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## Dissociation of Esterolytic and Clotting Activities of Thrombin by Trypsin-Binding Macroglobulin.* (30800)

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Recently, a protein from human plasma has been isolated which forms an enzymatically active complex with trypsin and is able to protect trypsin from inhibition by soybean trypsin inhibitor(1). Preparations of this material have demonstrated it to be an alpha-2 macroglobulin which may be identical with the component previously described as 19S-glycoprotein(2). This binding protein differs from serum trypsin inhibitors which appear to reversibly inactivate the proteolytic and esterolytic activities of the enzyme(3,4).

Trypsin-binding macroglobulin (TBM) is also capable of binding chymotrypsin as an enzymatically active complex(4). Because of the similarities in certain physical and kinetic properties of these estero-proteolytic enzymes with those of thrombin(5), it was of interest to determine if TBM might also bind thrombin and thereby be implicated in the blood coagulation mechanism.

Evidence is presented here for the binding of human thrombin by preparations of TBM. The nature of the binding is unusual, however, in that the complex formed is enzymatically inactive against fibrinogen but readily hydrolyzes synthetic esters such as N-p-tosyl-L-arginine methylester (TAMe) and N-carbobenzoxy-L-tyrosine p-nitrophenyl ester (CTN). This dissociation of the esterolytic and clotting activities of thrombin may have important implications concerning the "binding" and/or "active" site of this enzyme as well as the role this complex may have in blood clotting.

*Materials and methods.* Prothrombin was prepared from human plasma(6) and converted to thrombin in 25% (w/v) sodium citrate(7). Two human thrombin preparations were used in these studies; one had a specific activity of 1,280 N.I.H. clotting units and 256 TAMe units/mg protein, and the other, which had been partially purified by ion exchange chromatography(8), was about 2,500 clotting units and 410 TAMe units/mg. Bovine thrombin (specific activity = 2,900 clotting units/mg) was purified from a commercial source (Parke-Davis & Co.) by chromatography(8) and used in some experiments. Clotting assay procedures at 28°C for prothrombin (2-stage method) and thrombin (N.I.H. method) were as described pre-

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viously using N.I.H. bovine thrombin (Lot 3B) as reference(7).

TAMe esterase activity was determined at 28°C with the aid of an expanded scale thermostatic recording pH stat (E. H. Sargent & Co.) by the method of Ehrenpreis and Scheraga(9). The rate of TAMe hydrolysis was determined from the slope of the zero order curve obtained for the initial 10-15% hydrolysis of the substrate as calculated from the NaOH uptake. The unit of TAMe esterase activity is defined as the number of 0.1 $\mu$m $H^+$ titrated/min/ml of 0.01 M TAMe/ml of sample at pH 8.0, 28°C, $\mu = 0.15$ (8,9).

The method of Martin, Golubow and Axelrod(10) was used to determine CTN esterase activity at 32°C by measurement of the rate of appearance of p-nitrophenol at 400 m$\mu$ (Beckman DU spectrophotometer). The final reaction mixture, in which the organic solvent was eliminated, had an initial CTN substrate concentration of $2.4 \times 10^{-5}$ M. A unit of CTN esterase activity is arbitrarily defined in this paper as the increase in one absorbancy unit ($\Delta$ A)/minute at 400 m$\mu$ due to the release of p-nitrophenylate ion from the substrate.

Trypsin-binding macroglobulin was prepared by Miss Sally Howard, and we are also indebted to her and to Mr. John DeGroot and Dr. John W. Mehl for some of the characterization of this material. The macroglobulin was obtained from Cohn Plasma Fraction III-O as described previously(1), and with further steps which will be reported later. Several preparations, representing different degrees of purification, were used. The ultracentrifuge patterns are presented in Fig. 1. The best preparation (Fig. 1,C) has a major peak of 17S macroglobulin, with a minor component of 19S responsible for the skewed leading edge. Additional details including the method of assay of trypsin-binding capacity of these products are presented elsewhere(1).

Gel filtration (exclusion chromatography) on 1 × 60 cm columns of Sephadex G-100 (Pharmacia) was carried out as previously detailed(7) except the equilibrating solvent was either 0.5 M NaCl or 0.15 M $CaCl_2$.

