Activation of Chymotrypsinogen-A by Thrombin Preparations*

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ABSTRACT: Earlier demonstration that thrombin resembles trypsin in the ability to convert trypsinogen and plasminogen to their respective proteases prompted investigation of its effect on chymotrypsinogen A. Purified thrombin added to the zymogen induced rapid and progressive appearance of esterolytic activity toward acetyltyrosyl ethyl ester. The addition of indole to the zymogen–thrombin mixture after interaction had proceeded obviated esterolysis, indicating that the evolved activity was due to chymotrypsin. Elaboration of the active enzyme from its precursor appeared to take place in accord with enzyme kinetics, the thrombin functioning enzymatically on the zymogen. That this was not due to a possible trypsin contaminant of the thrombin was demonstrated with appropriate inhibitors, by procedures known to block thrombic action, and by separation of certain known contaminants, which were inactive. During zymogen activation trichloroacetic acid soluble ninhydrin-reacting material appeared, but not in parallel with elaboration of the protease. The presence of β-phenylpropionate in the zymogen–thrombin mixture during interaction markedly reduced elaboration of ninhydrin-reacting material whereas esterolytic activity appeared faster. Activation by thrombin was generally about 40% of that by trypsin, evidently because of concurrent degradation of the zymogen.

Similar activation was 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. Compared with the native and acetylated thrombin the insoluble derivative showed strikingly higher activation activity.

Thrombin resembles trypsin in many enzymatic properties (Sherry and Troll, 1954; Miller and Van Vunakis, 1956; Gladner and Laki, 1956; Laki *et al.*, 1958; Martin *et al.*, 1959; Ronwin, 1960; Laki and Gladner, 1964; Sherry *et al.*, 1965; Kézdy *et al.*, 1965), including the ability to activate trypsinogen and plasminogen (Engel *et al.*, 1966). Since cleavage of the ester and peptide bonds of many synthetic substrates (Ehrenpreis *et al.*, 1957; Lorand *et al.*, 1962; Elmore and Curragh, 1963; Baird *et al.*, 1965) and proteins[1] are catalyzed by both enzymes, and since trypsin activates chymotrypsinogen, the question whether thrombin can similarly activate this zymogen was explored.

The cleavage of one particular peptide bond in chymotrypsinogen A, an arginylisoleucine linkage, is paralleled by the appearance of enzymatic activity (Neurath, 1957; Desnuelle, 1960). The initial product obtained is π-chymotrypsin, which, in a subsequent autocatalytic step, is transformed to δ-chymotrypsin. Evidence herewith presented indicates that thrombin also can activate chymotrypsinogen, as might be anticipated from the known specificity of thrombin for certain arginyl ester or peptide linkages.

Materials and Methods

Bovine thrombin (*citrate thrombin*) was derived from purified factor II (*prothrombin*) obtained from oxalated plasma, according to the procedure of Goldstein *et al.* (1959). The factor II was then converted to thrombin in 25% sodium citrate (Seegers *et al.*, 1950), which was subsequently removed by dialysis. The specific activity of the thrombin was 800–1200 NIH clotting units/mg. Some of this material was further purified by starch gel electrophoresis, according to Tishkoff *et al.* (1960).

Experiments were also performed with purer thrombin kindly provided by Dr. Staffan Magnusson of the Karolinska Institute, Stockholm. Prepared by his published procedure (Magnusson, 1965)[2] except for

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[1] Proteolytic effects of thrombin have been observed on proteins other than fibrinogen, *i.e.*, α-casein and β-lactoglobulin (Pantlitschko and Gründig, 1957; Magnusson, 1965; Engel *et al.*, 1966), gelatin (Miller, 1958), secretin (Mutt *et al.*, 1965), and the B chain of insulin (Ehrenpreis *et al.*, 1957). In the last instance, curiously, thrombin catalyzes hydroylsis of a lysyl-alanyl bond, but not arginyl-glycyl (Bailey and Bettelheim, 1955; Laki and Gladner, 1964), nor does it split the arginyl-alanyl bond of glucagon (Gladner *et al.*, 1959).

