Activation of Trypsinogen and Plasminogen by Thrombin Preparations*

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ABSTRACT: The remarkable similarities between trypsin and thrombin in their action on certain synthetic substrates and proteins prompted investigation of the effects of thrombin on trypsinogen and plasminogen, zymogens which are activated by trypsin. Purified thrombin was added to the zymogens, and the mixtures were assayed for esterolytic and caseinolytic activities. Both zymogens were rapidly and progressively activated, in accordance with enzyme kinetics. That this was due to thrombin rather than to possible contaminants in the thrombin preparations was demonstrated by means of appropriate inhibitors, by procedures known to terminate thrombic action, and by separation of certain known contaminants. During trypsinogen activation trichloroacetic acid soluble ninhydrin-staining material was released in parallel with the appearance of esterolytic activity.

In the instance of trypsinogen, activation was about two-thirds of that by trypsin, but plasminogen activation by thrombin was much higher, and equalled that induced by streptokinase and euglobulin. Similar effects were obtained with two thrombin derivatives: acetylated thrombin, and a water-insoluble preparation obtained by coupling thrombin to a diazonium salt of *p*-amino-DL-phenylalanine–L-leucine copolymer. The observations indicate another biologic role for thrombin, which suggests the pathway whereby clot lysis can be linked to coagulation.

Thrombin, trypsin, and plasmin are strikingly similar (Laki *et al.*, 1958; Ronwin, 1960; Laki and Gladner, 1964). They catalyze hydrolysis of TAMe,[1] BAMe, BAEe, LMe (Sherry *et al.*, 1965), their respective amides (Sherry and Troll, 1954), as well as LEe (Ehrenpreis *et al.*, 1957). Thrombin and trypsin also accelerate the release of *p*-nitrophenol from carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester and from carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Martin *et al.*, 1959; Lorand *et al.*, 1962), and catalyze hydrolysis of the esters of γ-guanidino-L-α-toluene-*p*-sulfonamidobutyric acid (Baird *et al.*, 1965). Moreover, at least one biologic substrate, fibrinogen, is highly vulnerable to these three enzymes (Pechet and Alexander, 1962), certain biologically critical peptide bonds apparently being readily susceptible to their action. The enzymes also digest fibrin (Alkjaersig *et al.*, 1959; Guest and Ware, 1950; Seegers, 1961; Brakman *et al.*, 1964) as well as casein (Kunitz, 1947; Remmert and Cohen, 1949; Pantlitschko and Gründig, 1957; Magnusson, 1965).

Since trypsin can convert trypsinogen and plasminogen into the respective proteases (Kunitz, 1939; Alkjaersig *et al.*, 1958), we were prompted to investigate whether thrombin also could activate these zymogens. The data indicate that it does, further indicating similarity of thrombin to trypsin.

Materials and Methods. Purified bovine factor II (prothrombin), obtained by the procedure of Goldstein *et al.* (1959), was converted to thrombin by 25% sodium citrate (Seegers *et al.*, 1950), which was subsequently removed by dialysis. The specific activity of the 19 preparations used (citrate thrombin) was 200–500 NIH units/mg. Acetylated thrombin (three preparations) was prepared from this material, according to Landaburu and Seegers (1959). Water-insoluble thrombin was prepared by B. Alexander in collaboration with Dr. A. Rimon (Weizmann Institute of Science) by the technique of Hussain and Newcomb (1964). The final preparation, containing 4.8% protein,[2] was suspended in 0.1 M phosphate buffer (pH 7.4), and stored in the frozen state. Its esterase activity did not change during 7 months. Trypsin (Worthington) was twice crystallized, salt-free. SK (Varidase R) and EACA were generously provided by Lederle Laboratories. Trypsinogen (Worthington), one time crystallized, contained 46% protein and 54% $MgSO_4$, and <0.16 μg of trypsin/mg of protein. Plasminogen (Lot No. 2259–164–28), derived from

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[1] Abbreviations: TAMe, *p*-toluenesulfonyl-L-arginine methyl ester; BAMe, benzoyl-L-arginine methyl ester; BAEe, benzoyl-L-arginine ethyl ester; BAA, benzoyl arginine amide; LEe, lysine ethyl ester; SK, streptokinase; SBI, soybean trypsin inhibitor; EACA, ε aminocaproic acid; DFP, Thrombin Z'g'n Unit, thrombin unit in terms of zymogen activation activity.

[2] The protein was determined by measuring the valine content, as has been applied to water-insoluble trypsin (Bar-Eli and Katchalski, 1963).