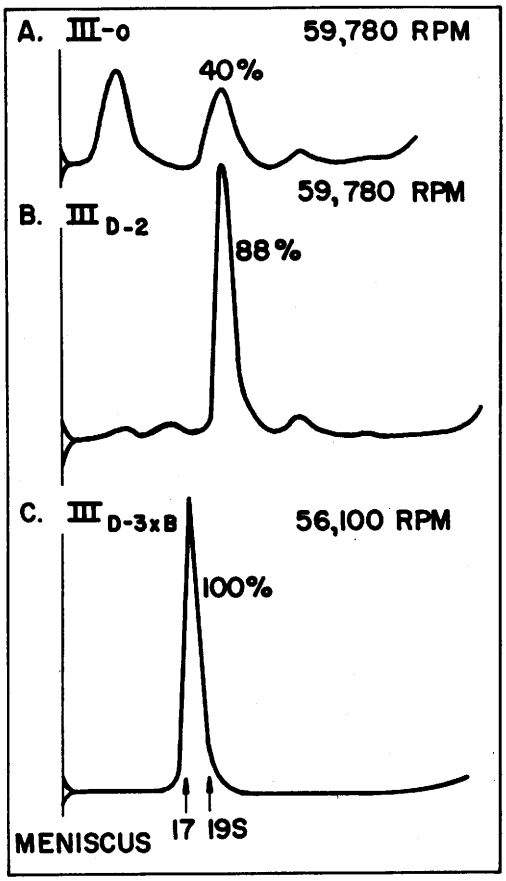


FIG. 1. Ultracentrifuge analysis of fractions obtained during purification of trypsin-binding macroglobulin from Cohen Plasma Fraction III. A. Lipid poor euglobulin from Cohen Fraction III. B. After DEAE chromatography and precipitation at low ionic strength. C. After treatment with Bentonite. Concentration was 5 mg protein/ml of NaCl-phosphate buffer pH 7.0, $\mu = 0.15$. Drawings made from photographs taken at 16 min after rotor reached full speed. Sedimentation is from left to right.

Protein was measured by the Lowry procedure(11) and sedimentation velocity analysis was performed in a Spinco Model E analytical ultracentrifuge as described elsewhere (1,7).

*Results.* Fig. 2 represents data obtained from a typical experiment in which TBM was incubated with thrombin and clotting and TAMe esterase activities of the enzyme measured at intervals. The final concentration of TBM in the incubation system is approximately equal to that in normal plasma and the thrombin activity used is equivalent

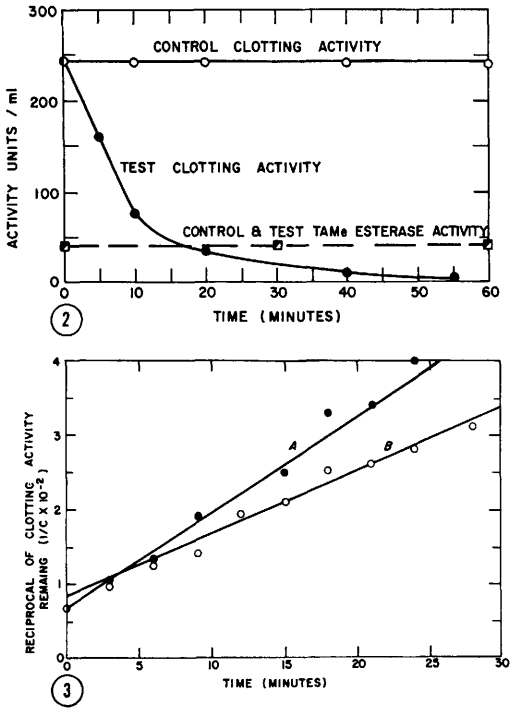


FIG. 2. Clotting and TAMe esterase activity of human thrombin during incubation with trypsin-binding macroglobulin. Test system consisted of 2 mg of TBM Fraction III D-2 and 0.19 mg of thrombin in a total volume of 1 ml, pH 7.2, $\mu = 0.2$. Control system did not include TBM. Aliquots taken at intervals indicated and assayed for clotting and TAMe esterase activity.

FIG. 3. Reciprocal second order rate plot for thrombin clotting inactivation by trypsin-binding macroglobulin. A. Incubation system similar to test system in Fig. 2 except 0.1 mg of thrombin/ml used. B. System similar to A, but TBM heated to 56° for 30 minutes before analysis. Best fitting line through experimental points calculated by method of least squares.

to that potentially available as plasma prothrombin(6).