[2] Preparations so obtained are essentially devoid of plasmin activity, have slight caseinolytic activity, and have been used as a protease in studies on the structure of secretin by proteolytic degradation (Mutt *et al.*, 1965).

final lyophilization from a solution of pH 5.5 instead of 6.0–6.5, it had a specific activity of 2130 NIH clotting units and 24.3 TAMe units/mg of protein.

Acetylated thrombin preparations were obtained from citrate thrombin by the method of Landaburu and Seegers (1959). They had TAMe[3] esterase activities of 1.6–7.7 μmoles/min mg of protein but were essentially devoid of clotting activity.[4] *Water-insoluble thrombin* was prepared by coupling citrate thrombin to diazotized *p*-amino-DL-phenylalanine-L-leucine copolymer (Hussain and Newcomb (1964); Engel *et al.* (1966)). *Trypsin* (twice crystallized and salt free), *chymotrypsinogen A* (chromatographically homogeneous), *α-chymotrypsin* (three times crystallized), δ-chymotrypsin, and γ-chymotrypsin were from Worthington. *ATEe* was from Calbiochem; *indole*, from Nutritional Biochemical Corp.; *β-phenylpropionate* and *benzamidine-HCl*, from K and K Laboratories; *SBI* and *PTI* (both crystallized), from Worthington.

General Procedure. Chymotrypsinogen was mixed in 0.05 M phosphate buffer (pH 7.8) except when otherwise indicated, with thrombin (or its derivatives), or with trypsin at 0°. The enzyme–zymogen concentrations and ratios in terms of protein and TAMe units are given in the text and in the particular experiments presented in the figures. At selected intervals thereafter aliquots were removed and added to an equal volume of 0.001 N HCl to stop the activation reaction (Neurath *et al.*, 1956), and this mixture was assayed for esterolytic activity in a system containing ATEe, $CaCl_2$ (0.4 M), and Tris buffer, pH 8.0 (Cunningham, 1954). Esterolysis was calculated from the continuously recorded uptake of NaOH (0.1 N) in the pH-Stat attached to an automatic buret (Radiometer, Copenhagen). The enzyme–substrate (ATEe) concentration ratios were so selected as to assure a zero-order reaction, assuming complete conversion of chymotrypsinogen to chymotrypsin. The net activity was calculated from the difference between the base uptake of the zymogen–thrombin–ester mixture inhibited by the addition of HCl at zero time and the uptake of the zymogen–thrombin–ester mixture similarly inhibited by HCl addition at sequential intervals of interaction. This difference, expressed as the amount of chymotrypsin present, was assumed to reflect the amount of chymotrypsinogen activated.

The assay system was as follows: 2.0 ml of 0.02 M ATEe, 0.9 ml of 0.005 M Tris buffer (pH 8), 0.9 ml of 0.4 M $CaCl_2$, and 0.1 ml of the enzyme–zymogen combination were adjusted with NaOH in the pH-Stat to pH 7.9 ± 0.05. Distilled water was then added to a final volume of 4.0 ml. This comprised the *reaction mixture*. To determine the spontaneous ATEe hydrolysis, 0.1 ml of the buffer was used instead of the test solution. Final concentrations of other interactants in this mixture, called for by experimental conditions, were, per milliliter: thrombin, 0.28–9.3 TAMe units;[5] trypsin, 0.25–1.0 μg (0.6–2.4 TAMe units); α-chymotrypsin, 0.25 μg; δ-chymotrypsin, 0.37 μg; γ-chymotrypsin, 0.37 μg; chymotrypsinogen, 4.1–62.5 μg; indole, 1.75 μg; β-phenylpropionate, 0.0025 mmole; SBI, 2.5 μg; PTI, 1 μg; benzamidine-HCl, 0.005–10 μg.