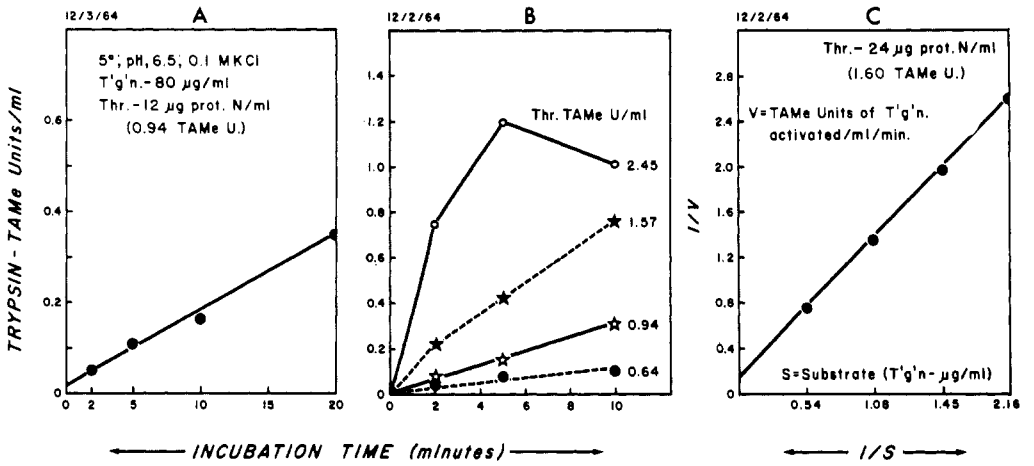

FIGURE 1: Activation of trypsinogen by thrombin. Interaction in 0.15 M KCl (pH 6.5) at 5°. Equal volumes of 0.005 N HCl were added to aliquots removed at various intervals, and they were then assayed for esterase activity (see Methods), which are expressed as trypsin TAMe units/ml. A: Trypsinogen, 2.0 μg/ml, and thrombin, 0.94 TAMe units/per ml. B: Trypsinogen, 2.0 μg/ml. Thrombin TAMe units/ml: ●---●, 0.64; ☆—☆, 0.94; ★---★, 1.6; ○—○, 2.5. C: Thrombin, 1.6 TAMe units/ml. Trypsinogen concentration (S) in micrograms of protein per milliliter. Reaction stopped after 10 min by addition of 0.005 N HCl. V represents the zymogen activated in TAMe units per minute per milliliter.

human Cohn plasma fraction III, and plasmin obtained from this same lot of plasminogen by spontaneous activation in glycerol, were kindly provided by Dr. A. J. Johnson of New York University. Euglobulin was prepared from human serum according to Norman's procedure (1957). α-Chymotrypsinogen and SBI were from Worthington. Albumin (bovine, 1%) was from Armour Co. TAMe was procured from K and K Laboratories; BAA was from Mann Research Laboratories. Hammarsten Casein (General Biochemicals), was purified according to Norman (1957). Benzamidine HCl and DFP were from K and K Laboratories.

General Procedure. TAMe (0.08 M) (2 ml) plus 1.6 ml of 0.15 M KCl solution was adjusted with 0.1 N NaOH in the pH-Stat to pH 7.9, and KCl solution was then added to a final volume of 3.9 ml. To this reaction mixture was added 0.1 ml of the solution to be tested. To determine spontaneous TAMe hydrolysis, 0.1 ml of the KCl solution was added instead. Following are the final concentrations of enzyme, zymogen, and inhibitors (per milliliter): thrombin, 0.64–3.4 TAMe units; trypsin, 0.86 and 1.7 TAMe units (0.2 and 0.4 μg); plasmin, 1.4 TAMe units; trypsinogen, 2 μg; plasminogen, 40 μg; SBI, 0.8 μg; and benzamidine HCl, 0.4 μg.

Enzyme–substrate concentrations were those required for a zero-order reaction. TAMe hydrolysis was determined at 25°, maintained by a double-walled reaction vessel perfused with water circulated from a thermostatically controlled bath, and protons liberated were measured by automatically recorded titration with 0.1 N NaOH in the pH-Stat (Radiometer, Copenhagen). The reaction was followed for at least 10 min. Zymogen activation is expressed in terms of TAMe units of the active enzyme elaborated from the respective zymogens.

The experiments were performed as follows: the enzyme, the zymogen, or a combination of both was added to the reaction mixture directly; or the enzyme and the zymogen were incubated at 5° or 37°, at pH 6.5[3] except where otherwise indicated. At selected intervals aliquots were removed, an equal volume of HCl (0.005 N) was added to terminate the thrombin action in the trypsinogen activation experiments, and 0.1 ml of the mixture was then added to the reaction mixture.

Caseinolytic Assay. Zymogen and enzyme in appropriate dilutions were combined and incubated as above at 5 or 37° for varying intervals, at the end of which aliquots were removed, and in the instance of the trypsinogen activation experiments the reaction was stopped by the addition of an equal volume of HCl (0.005 N). No attempt was made to stop the reaction in the plasminogen experiments. Of these zymogen–enzyme mixtures, 1.0 ml was mixed with 1.0 ml of casein solution previously warmed to 37°, and after 30 min at 37°, 1.0 ml of trichloroacetic acid (15%) was added. Ten minutes later another 2.0 ml of trichloroacetic acid was added, the mixtures were left for 1 hr at room temperature, then centrifuged at 4° for 10 min at 1500 rpm, and the supernatants were filtered and measured for absorbance at 280 mμ. One caseinolytic unit is defined as that activity which under the experimental conditions increased the optical density by 1 unit/min.

[3] In preliminary experiments pH 6.5 was found to be optimal for activation of trypsinogen and plasminogen by thrombin.

Factor VII (convertin) was determined according to Owren and Aas (1951); factor X (Stuart), according to Bachmann *et al.* (1958); thrombin clotting activity, according to Seegers and Smith (1942). Ammonia arising from BAA hydrolysis was determined by the Conway method (Davis and Smith, 1955), modified by automatically recording the uptake of acid (HCl, 0.01 N) in the pH-Stat at pH 6.6.

Results

The Effect of Thrombin on Trypsinogen. As evident in a typical experiment (Figure 1A) the addition of thrombin to trypsinogen induced progressive elaboration of TAMe esterolytic activity. In 43 experiments with all the thrombin preparations the mean maximal enhancement in activity was 0.44 (std dev ±0.13) TAMe unit of trypsin/TAMe unit of thrombin. After reaching a maximum, the activity declined during prolonged interaction, especially at 37°. Under the same conditions the thrombin solution alone loses *ca.* 30% of its esterase activity in 30 min. The initial velocity of activation was closely related to thrombin concentration over the range of 0.64–2.45 TAMe units/ml (Figure 1B). With fixed thrombin concentration and varying trypsinogen the degree of activation at a given incubation time was rectilinear with zymogen concentration over the range of 0.46–1.84 μg of zymogen/ml (Figure 1C). Activation was not enhanced by $CaCl_2$ (0.2 M).

During activation trichloroacetic acid soluble material, measured according to Moore and Stein (1954), was released in parallel and in equivalent amounts on a molar basis, with the appearance of tryptic esterolytic activity (Figure 2). Some thrombin clotting activity (44%) disappeared from the mixture during activation, but substantial amounts were still demonstrable.