As in all experiments using TBM of varying degrees of purity, the thrombin clotting activity was progressively inactivated with time but the TAMe esterase activity was not. Similar results were obtained if clotting and CTN esterase activity were measured. Addition of heparin (0.1 mg/ml) to the incubation system in Fig. 2 did not increase the degree of clotting inactivation, but rather to some extent prevented it.

Fig. 3 is a second-order rate plot of the data obtained in an experiment similar to Fig. 2 which indicates the inactivation of thrombin clotting activity follows an apparent bimolecular reaction. Also included in the Figure are data obtained for a heat-treated TBM preparation.

Table I presents data obtained on the trypsin-binding capacity and apparent second order rate constants for clotting inactivation for the various TBM preparations examined. The data thus obtained indicate that in spite of the varying purity of the TBM examined, the rate of clotting inactivation correlates reasonably well with the concentration of the 17-19S components in the products as well as their trypsin-binding capacity. (Table Ib,f,k).

In addition, it is also evident that bovine thrombin is inactivated by TBM at about the same order of magnitude as the human enzyme (Table Ic,g) and that the rate of inactivation is temperature dependent (Table Id). Adding small amounts of pooled serum to the TBM-thrombin incubation system increases the rate of thrombin inactivation (Table Ia and e, a and h). In comparing the best preparation of TBM available (Table Ik) with that of serum (Table Ia) and assuming TBM to be about 2 mg/ml of normal serum, TBM could account for a substantial amount of the "antithrombin" activity of serum.

To study the nature of the dissociation of the esterolytic and clotting activity of thrombin by TBM, several experiments were performed in which the enzyme macroglobulin incubation system was subject to gel filtration analysis. An example of such an experiment is given in Fig. 4,A. In this experiment, thrombin was incubated with TBM until about 80% of the clotting activity had been inactivated before the system was placed on the gel column. A control experiment consisting of thrombin and buffer was also run for comparison purposes (Fig. 4,B). Inspection of the protein and enzyme elution pattern presented in Fig. 4,A indicates that better than 70% of the esterase activity is bound to the TBM and appears at the void volume of the column (Vo = 14 ml). The remaining esterase activity appears at the elution volume (Ve = 23 ml) anticipated for thrombin alone from the control experiment (Fig. 4,B). No clotting activity could be detected after

TABLE I. Relationship Between Thrombin-Binding Capacity as Measured by Clotting Inactivation and Trypsin-Binding Capacity of Several Trypsin-Binding Macroglobulin Preparations.

| Fraction No.* | 17-19S macroglobulin, % of total protein | No. of experiments | Apparent second order rate of thrombin-binding (k)† Avg | Range | Trypsin-binding capacity, μg trypsin/mg | Remarks |
|---|---|---|---|---|---|---|
| a) Serum (3.5 mg)‡ | — | 2 | $2.0 \times 10^{-4}$ | $1.5–2.5 \times 10^{-4}$ | — | — |
| b) $III_{0-2}$ | 40 | 4 | $4.1 \times 10^{-5}$ | $1.1–7.5 \times 10^{-5}$ | 34 | — |
| c) $III_{0-2}$ | " | 1 | $8.0 \times 10^{-5}$ | — | — | Bovine thrombin |
| d) $III_{0-2}$ | " | 1 | $1 \times 10^{-4}$ | — | — | Incubated 37°C |
| e) $III_{0-2}$ | " | 1 | $7 \times 10^{-4}$ | — | — | +7 mg serum/ml |
| f) $III_{0-3}$ | 44 | 1 | $9.0 \times 10^{-5}$ | — | 47 | — |
| g) $III_{0-3}$ | " | 1 | $6.0 \times 10^{-5}$ | — | — | Bovine thrombin |
| h) $III_{0-3}$ | " | 1 | $2.3 \times 10^{-3}$ | — | — | +3.5 mg serum/ml |
| i) $III_{D-2}$ | 88 | 3 | $2.0 \times 10^{-3}$ | $1.3–2.6 \times 10^{-3}$ | 60 | — |
| j) $III_{D-2}$ | " | 1 | $8.0 \times 10^{-4}$ | — | — | TBM heated at 56°C for 30 min |
| k) $III_{D-3\,BX}$ | 100 | 1 | $2.6 \times 10^{-3}$ | — | 80 | — |

* Unless otherwise stated, the incubation system was identical to that given in Fig. 2, in which 2 mg TBM and human thrombin varying from 240 to 8 NIH units in a final volume of 1 ml at 28°C was assayed at intervals for clotting activity.