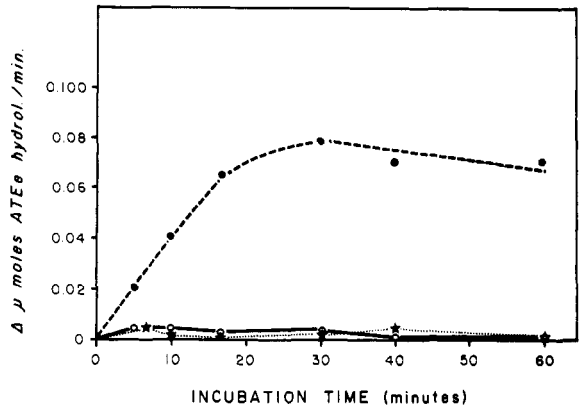

FIGURE 1: ATEe hydrolysis by a citrate thrombin preparation, by chymotrypsinogen, or by a combination of both, incubated in 0.05 M phosphate buffer (pH 7.8) at 0°. At sequential intervals an equal volume of 0.001 M HCl was added to aliquots, which were then assayed for esterolytic activity (see Methods). The concentrations were, per milliliter of reaction mixture: thrombin, 2.32 TAMe units (0.58 μg of protein); chymotrypsinogen, 8.2 μg. ●---●, thrombin plus chymotrypsinogen; O——O, chymotrypsinogen; ★---★, thrombin.

The experiments were performed in two ways: either the enzyme, zymogen, or both were added directly to the reaction mixture, or the enzyme and zymogen were preincubated for varying intervals at 0°, following which HCl was added. Aliquots were then assayed for esterase activity at 25°, maintained by a double-walled reaction vessel perfused with water from a constant-temperature bath. The amount of protons liberated, indicated by the base uptake, was measured for at least 15 min. Caseinolytic activity of the thrombin preparations was determined by the method of Norman (1957).

Factor VII (convertin) was assayed according to Owren and Aas (1951); factor X (Stuart), according to Bachmann *et al.* (1958); and thrombin clotting activity, according to Seegers and Smith (1942). Trichloroacetic acid soluble material which was released during zymogen activation and which reacted with

[3] Abbreviations used: TAMe, *p*-toluenesulfonyl-L-arginine methyl ester; ATEe, acetyl-L-tyrosine ethyl ester; SBI, soybean trypsin inhibitor; PTI, pancreatic trypsin inhibitor.

[4] The range of their clotting activities was 105 sec to 60 min, values which reflect only trace activities which cannot be converted to NIH clotting units.

[5] One TAMe unit is that amount of enzyme which within the first 15 min releases from TAMe (0.04 M) 1 μmole of acid/ml of reaction mixture at 25° and pH 7.9.

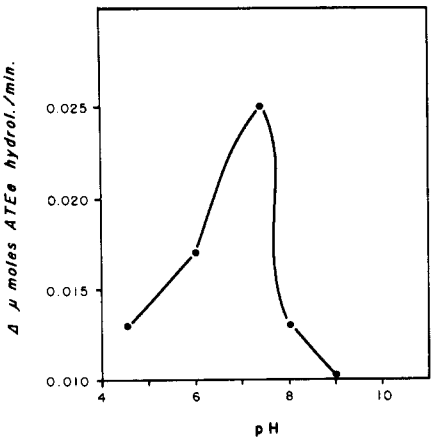

FIGURE 2: Thrombin activation of chymotrypsinogen at varying pH. Thrombin (1.5 TAMe units/ml of reaction mixture) and chymotrypsinogen (8.2 μg/ml) were interacted at 0° for 15 min, and the mixtures then assayed. Activation is expressed as increase in micromoles of ATEe hydrolyzed per minute per milliliter of reaction mixture.

ninhydrin was measured according to Moore and Stein (1954).

Results

As evident in a typical experiment (Figure 1), thrombin added to chymotrypsinogen promptly and progressively induces an increase in ATEe esterase activity. In 37 experiments with all the thrombin preparations the mean maximal increase was 0.66 μg of chymotrypsin (range 0.1–2.4; std dev ±0.41)/TAMe unit of thrombin. The much more highly purified preparation of Magnusson produced ATEe esteratic activity equivalent to 1.6 μg of chymotrypsin (expressed in terms of δ-chymotrypsin)/TAMe unit. In an experiment run concurrently with one of our citrate thrombin preparations, it was observed that 1.8 μg of chymotrypsin evolved/TAMe unit.