Activation by thrombin was compared with that by trypsin. A trypsin solution (0.1 ml) containing 0.40 μg, 0.1 ml of trypsinogen solution containing 8.0 μg in 0.15 M KCl, and 0.2 ml of 0.2 M $CaCl_2$ were mixed and incubated at 5°, pH 6.5. At selected intervals an equal volume of 0.005 N HCl was added to aliquots to stop the reaction (Neurath *et al.*, 1956), and esterolysis was measured. An S-shaped activation curve indicative of an autocatalytic reaction was obtained, but its contour was lower and more extended than that observed by Neurath and Dixon (1957). This we attribute to the suboptimal experimental conditions.[4] The highest rate of activation induced by trypsin was 2.4×10^{-6} μmole of trypsinogen (assumed mol wt, 24,000)/min per TAMe unit of trypsin. This is to be compared with 1.6×10^{-6}/TAMe unit of thrombin. Plasmin, substituted for thrombin and trypsin in equivalent esterase activity, had no effect.

Heating the thrombin preparation at 60–70° in 0.01 N HCl for 5 min followed by rapid cooling and pH restoration to 8.0 (Ronwin, 1956) was found by us to abolish its ability to activate trypsinogen and its esterolytic activity. Under the same conditions trypsin lost only 20% of its esterolytic activity.

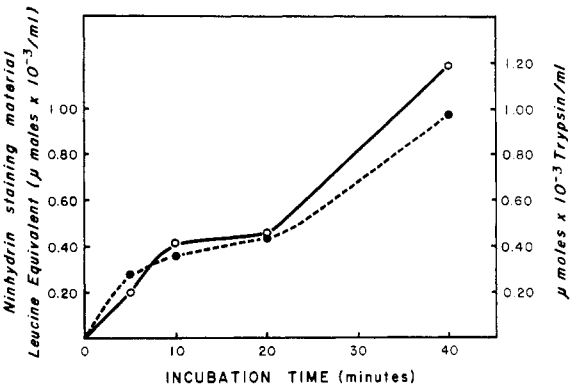

FIGURE 2: Release of ninhydrin-reacting trichloroacetic acid soluble material during activation of trypsinogen by thrombin. Thrombin (1.4 TAMe units/ml) and trypsinogen (6 mg/ml) were incubated at 5°, and at various intervals aliquots were removed to determine the trichloroacetic acid soluble ninhydrin-reacting material (Moore and Stein, 1954) and the evolved esterolytic activity. For the latter, 0.1 ml of the zymogen–thrombin mixture was added to 3.9 ml of 0.005 N HCl. O—O indicates amount of trypsin evolved per milliliter of zymogen–thrombin mixture, calculated on the basis of 0.23 μg of trypsin being equivalent to 1 TAMe unit. ●—● indicates the amount of trichloroacetic acid soluble ninhydrin-reacting material per milliliter, in terms of equivalent amount of ninhydrin-reacting leucine.

The activity evolved in the trypsinogen–thrombin mixture was not attributable to newly produced thrombin or enhancement in the original thrombin activity since it persisted despite the addition of an equal volume of 0.005 N HCl, a procedure found by us to block thrombin esterolysis. Moreover, in other experiments SBI was added instead of HCl. This agent, which does not inhibit thrombin (Tagnon and Soulier, 1946), abolished the evolved esterolytic activity, but did not affect the esterolytic activity of the original thrombin.

Trypsinogen activation was also demonstrated by increases in caseinolytic activity, in seven experiments averaging 3.1×10^{-3} caseinolytic unit (range 2.1–4.8)/ml, equivalent to the activity of 2.7 μg of trypsin. Peak activity was generally attained in 10 min. Also with casein as substrate, activation by thrombin was compared with that by trypsin. Maximal activation by the latter was equivalent to 6.4×10^{-3} unit of tryptic caseinolytic activity/ml; by thrombin, 4.8×10^{-3}.

All of our citrate thrombin preparations contain factors VII and X, without which the parent factor II cannot be activated by citrate (Goldstein *et al.*, 1959; Tishkoff *et al.*, 1960). In order to exclude their possible role in trypsinogen activation, their removal was attempted by starch gel electrophoresis (Tishkoff *et al.*,

[4] Optimal conditions, according to Pechère and Neurath (1957), are: 0.1 M Tris buffer (pH 8.0); $CaCl_2$, 0.05 M; 0°.

TABLE I: Trypsinogen Activation and Esterolytic Activity of Various Thrombin Preparations.

Thrombin Prepn	Mg of Protein/ml of Reaction Mixture	Thrombin Z'g'n Units[a]	Thrombin TAMe Units[b]	Ratio, Thrombin Z'g'n Units: TAMe Units
Citrate	0.006	2.7	0.9	2.9
Prepd by electrophoresis	0.022	2.3	1.1	2.1
Acetylated	0.073	2.0	0.9	2.2
Insol	0.015	2.9	0.3	9.7

[a] One thrombin Z'g'n unit is the amount of thrombin which induced 1% activation/min per ml of reaction mixture, as measured with the pH-Stat after 5 min of incubation at 5°. [b] One TAMe unit is defined as that which within the first 15 min releases from TAMe 1 μmole of acid/ml of reaction mixture (see Methods).