† Reciprocal rate of thrombin inactivation in N.I.H. units/min.

‡ A single pool of human serum was used in these experiments.

gel filtration of the enzyme-TBM complex although this could readily be measured in the control experiment. Similar results were obtained using an incubation system consisting of the purest TBM available (Fig. 1,C) and a thrombin preparation (specific activity = 2,500 NIH units/mg) followed by gel filtration on a column equilibrated with calcium chloride. These experiments indicate that both clotting and esterase activity of thrombin are bound to TBM; the former being inactivated and the latter being active in the protein complex thus formed.

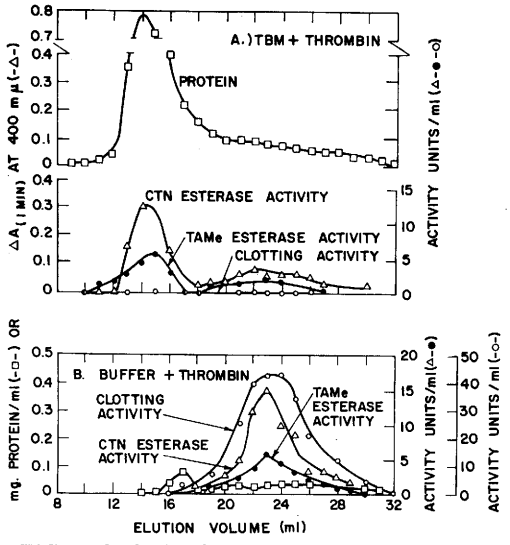


FIG. 4. Sephadex G-100 elution pattern for thrombin after incubation in the presence and absence of trypsin-binding macroglobulin for 1 hr. A. Thrombin-TBM complex with initial activity of 34.4 clotting units and 75 TAMe units/ml. B. Control, without TBM, with initial activity of 176 clotting units and 70 TAMe units/ml. Column (1 x 60 cm) was equilibrated with 0.5 M NaCl; 1.9 ml samples applied and 1 ml effluent fractions collected and analyzed as indicated.

Attempts to determine whether or not the thrombin-TBM complex can be dissociated to free the bound clotting activity of thrombin have thus far been unsuccessful. Preparations of commercial trypsin (Mann Research Laboratories) when added in small amounts and which are generally rapidly bound to TBM (Table I) do not regenerate clotting activity from the complex. This is probably due to technical difficulties since trypsin rapidly proteolyzes thrombin and trypsin is still enzymatically active when bound to the macroglobulin. Partial success has been obtained in dissociating the esterase activity from the complex. This was achieved by pooling the major protein peak shown in Fig. 4 and dialyzing against water. Under these conditions TBM is insoluble and about 70% of the bound esterase activity can be recovered in the aqueous supernatant.

In other experiments TBM had no effect on the 2-stage prothrombin assay procedure. After incubation of TBM and a prothrombin preparation followed by gel filtration through

Sephadex G-200, there was complete separation of the 2 proteins indicating that no binding complex was formed. In one experiment a TBM thrombin complex isolated after gel filtration was tested for its corrective ability on Factor VIII and Factor IX deficient plasmas using the partial thromboplastin test system(12). In spite of the relatively high esterase activity and bound, but inactive clotting activity of this complex, neither clotting factor activity could be detected in this test system. There was also no evidence that TBM interfered with the fibrinogen-fibrin transformation. Incubation of TBM (2 mg) with fibrinogen (0.1 mg) in a total volume of 1 ml for over 2 hours did not effect its coagulation by thrombin.

*Discussion.* While this study was in progress, a report appeared by Steinbuch *et al* (13) in which they studied the effect on various clotting tests of their preparations of alpha-2 macroglobulin isolated by a different method than ours. They found that their preparations had no influence on coagulation time, recalcified clotting time, one stage prothrombin (Quick), cephalin time or prothrombin consumption. However, their preparations did inhibit the proteolytic action of plasmin (fibrinolysin). With the exception of the latter finding, most of these negative results might be explained by the fact that the inactivation of thrombin clotting activity by TBM seems to be a time dependent mechanism as demonstrated in Fig. 2. The apparent bimolecular nature of this reaction (Fig. 3,A) indicates it to be a function of the concentration of the enzyme and TBM and consequently the number of available binding sites on the macroglobulin. The fact that heat treatment of TBM alters these binding sites is also apparent from the change in the rate constant of inactivation. (Fig. 3,B and Table Ij.)