Prior heating of the thrombin preparations at 65° for 5 min in 0.01 N HCl, followed by prompt cooling and pH adjustment to 8.0, obviated their effects. In contrast, similarly treated trypsin retained its ability to activate chymotrypsinogen. Moreover, when an equal volume of HCl was initially incorporated in the thrombin–chymotrypsinogen mixture, esterolysis was negligible. This excluded esterolysis as being due independently to the thrombin or the zymogen.

Between pH 4.5 and 9.0 activation was optimal at 7.4 (Figure 2), the optimal temperature was 0° (range 0–37°), and the kinetics were consistent with enzymatic behavior of the thrombin (Figure 3). Although the zymogen as well as the thrombin alone exhibited slight esterase activity, their respective activities remained constant (Figure 1).

With trypsin as activator, protease elaboration proceeded as described by Neurath and Dixon (1957). No autocatalysis was observed. Compared with trypsin activation which was considered to be 100%, activation by thrombin attained approximately 40% within 15 min, after which the curve of activation became level, or declined in some instances. In a typical experiment the specific activating activity of the thrombin was 2.3 × 10^{-6} μmole of chymotrypsinogen activated/min per TAMe unit of thrombin, compared with 6.5 × 10^{-6}/TAMe unit of trypsin. Here the enzyme:zymogen protein ratios were approximately the same (1:35), but the TAMe unit:zymogen ratio for thrombin was 1.43 times that of trypsin:zymogen (0.86 thrombin TAMe unit:8.2 μg of chymotrypsinogen; 0.6 trypsin TAMe unit:8.2 μg of chymotrypsinogen). In another experiment in which the zymogen was concurrently activated under identical conditions by approximately the same number of TAMe units of each enzyme in a ratio to zymogen of 1:48, the amount of zymogen activated by thrombin was 38% of that activated by trypsin, calculated in terms of concurrently assayed ATEe hydrolysis by δ-chymotrypsin.

Addition of more thrombin to the chymotrypsinogen–thrombin mixture after 60-min interaction produced

TABLE 1: Disappearance of Chymotrypsinogen during Its Activation by Thrombin.[a]

Incubation Time (min)	Evolved Esterase Activity (μmole/min) Thrombin + Chymotrypsinogen A	Thrombin + Chymotrypsinogen + Trypsin B	Residual Chymotrypsinogen C (B−A)	Chymotrypsinogen Unaccounted for D (0.062−B)
0	0	0.062	0.062	0
10	0.025	0.057	0.032	0.005
20	0.031	0.055	0.024	0.007
40	0.031	0.037	0.006	0.025
60	0.023	0.038	0.015	0.024

[a] Reactions were run at pH 7.8 in 0.05 M phosphate buffer at 0° for varying intervals following which aliquots were removed, the interaction in them was stopped by the addition of an equal volume of 0.001 N HCl, and they were then assayed for ATEe esterase activity (column A) (see Methods). To measure residual chymotrypsinogen an equal volume of trypsin solution plus $CaCl_2$ (0.4 M) was added to other aliquots of the thrombin–zymogen mixture, the incubation was continued for 60 more min at 0°, an equal volume of 0.001 N HCl was then added, and the esterase activity was measured. The residual chymotrypsinogen (C) is calculated from the difference between A and B. Unaccounted for zymogen is calculated from the difference between the value at zero time and the other values in B.