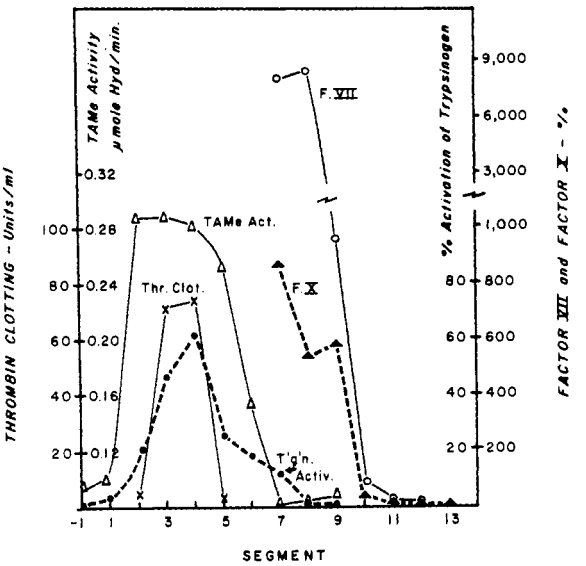

FIGURE 3: Thrombin, factor VII, factor X, TaMe esterase, and trypsinogen activation activities of expressates obtained from starch gel segments of electrophoresis run of a thrombin preparation, assayed as indicated in Methods. Segment designated −1 represents the control, having been taken on the contralateral side of the point of application of the original material. Segments 2–7 were not assayed for factors VII and X because the determination in the presence of thrombin is invalid. The activities of the factors are expressed as per cent based on normal plasma containing 100%. Trypsinogen activation is expressed as enhancement in per cent of the original TAMe esterolytic activity of the enzyme–thrombin mixture obtained at 0 time in the presence of 0.005 N HCl.

1960). No thrombin was lost. The expressates from starch segments that were devoid of thrombin but rich in factors VII and X were inert on both trypsinogen and TAMe (Figure 3). In contrast, expressates which were rich in thrombin clotting and esterolytic activity were highly potent in trypsinogen activation. It cannot be claimed, however, that they were actually devoid of factors VII and X since these factors cannot be assayed in the presence of thrombin. Acetylated thrombin, devoid of clotting activity yet retaining esterolytic activity, activated trypsinogen similarly to its nonacetylated counterpart (Table I).

The insoluble thrombin derivative also activated trypsinogen. It had less clotting activity than native thrombin but it still exhibited relatively good zymogen

TABLE II: Thrombin Activation of Trypsinogen and Zymogen Disappearance.[a]

Incubn Time (min)	Evolved Esterase Activity (Δμmole of TAMe/min) A: Trypsinogen + Thrombin	B: Trypsinogen + Thrombin + Trypsin	C: (B − A) Residual Trypsinogen	D (0.25 − B) Trypsinogen Unaccd for
0	0	0.25	0.25	0
0.5	0.09	0.25	0.16	0
2	0.11	0.16	0.05	0.09
5	0.06	0.11	0.05	0.14
20	0.04	0.10	0.06	0.15
40	0.04	0.09	0.05	0.16

[a] Thrombin and trypsinogen incubated at 5° for varying intervals following which aliquots were removed; the interaction was then stopped by the addition of equal volume of 0.005 N HCl, and then assayed for esterase activity (column A). To measure the residual trypsinogen an equal volume of trypsin plus $CaCl_2$ (0.4 M) was added to other aliquots of the incubating thrombin–zymogen mixture, interaction was permitted for an additional 10 min at 5° at the end of which an equal volume of 0.005 N HCl was added, and the esterase activity was measured (column B).

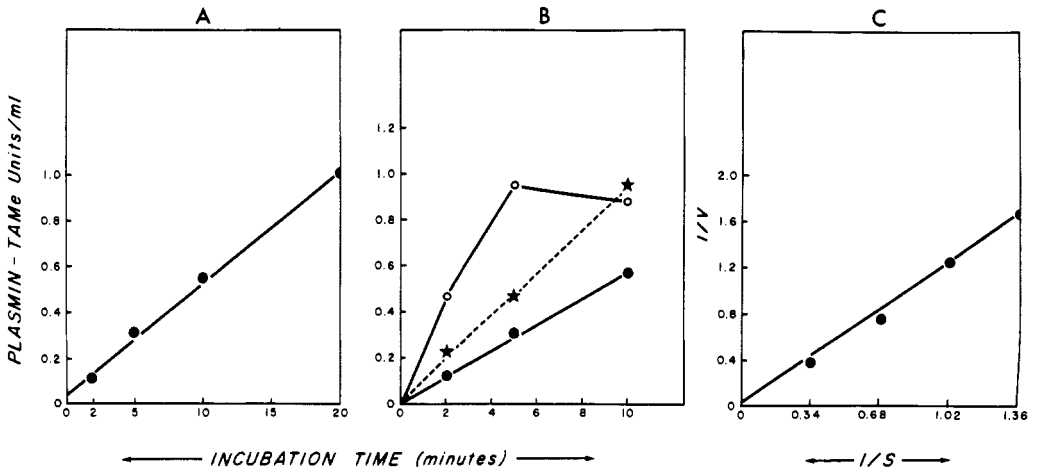

FIGURE 4: Activation of plasminogen by thrombin. Zymogen and thrombin incubated at 0.15 M KCl (pH 6.5) at 5° (A and B), or 37° (C). Aliquots (0.1 ml) of mixture were removed at various intervals and assayed for TAMe esterolytic activity, as described in Methods. A: Plasminogen, 40 μg/ml, and thrombin, 0.94 TAMe unit/ml. B: Plasminogen, 40 μg/ml; thrombin TAMe units per milliliter: ●—●, 0.94; ★----★, 1.6; ○—○, 2.5. C: Thrombin, 1.6 TAMe units/ml. Plasminogen concentration (*S*) in micrograms of protein per milliliter. Interaction at 37° for 2 min, and the mixture was then assayed for esterolytic activity. *V* represents the zymogen activated in terms of TAMe units per minute per milliliter.

activation and esterolytic activity (Table I). Except for the insoluble derivative there was good correlation between the various preparations in both esterolytic activity and trypsinogen activation.