Seegers has summarized the various antithrombins which have been reported to exist in serum into 6 broad categories(14). It is difficult to decide whether or not TBM might fall into one of these classifications, particularly since it inactivates the clotting but not the esterase activity of thrombin. Previous work by others seems to indicate that the anti-thrombin activity of whole serum progressively inactivates both enzymic activities of thrombin. However, there is no information as to whether the rates of inactivation are identical. We have some preliminary experiments which seem to indicate that they are not. If confirmed, this would seem to suggest that TBM might play an important role in the clotting process particularly in regard to the possibility of residual thrombin existing in an active esterolytic form in serum. It should also be pointed out that many methods used for isolation and purification of the various serum or plasma antithrombins are based on ammonium sulfate fractionation. Trypsin-binding macroglobulin is inactivated by exposure to this salt and almost totally destroyed at concentrations exceeding 0.2 M(1). It remains to be determined if ammonium sulfate has a similar effect on the binding of thrombin by TBM.

In spite of the extensive purification and characterization of the thrombin preparations used(8), the possibility exists that they might be contaminated with another esterolytic enzyme which must be considered in interpretation of these results. This contaminant is likely to be thrombokinase (activated Factor X) described by Milstone(15) which, with the exception of clotting activity, has many properties similar to thrombin. However, the esterolytic activity of thrombokinase is inhibited by soybean trypsin inhibitor while the esterase activity of our purified thrombin preparations is not(8). In this respect, and in view of the fact that no other esterolytic enzyme could be detected in our preparations under a variety of experimental conditions(8), it would seem to preclude the possibility of thrombokinase playing a significant role in the results obtained.

Finally, we would like to draw attention to the fact that it is generally believed that thrombin shares an "active" site similar in structure and properties to several other estero-proteolytic enzymes (*i.e.*, trypsin, chrymotrypsin)(5). The dissociation of the estero-proteolytic activity of thrombin by TBM may indicate that this active site is masked in some manner so that the complex is capable of acting on small synthetic esters (*i.e.*,

TAMe, CTN) but is incapable of hydrolyzing sensitive bonds on larger molecules (*i.e.*, fibrinogen). Alternatively, these enzymatic activities of thrombin may be associated with different "binding sites" if not different molecules.

*Summary.* Preparations of alpha-2 trypsin-binding macroglobulin purified from Cohn Plasma Fraction III-O have been found to bind human and bovine thrombin. The enzyme-macroglobulin complex formed is inactive against fibrinogen but readily hydrolyzes synthetic substrates such as N-p-tosyl-L-arginine methyl ester and N-carbobenzoxy-L-tyrosine p-nitrophenyl ester. Inactivation of thrombin clotting activity by binding to the macroglobulin follows an apparent bimolecular reaction. The complex has been isolated by Sephadex G-100 gel filtration and partial success has been obtained in dissociating the esterase activity but not the clotting activity from the macroglobulin. These findings may have important implications concerning the "binding" or "active site" of the estero-proteolytic activities of thrombin as well as the role which the enzyme-macroglobulin complex may have in blood clotting.

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## Endotoxin Cytotoxicity: Role of Cell-Associated Antibody.* (30801)

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The ability of bacterial endotoxins to damage cells has been demonstrated both *in vivo*(1) and *in vitro*(2,3). It has been suggested (*cf.* 3) that many of the biological effects of endotoxins, including the enhancement of host resistance to a large variety of microbial pathogens, may result from the release of intracellular materials from certain cells, such as macrophages, following their exposure to endotoxin. Among the active materials released may be oligodeoxyribonucleotides since it has been demonstrated that these oligomers, resulting from the enzymatic digestion of DNA, stimulate both antibody production, as measured by the Jerne technique(4), and phagocytosis, as measured by carbon clearance(5).

A test system(6) which utilizes monolayer cultures of peritoneal macrophages and permits a quantitation of the cytotoxicity of endotoxin *in vitro* has been developed. This test system has now been employed to de-

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