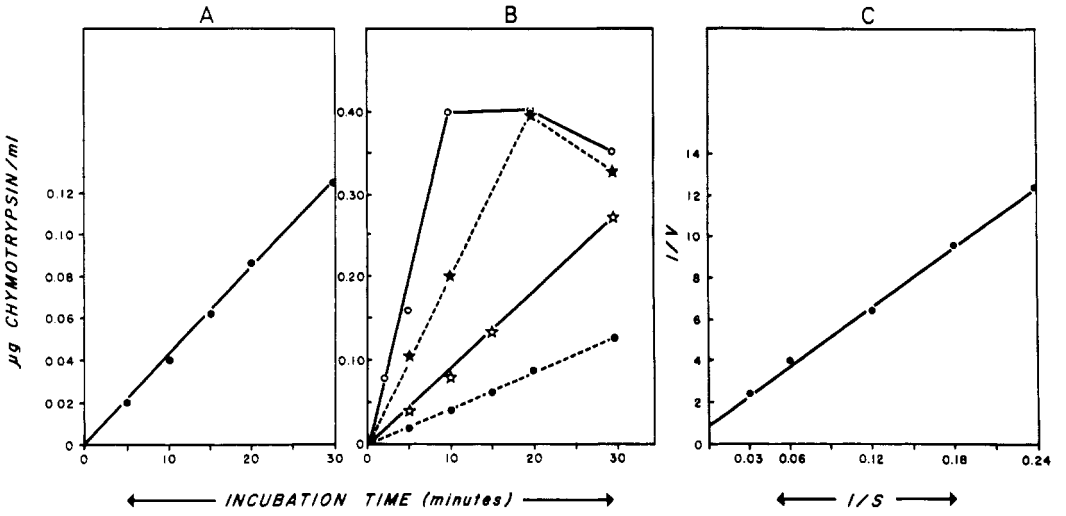

FIGURE 3: Activation of chymotrypsinogen by thrombin in 0.05 M phosphate buffer (pH 7.8) at 0°. Equal volumes of 0.001 N HCl were added to aliquots removed at various intervals (Neurath *et al.*, 1956). They were then assayed for esterase activities (see Methods), which are expressed in terms of micrograms of chymotrypsin/ml of reaction mixture. (A) Chymotrypsingogen, 8.2 μg/ml, and thrombin, 1.16 TAMe units/ml (0.29 μg of protein). (B) Chymotrypsinogen, 8.2 μg/ml; thrombin TAMe units/ml: ●---●, 1.16 (0.29 μg of protein); ☆—☆, 2.32 (0.58 μg of protein): ★—★, 4.60 (1.16 μg of protein); ○——○, 9.3 (2.36 μg of protein). (C) Thrombin, 0.75 TAMe unit (0.19 μg of protein)/ml. Chymotrypsingoen concentration (*S*) is in micrograms per milliliter. Reaction stopped after 15 min by addition of 0.001 N HCl. *V* represents micromoles of chymotrypsinogen activated per minute per milliliter (mol wt 25,000) (Neurath and Dixon, 1957).

no additional esterolytic activity, whereas more zymogen comparable to the original amount produced more activity, in 15 min almost equal to that obtained initially. This indicates that the zymogen, rather than thrombin, had become the limiting factor in 60 min, although thrombin *per se* was found to lose 11–15% of its esterolytic activity under the same conditions.

Since the suboptimal activation by thrombin compared with trypsin could be due to degradation of the zymogen occurring simultaneously with activation, this possibility was explored by measuring evolving activity in parallel with zymogen disappearance. Chymotrypsinogen was mixed with thrombin, and at selected intervals aliquots were removed and assayed for esterolytic activity. At the same times other aliquots were mixed and incubated with trypsin for 60 min, and then assayed. Evidently more chymotrypsinogen disappeared than could be accounted for as chymotrypsin (Table I). This was not due to degradation of evolved chymotrypsin since in separate experiments the esterase activity of α-chymotrypsin as well as δ- and γ-chymotrypsin remained essentially unaltered during incubation with thrombin for at least 60 min.

As in similar studies with trypsinogen and plasminogen (Engel *et al.*, 1966), activation by several different thrombin preparations was compared. These were: (a) *citrate thrombin*, as described above; (b) *thrombin purified by electrophoresis;* (c) *purified thrombin of Magnusson;* (d) *acetylated thrombin;* (e) the *water-insoluble* thrombin–phenylalanine–leucine copolymer. The results (Table II) indicate that per TAMe unit of thrombic activity, the water-insoluble preparation was the most active, exhibiting 13-fold the activity of citrate thrombin, and ninefold the activity of the preparation obtained by electrophoresis.