Two possibilities were considered to explain the lower (*ca.* 70%) trypsinogen activation by thrombin than that by trypsin: (a) either activation by thrombin was incomplete; or (b) the total observed activity reflected both zymogen activation and concurrent degradation of thrombin, zymogen, or evolved trypsin. To test the first possibility, trypsinogen was sequentially measured during activation. The data (Table II) show that a substantial amount of the original zymogen could not be accounted for, either as thrombin-evolved trypsin or as trypsin evolved by thrombin plus added trypsin. Also, the discrepancy could not be attributed to spontaneous trypsinogen degradation since the zymogen incubated without thrombin could be fully activated by trypsin. Although some thrombin activity disappears under the experimental conditions, the discrepancy cannot be due to concurrent degradation or block of the original thrombin activity since substantial amounts of thrombin remain in the thrombin–zymogen mixture after maximal activation. Furthermore, a supplement of thrombin did not induce further activation.

To determine whether the system still possessed its activating potential, a supplement of trypsinogen equivalent to the original zymogen was added after 40 min of thrombin–trypsinogen interaction, when maximal activation had occurred. Within 10 min total esterolytic activity increased by 0.71 TAMe unit, an amount within the range evolved *de novo* from the original zymogen. Although the system thus appeared potentially intact, it remained uncertain whether activation of the trypsinogen supplement could be attributed to the original thrombin, to the evolved trypsin, or to both.

Trypsinogen is frequently contaminated with trypsin. To exclude the possibility that this might be involved in the zymogen activation by thrombin, the zymogen was treated with DFP (Gladner and Folk, 1958) which inhibits trypsin. Thrombin activated DFP-treated trypsinogen faster than the native zymogen (2.1×10^{-6} μmoles DFP–trypsinogen activated/min per TAMe unit of thrombin compared with 0.7×10^{-6} for the native zymogen). Under the same conditions the rate of activation of DFP–trypsinogen by trypsin was 1.8×10^{-6} μmoles/mln per TAMe unit of trypsin, compared with 2.4×10^{-6} for untreated trypsinogen.

Plasminogen Activation by Thrombin. The addition of thrombin to plasminogen induced a progressive increase in TAMe esterolytic activity, as evident in a typical experiment in Figure 4A. In 30 experiments the mean maximal activation was 0.46 TAMe unit of plasmin (std dev ±0.17)/TAMe unit of thrombin. Activation was rapid at 5°, faster at 37°, but at the latter temperature the evolved activity was more labile. Glycerol, known to stabilize plasmin (Alkjaersig *et al.*, 1958), in 17% concentration prevented this decline in activity. As with trypsinogen, thrombin activation of plasminogen appeared to be enzymatic (Figure 4B and C), and prior acid–heat treatment of the thrombin obviated its action.

Plasminogen activation by thrombin was compared with that induced by SK plus human euglobulin.[5] The

[5] The concentrations of reactants in the reaction mixture were, per milliliter: 0.73 mg of plasminogen in 0.15 M KCl at pH 4.5; 0.23 ml of a human euglobulin preparation diluted 1:10 with 0.15 M KCl, pH 7.4 (equivalent to euglobulin in 0.012 ml of serum); and 0.13 mg of SK (also in 0.15 M KCl).

maximal activity induced by these was equivalent to 0.90 TAMe unit of plasmin/ml, compared with 0.79 unit for thrombin. Plasminogen activation by SK–euglobulin was restricted to a pH range of 7.1–7.5, whereas thrombin was still active down to 5.5.

With trypsin as the activator (Alkjaersig *et al.*, 1958) the initial rate of activation was equivalent to 0.036 TAMe unit of plasmin/min per TAMe unit of trypsin. The comparative value for thrombin was 0.063. SBI, which inhibits plasmin (Kline, 1960) but not thrombin (Tagnon and Soulier, 1946), abolished the evolved esterolytic activity. EACA (0.1 M) failed to inhibit the esterolytic activity of thrombin, whereas it blocked 36% of the activity evolved in the thrombin–plasminogen mixture.

As with trypsinogen, residual plasminogen in the mixture was measured during activation by thrombin. Within 30 sec the zymogen decreased markedly, and after 2 min had practically disappeared (Table III, column C). Again as with trypsinogen, the activating potential of the system was challenged with a supplement of plasminogen (40 μg/ml). After an additional 10 min of incubation the esterolytic activity increased from 0.29 μmole of TAMe hydrolysis/min to 0.55. On the contrary, supplements of thrombin produced no further elaboration of activity.

TABLE III: Plasminogen Activation by Thrombin and Zymogen Disappearance.[a]

	Evolved Esterase Activity (Δμmole of TAMe/min)		
	A	B	C (B − A)
Incubn Time (min)	Plasminogen + Thrombin	Plasminogen + Thrombin + SK–Human Euglobulin	Residual Plasminogen
0	0	0.20	0.20
0.5	0.09	0.14	0.05
2	0.22	0.22	0
10	0.19	0.22	0.03
20	0.12	0.15	0.03
40	0.12	0.12	...

[a] Activation reactions run in 0.01 M phosphate buffer (pH 7.4) at 5° for varying intervals following which aliquots were removed for assay of evolved esterase activity (column A) (see general procedure in Methods). To measure the residual plasminogen, an equal volume of SK–euglobulin was added to other aliquots, the interaction was continued at 5° for an additional 15 min, and the esterase activity was measured (column B). Residual plasminogen (column C) is represented by the difference between columns A and B.

Plasminogen activation by thrombin was also studied with casein as substrate. The plasminogen preparation alone showed no caseinolysis. As observed by others (Pantlitshko and Gründig, 1957; Magnusson, 1965), thrombin itself exhibited significant caseinolysis. Since this was not blocked by SBI, or by 0.1 M EACA which partially blocks plasmin caseinolysis (Alkjaersig *et al.*, 1959), contamination of the thrombin with trypsin or plasmin could be excluded. In 13 experiments thrombin added to plasminogen increased caseinolytic activity by an average of 2.3×10^{-3} unit/ml (range, 0.65–3.9). This value was identical with that obtained under the same conditions by activating with SK–euglobulin. EACA (0.1 M) blocked 78% of the evolved activity; 0.5 M EACA blocked it completely.

