (19) therefore requires explanation. The first question is, does Ca$^{2+}$ inhibition account entirely for the failure of fragment 1 to appear? The data show that the rate of prothrombin degradation would be reduced by about 30-fold assuming a free Ca$^{2+}$ concentration in blood of about 1.5 mM (42). The amount of prothrombin attacked would thus be reduced to 2 nmol per ml which, while it is a significant reduction, would not prevent the reaction from being detectable. A second factor to be taken into account is that the generation of thrombin lowers the prothrombin concentration, and hence the rate at which it is cleaved. The maximum thrombin concentration reached during coagulation in glass is 10 units (0.1 NIH unit) per ml (41) before it dwindles as a result of the action of antithrombin III and other inhibitors. This accounts for consumption of 7% of the plasma prothrombin, not a substantial decrease. Actually, direct determinations of prothrombin consumption during coagulation show a rather

### Table I

<table>
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<th>Enzyme/substrate</th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
<th>$k_{cat}/K_m$</th>
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<tr>
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$^a$ I have assumed 1 NIH unit of thrombin is 10 pmol, as in present work.

$^b$ Data of Bando et al. (31) interpreted by van Nispen et al. (30).
greater drop than this, of about 20% (43). This would decrease the rate of attack by thrombin by about 20%, the reaction being approximately first order with respect to prothrombin when Ca²⁺ is present. Allowing for this, the conversion of prothrombin to fragment 1 should still occur to the extent of 1.6 nmol per ml.

It must therefore be concluded that, although prothrombin is significantly protected from proteolysis by the presence of Ca²⁺ in a purified system, this is not the only reason why fragment 1 is not detectable during coagulation. Some other mechanisms must be at work. It may be mentioned that no evidence of either substrate or product inhibition was seen in the present study. This leaves the possibility that other substrates of thrombin compete successfully with prothrombin. Among these, fibrinogen and also Factor XIII predominate, at least on the basis of their concentrations in plasma. The possible role of these substrates as inhibitors of the proteolysis of prothrombin by thrombin is under investigation.

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