To explore whether the evolved esterolytic activity was actually due to chymotrypsin, experiments were carried out with indole and β-phenylpropionate, specific chymotrypsin inhibitors (Huang and Niemann, 1953; Neurath and Dixon, 1957). Chymotrypsinogen was incubated with citrate thrombin, and at selected intervals aliquots were assayed. Duplicate samples obtained *after* interaction with thrombin were mixed with indole (final concentration in the zymogen–thrombin mixture, 0.6 M), and similarly assayed. Eighty-seven per cent of the activity that evolved without the inhibitor was inhibited by it. Under the same conditions indole inhibited 60, 62, and 74% of the esterolytic activity of α-, δ-, and γ-chymotrypsin, respectively.

Activation of chymotrypsinogen by trypsin proceeds stepwise (Desnuelle and Rovery, 1961), beginning with the formation of π-chymotrypsin by catalytic cleavage of a single specific Arg-Ile bond (Jacobsen, 1947; Abrams and Jacobsen, 1951; Rovery *et al.*, 1955; Bettelheim and Neurath, 1955; Desnuelle, 1960; Neurath, 1957), which reveals the active enzyme site. Apparently this occurs also with the *Streptomyces griseus* protease (Awad and Wilcox, 1963) or, for that matter, with any other known activating agent (Neurath, 1964). This single scission, rapid and rate determining, is followed sequentially by autolytic (chymotrypsin catalyzed) splitting of three more bonds (Ser-Leu;

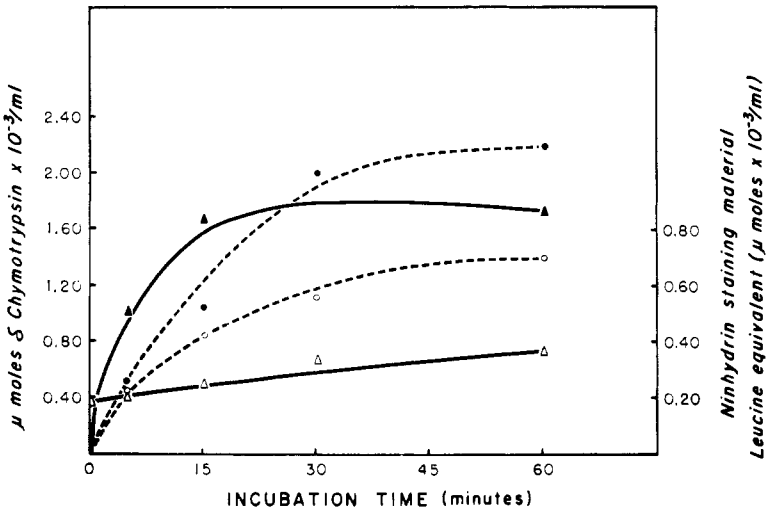

FIGURE 4: Release of ninhydrin-reacting trichloroacetic acid soluble material during activation of chymotrypsinogen by thrombin in the presence or absence of β-phenylpropionate. Thrombin and chymotrypsinogen were incubated at 0° without or with the inhibitor (0.1 M). At various intervals aliquots were 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 0.1 ml of 0.001 N HCl. Final concentrations per milliliter of the reaction mixture were: thrombin, 1.75 TAMe units (protein, 0.93 μg); chymotrypsinogen, 62.5 μg. O---O indicates amount of trichloroacetic acid soluble ninhydrin-reacting material in terms of μmoles of leucine $\times 10^{-3}$/ml of thrombin–zymogen incubation mixture in the absence of inhibitor; Δ—Δ, in the presence of inhibitor. ●---● indicates the amount of chymotrypsin evolved in the absence of the inhibitor per milliliter of zymogen–thrombin mixture, calculated on the basis of the activity of δ-chymotrypsin; ▲—▲, the amount evolved in the presence of the inhibitor.