Acetylated thrombin containing less than 0.1 NIH unit of clotting activity/ml could still activate plasminogen (Table IV). The insoluble thrombin derivative also was active (Table IV).

As in the trypsinogen experiments, fractions obtained during further purification of thrombin by starch gel electrophoresis were assayed for plasminogen activation and esterase activity (Figure 5). Here also, only the expressates from segments containing maximum clotting activity were active on TAMe and plasminogen. Fractions containing factors VII and X but devoid of thrombin were inactive.

Since trypsin or trypsinlike entities exist in plasma (Gullick, 1963), and since commercial as well as purified thrombin may contain fibrinolytic contaminants (Hudemann, 1940; Guest and Ware, 1950; Brakman *et al.*, 1964), an attempt was made to exclude such possible contaminants as underlying the observed effects. Benzamidine inhibits trypsin (Mares-Guia and Shaw, 1963, 1965). We found that it was inert toward esterolytic activity of thrombin and only slightly inhibitory on plasmin. Within 20 min at 5° an equal weight of benzamidine added to trypsin reduced its esterolytic activity by 80%, and its caseinolytic activity by 43%. Conversely, benzamidine added to the thrombin–plasminogen mixture had no effect on elaboration of the esterolytic and caseinolytic activity, or on the activities after they evolved. Obviously this inhibitor could not be used in the trypsinogen activation experiments because it inhibits trypsin.

The question of contaminants in the thrombin preparations was also explored with BAA, which is readily hydrolyzed by trypsin but not by thrombin (within certain concentrations) (Sherry and Troll, 1954; Gullick, 1963). Up to 1.6 TAMe units of thrombin[6] were inert toward BAA, yet this amount readily activated both zymogens.

The action of thrombin on albumin and chymotrypsinogen A was also studied. No increase in esterase activity was observed with albumin, whereas thrombin

[6] This particular preparation contained 9.4 NIH thrombin clotting units/TAMe unit.

TABLE IV: Plasminogen Activation and Esterolytic Activities of Various Thrombin Preparations.

Thrombin Prepn	Mg of Protein/ml of Reaction Mixture	Thrombin Z'g'n Units[a]	Thrombin TAMe Units[a]	Ratio, Thrombin Z'g'n Units: TAMe Units
Citrate	0.012	2.1	1.8	1.2
Prepd by electrophoresis	0.022	4.1	1.3	3.2
Acetylated	0.065	3.9	1.4	2.8
Insol	0.015	3.1	0.3	10.0

[a] Thrombin Z'g'n Unit and TAMe Unit are defined in Tables I and II.

added to chymotrypsinogen produced chymotryptic activity, as measured with *N*-acetyl-L-tyrosine ethyl ester as substrate, but no TAMe activity (Engel and Alexander, 1964). This will be reported separately.

Discussion

Although the proteases evolved by thrombin were not separated and identified, the evidence indicates that trypsinogen and plasminogen are activated by this enzyme, a protease generally considered to be highly specific for fibrinogen (Lorand, 1952; Bettelheim and Bailey, 1952; Blombäck, 1963; Laki, 1963; Laki and Gladner, 1964). SBI, an inhibitor of both trypsin and plasmin but not thrombin, abolished the evolved activity. In respect to plasminogen activation, inhibition of evolved activity was similarly obtained with EACA, an agent also inert on thrombin.

Maximal trypsinogen activation was *ca.* two-thirds that obtained with trypsin. Plasminogen activation was much higher than that induced by trypsin, and was equal to that obtained with SK–euglobulin. The discrepancy with trypsinogen is attributable to concurrent degradation of the zymogen during its activation, of the product evolved, or of both. A similar discrepancy has been observed with cathepsin B as activator (Greenbaum *et al.*, 1959), and in activation by mold proteases (Desnuelle and Rovery, 1961). In the instance of plasminogen essentially all the zymogen was activated, accounted for as the active protease.

The possibility that activation induced by the thrombin preparations was caused by trypsin or trypsin-like contaminants appears most unlikely. Certain observations regarding activation by thrombin *vis-à-vis* trypsin or SK–euglobulin are relevant and noteworthy. In contrast to trypsin, trypsinogen activation by thrombin does not proceed autocatalytically. This is true also for activation by cathepsin B (Greenbaum *et al.*, 1959) and by mold proteases (Desnuelle and Rovery, 1961; Hofmann, 1960).

Thrombin works more rapidly, and over a broader pH range (5.5–8) than either trypsin or SK–euglobulin. Furthermore, the trypsin inhibitor, benzamidine, did not prevent thrombin action. Moreover, our thrombin

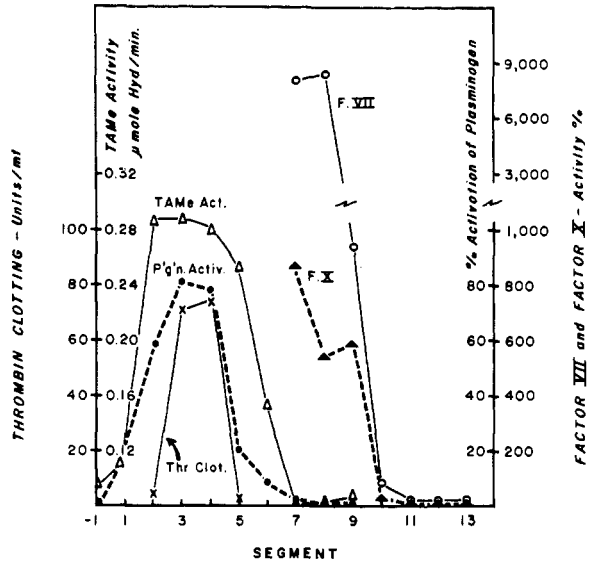

FIGURE 5: Thrombin, factor VII, factor X, TAMe esterase, and plasminogen activation activities of expressates obtained from starch gel segments of electrophoresis run of a thrombin preparation, assayed as indicated in Methods. Segment designated −1 represents the control, having been taken on the contralateral side of the point of application of the original material. Segments 2–7 were not assayed for factors VII and X because the determination in the presence of thrombin is invalid. Plasminogen activation is expressed as enhancement in esterolytic activity in per cent of the thrombin–zymogen mixture, obtained at 0 time.

was inert on BAA whereas trypsin readily catalyzed its hydrolysis.

The experiments with DFP-treated trypsinogen also exclude a possible contaminant of the thrombin as responsible for activation since trypsin acts on it more slowly than on the native zymogen. The data also exclude a trypsin contaminant of the zymogen as being involved because the thrombin could activate the treated zymogen even better than the parent material.

Trypsinogen activation by trypsin and some other proteases is induced by cleavage of a specific lysyl–isoleucine bond (Neurath, 1964). Presumably thrombin acts at the same site since this enzyme influences hydrolysis of lysyl esters (Ehrenpreis *et al.*, 1957), lysyl–amide bonds (Köbele, 1962), and lysyl–alanyl bonds (Scheraga, 1961). Assuming that thrombin, like trypsin, can catalyze cleavage of carbon terminal lysyl and carbon terminal arginyl peptide bonds in proteins, one may conclude from what is known of the structure of trypsinogen (Walsh *et al.*, 1964) that 16 bonds (14 lysyls and 2 arginyls) are potentially susceptible to thrombin. Which one, or combination, of these is involved in activation by thrombin remains to be determined by terminal amino acid analysis or other characterization of the products. For this purpose the insoluble thrombin derivative is admirably suited by virtue of its ready removal from the zymogen–thrombin mixture.

Also, thrombin can now be included with the group of agents known to activate plasminogen–trypsin, plasmin, SK, and urokinase. As to its mode of action certain points are noteworthy. That activation is only partially inhibited by 0.1 M EACA suggests that the enzyme activates plasminogen *via* a pathway different from SK–euglobulin (Alkjaersig *et al.*, 1959). The action of the thrombin preparation, moreover, cannot be due to a *plasmin* contaminant of thrombin, since in our experience and that of Alkjaersig *et al.* (1959) plasmin activates the zymogen much more slowly than we observed with thrombin, and much more plasmin is required. The same considerations hold for a possible trypsin contaminant (Alkjaersig *et al.*, 1959).

Our data on plasminogen activation provide no information as to whether only one, or several plasmin(s) evolve, as has been observed in activation by SK–euglobulin (Markus and Ambrus, 1960). Here, too, the insoluble thrombin derivative would be extremely useful in further study of the activation products.

The observation on plasminogen also can explain the well known phenomenon of clot lysis occurring in parallel with, or consequent to, coagulation. Although most of the thrombin evolving during coagulation is rapidly neutralized by plasma antithrombin, sufficient enzyme can exist not only to clot fibrinogen but also to activate plasminogen.

References

Alkjaersig, N., Fletcher, A., and Sherry, S. (1958), *J. Biol. Chem. 233*, 86.

Alkjaersig, N., Fletcher, A., and Sherry, S. (1959), *J. Biol. Chem. 234*, 832.

Bachmann, F., Duckert, F., and Koller, F. (1958), *Thromb. Diath. Haemorrhag. 2*, 24.

Baird, J. B., Curragh, E. E., and Elmore, D. T. (1965), *Biochem. J. 96*, 733.

Bar-Eli, A., and Katchalski, E. (1963), *J. Biol. Chem. 238*, 1690.

Bettelheim, F. R., and Bailey, K. (1952), *Biochim. Biophys. Acta 9*, 578.

Blombäck, B. (1963), *Thromb. Diath. Haemorrhag., Suppl. 13*, 29.

Brakman, P., Klug, P., and Astrup, T. (1964), *Thromb. Diath. Haemorrhag. 11*, 234.

Davis, N. C., and Smith, E. L. (1955), *Methods Biochem. Analy. 2*, 232.

Desnuelle, P., and Rovery, M. (1961), *Advan. Protein Chem. 16*, 139.

Ehrenpreis, S., Leach, S. J., and Scheraga, H. (1957), *J. Am. Chem. Soc. 79*, 6086.

Engel, A. M., and Alexander, B. (1964), *Federation Proc. 23*, 299.

Engel, A. M., Pechet, L., and Alexander, B. (1963), *Federation Proc. 22*, 561.

Gladner, J., and Folk, J. E. (1958), *J. Biol. Chem. 231*, 393.

Goldstein, R., Le Bolloc'h, A., Alexander, B., and Zonderman, E. (1959), *J. Biol. Chem. 234*, 2857.

Greenbaum, L., Hirshkowitz, A., and Shoichet, I. (1959), *J. Biol. Chem. 234*, 2885.

Guest, M., and Ware, A. (1950), *Science 112*, 21.

Gullick, H. (1963), *New Engl. J. Med. 268*, 851.

Hofmann, T. (1960), *Biochem. J. 74*, 41.

Hudemann, S. (1940), *Kolloid-Z. 92*, 289.

Hussain, Q. Z., and Newcomb, T. (1964), *Proc. Soc. Exptl. Biol. Med. 115*, 301.

Kline, D. (1960), *Thromb. Diath. Haemorrhag. 6*, 118.

Köbele, B. (1962), Ph.D. Thesis, Tübingen.

Kunitz, M. (1939), *J. Gen. Physiol. 22*, 293.

Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.

Laki, K. (1963), *Thromb. Diath. Haemorrhag., Suppl. 13*, 59.