Tyr-Thr; and Asp (NH_2)-Ala), associated with the formation of three other active species of chymotrypsins, δ, γ, and α, and the appearance of peptide fragments (Jacobsen, 1947; Rovery *et al.*, 1955; Bettelheim and Neurath, 1955; Dreyer and Neurath, 1955; Neurath and Dixon, 1957; Desnuelle, 1960). These chymotrypsin-catalyzed steps can be inhibited by β-phenylpropionate.

To investigate whether activation by thrombin similarly proceeds stepwise, activation experiments were performed with and without this inhibitor in the thrombin–zymogen interacting mixture (final concentration, 0.1 M). When added to the thrombin and chymotrypsinogen solutions *before* they were admixed, elaboration of esterolytic activity was if anything even faster than in the absence of the inhibitor (Figure 4). At the same time appearance of trichloroacetic acid soluble ninhydrin-reacting material was relatively much slower and much smaller compared with that released during thrombin–chymotrypsinogen interaction without the inhibitor.

Addition of β-phenylpropionate *after* interaction of thrombin with chymotrypsinogen decreased the final measurable esterolytic activity only slightly (8%). Under the same conditions the same concentration of the inhibitor inhibited the esterolytic activity of δ- and γ-chymotrypsin by 15 and 5%, respectively. Esterolysis of α-chymotrypsin was *increased* by about 20%.

In view of the possibility (see below in Discussion) that the thrombin preparations may have been contaminated with trypsin, studies were carried out with various trypsin inhibitors. Since under our conditions SBI and PTI[6] were found to inhibit the product of activation, namely chymotrypsin (45 and 79%, respectively), these were of no value in distinguishing between activation by thrombin *per se* or trypsin. On the other hand the trypsin inhibitor, benzamidine hydrochloride (Mares-Guia and Shaw, 1963, 1965), did not inhibit the TAMe esterase activity of thrombin or the ATEe activity of chymotrypsin over a wide concentration range. Accordingly, it was selected for study. Chymotrypsinogen activation by thrombin was unaffected by the inhibitor whereas it almost completely blocked activation by trypsin (Figure 5). These data eliminate a trypsin contaminant of the thrombin as accounting for activation.

To see whether activation could be due to factors VII and X, known contaminants of citrate thrombin, a preparation was subjected to electrophoresis on starch gel, according to Tishkoff *et al.* (1960). The expressates from individual gel segments were assayed for TAMe esterolytic activity, fibrinogen clotting activity, factor

[6] Considerable inhibition of chymotrypsin esterase and caseinolytic activity by PTI has also been observed by Wu and Laskowski (1955).

TABLE II: Comparative Chymotrypsinogen Activation and Esterolytic Activities of Thrombin Preparations.

Preparation	μg of Thrombin–Protein/ml of Reaction Mixture	Thrombin–Zymogen Units/ml[a]	Thrombin TAMe Units/ml[d]	Thrombin–Zymogen Units: TAMe Units
Citrate[b]	8.1	0.83	4.6	0.18
Prepared by electrophoresis	5.5	0.30	1.1	0.27
Magnusson	4.3	0.52	1.7	0.31
Acetylated[b]	7.8	1.22	4.7	0.26
Insoluble	3.7[c]	0.73	0.28	2.60

[a] One thrombin unit in terms of zymogen activation (thrombin–zymogen unit) is the amount of enzyme which produced a 1% activation of chymotrypsinogen per minute per milliliter of reaction mixture, the evolved ATEe esterolytic activity being measured in the pH-Stat during 15 min at 25°. [b] The acetylated preparation was derived from the same citrate thrombin. With this material the fibrinogen clotting time was 60 min, reflecting only trace clotting activity, whereas the parent material had 210 NIH clotting units/mg. The thrombin obtained by electrophoresis and the insoluble thrombin derivatives were from different citrate thrombin preparations. [c] Protein of the water-insoluble thrombin was determined by measuring the valine content, according to the procedure of Bar-Eli and Katchalski (1963) applied to water-insoluble trypsin. [d] Definition of TAMe unit is in footnote 5.