Laki, K., and Gladner, J. A. (1964), *Physiol. Rev. 4*, 127.

Laki, K., Gladner, J., Folk, J. E., and Komins, D. R. (1958), *Thromb. Diath. Haemorrhag. 2*, 3.

Landaburu, R., and Seegers, W. (1959), *Can. J. Biochem. Physiol. 37*, 1361.

Lorand, L. (1952), *Biochem. J. 52*, 200.

Lorand, L., Brannen, W. T., and Rule, N. G. (1962), *Arch. Biochem. Biophys. 96*, 147.

Magnusson, S. (1965), *Arkiv Kemi 24*, 349.

Mares-Guia, M., and Shaw, E. (1963), *Federation Proc. 22*, 528.

Mares-Guia, M., and Shaw, E. (1965), *J. Biol. Chem. 240*, 1579.

Markus, G., and Ambrus, C. M. (1960), *J. Biol. Chem. 235*, 1673.

Martin, C. J., Golubow, J., and Axelrod, A. E. (1959), *J. Biol. Chem. 234*, 1718.

Moore, S., and Stein, W. (1954), *J. Biol. Chem. 211*, 907.

Neurath, H. (1964), *Federation Proc. 23*, 1.

Neurath, H., and Dixon, G. (1957), *Federation Proc. 16*, 791.

Neurath, H., Rupley, J., and Dreyer, W. (1956), *Arch. Biochem. Biophys. 65*, 243.

Norman, D. (1957), *J. Exptl. Med. 106*, 423.

Owren, P. A., and Aas, K. (1951), *Scand. J. Clin. Lab. Invest. 3*, 201.

Pantlitshko, M., and Gründig, E. (1957), *Monatsh. Chem. 88*, 253.

Pechère, J. F., and Neurath, H. (1957), *J. Biol. Chem. 229*, 389.
Pechet, L., and Alexander, B. (1962), *Biochemistry 1*, 875.
Remmert, L. F., and Cohen, P. P. (1949), *J. Biol. Chem. 181*, 431.
Ronwin, E. (1956), *Can. J. Biochem. Physiol. 34*, 1169.
Ronwin, E. (1960), *Biochim. Biophys. Acta 38*, 258.
Scheraga, H. (1961), Protein Structure, New York, N. Y., Academic, p 144.
Seegers, W. H. (1961), *Thromb. Diath. Haemorrhag. 6, Suppl. 1*, 101.
Seegers, W. H., and Smith, H. P. (1942), *Am. J. Physiol. 137*, 348.
Seegers, W. H., McClaughry, R., and Fahey, J. (1950), *Blood 5*, 421.
Sherry, S., Alkjaersig, N., and Fletcher, A. P. (1965), *Am. J. Physiol. 209*, 577.
Sherry, S., and Troll, W. K. (1954), *J. Biol. Chem. 208*, 95.
Tagnon, H., and Soulier, J. (1946), *Proc. Soc. Exptl. Biol. Med. 61*, 440.
Tishkoff, G., Pechet, L., and Alexander, B. (1960), *Blood 15*, 778.
Walsh, K. A., Kauffman, D. L., Sampath-Kumar, K. S. V., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 301.

The Effects of Pressure on F–G Transformation of Actin*

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ABSTRACT: The effects of pressure on F- and G-actin were measured in a range up to 4000 kg/cm^2 at room temperature. F-actin in the absence of adenosine triphosphate (ATP) started to undergo irreversible denaturation at a pressure of *ca.* 1500 kg/cm^2, and complete denaturation at 3000 kg/cm^2, whereas in the presence of ATP the pressure where denaturation began was 2500 kg/cm^2, showing a significant protective effect of ATP *vs.* pressure denaturation of F-actin. The addition of EDTA to the system had no effect on the pressure denaturation curve of F-actin in the absence of ATP. On the other hand, for the system containing both ATP and EDTA, the pressure denaturation curve became almost the same as that without ATP (*i.e.*, irreversible denaturation of F-actin began at *ca.* 1500 kg/cm^2 of applied hydrostatic pressure), suggesting that reversible depolymerization occurred in the presence of ATP, since G-actin is known to denature easily in the presence of EDTA, while F-actin does not. Repolymerization of the depolymerized actin after the release of pressure could be observed under suitable conditions, a result which indicates that the transformation of F- to G-actin had occurred under applied pressure. Therefore, it is inferred that the denaturation curve without ATP would represent a reversible F–G transformation caused by pressure. A volume change estimated from the pressure denaturation curve is calculated as −84 ml/mole of monomer, indicating that a volume increase occurs on polymerization of G-actin, presumably due to hydrophobic bonding between monomers. The effect of ATP to protect F-actin *vs.* pressure denaturation is significant compared with the effects of ADP, Mg^{2+}, and Ca^{2+}, suggesting that the protein's structure is stabilized by an appropriate sterical arrangement of a nucleotide binding site fitted for an ATP molecule. Similar experiments performed on G-actin showed that G-actin which requires low salt solutions is, nevertheless, less stable *vs.* pressure denaturation than is F-actin. The concentration dependence for pressure denaturation of F-actin showed a linear but nonproportional relationship, that is, the amount of protein denatured by pressure is dependent upon the initial protein concentration.

Many studies have been reported on the G–F transformation and on the molecular structure of actin. Recent experiments show that an actin molecule composed of two subunits has a rigid hard core (Mihashi and Ooi, 1965a), and that some structural change of the molecule has occurred in the course of the G–F transformation as judged from observations of the difference spectrum between G- and F-actin (Higashi and Oosawa, 1965). Therefore, on the addition of salts, the G-actin which existed in salt-free solution undergoes a specified change in its conformation necessary for polymerization into the double-stranded helical structure shown by electron microscopic studies (Hanson and Lowy, 1963).

Another important feature of actin is the role played by the divalent cations, Ca^{2+} and Mg^{2+}, and nucleo-

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