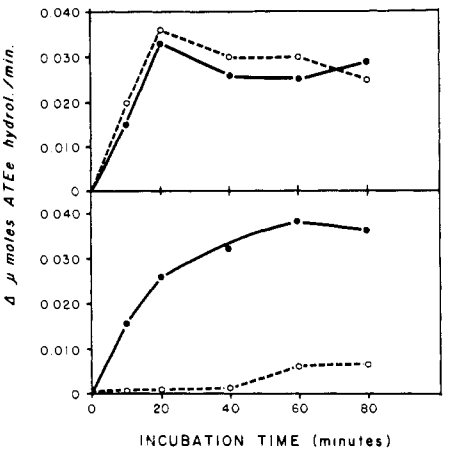

FIGURE 5: Activation of chymotrypsinogen by thrombin and by trypsin, with and without benzamidine. The enzymes were incubated with the zymogen in phosphate buffer (0.05 M, pH 7.8) at 0°. At preselected intervals equal volumes of 0.001 N HCl were added to aliquots, and these mixtures were then assayed for esterolytic activities. Concentration of reactants, per milliliter of reaction mixture, were: thrombin, 1.35 TAMe units (0.37 μg of protein); trypsin, 0.6 TAMe unit (0.25 μg); chymotrypsinogen, 8.2 μg; benzamidine-HCl, 6.25 μg/TAMe unit of trypsin and 6.47 μg/TAMe uni tof thrombin. Upper graph: O---O, thrombin plus benzamidine plus chymotrypsinogen; ●——●, thrombin plus chymotrypsinogen. Lower graph: O---O, trypsin plus benzamidine plus chymotrypsinogen; ●——●, trypsin plus chymotrypsinogen.

VII and X content, and ability to activate chymotrypsinogen. The expressates containing maximal thrombin activity were highly potent in zymogen activation whereas those that contained factors VII and X, but which were devoid of thrombin, were inert (Figure 6).

Discussion

Chymotrypsinogen A can be activated by several proteases: by trypsin (Northrop *et al.*, 1948; Jacobsen, 1947; Neurath, 1957; Desnuelle, 1960; Desnuelle and Rovery, 1961); by a protease from *Bacillus subtilis* (Abrams and Jacobsen, 1951); by *Aspergillus saitoi* protease (Gabeloteau and Desnuelle, 1960); by an enzyme from *Aspergillus oryzae* (Awad and Wilcox, 1963); by an enzyme from a species of *Penicillium* (Kunitz, 1939); and by a protease from *S. griseus* (Awad and Wilcox, 1963; Hashizume and Imahori, 1964). Our observations indicate that thrombin also can be included.

This enzyme, formerly thought to function exclusively in fibrinogen clotting by proteolysis that is limited to four specific arginylglycyl peptide bonds (Bailey *et al.*, 1951; Lorand and Middlebrook, 1952; Scheraga, 1961; Blombäck, 1963), can also activate trypsinogen and plasminogen (Engel *et al.*, 1966). In these respects as well as in the coagulation of fibrinogen (Alexander *et al.*, 1965) and in the hydrolysis of C-terminal arginyl, C-terminal lysyl ester, amide, and peptide bonds of certain synthetic substrates, thrombin and trypsin are strikingly similar (Sherry and Troll, 1954; Sherry *et al.*, 1965; Kézdy *et al.*, 1965).

Moreover, the action of thrombin is not limited to "basic" amino acid ester or peptide bonds. It can also catalyze the hydrolysis of L-tyrosine- and L-leucine-β-naphthylamide (Köbele, 1962), and carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester (Martin *et al.*, 1959), in these regards resembling chymotrypsin (Kézdy *et al.*, 1965).

As has been mentioned, activation of chymotrypsinogen by trypsin and some other proteases involves initial cleavage of one specific Arg-Leu linkage. Since thrombin acts enzymatically, and since the optimal conditions for its action (pH, temperature, and buffer) are so similar to those of trypsin, thrombin most likely acts on this same bond, although theoretically cleavage of any one, or combination, of the 17–19 thrombin sus-

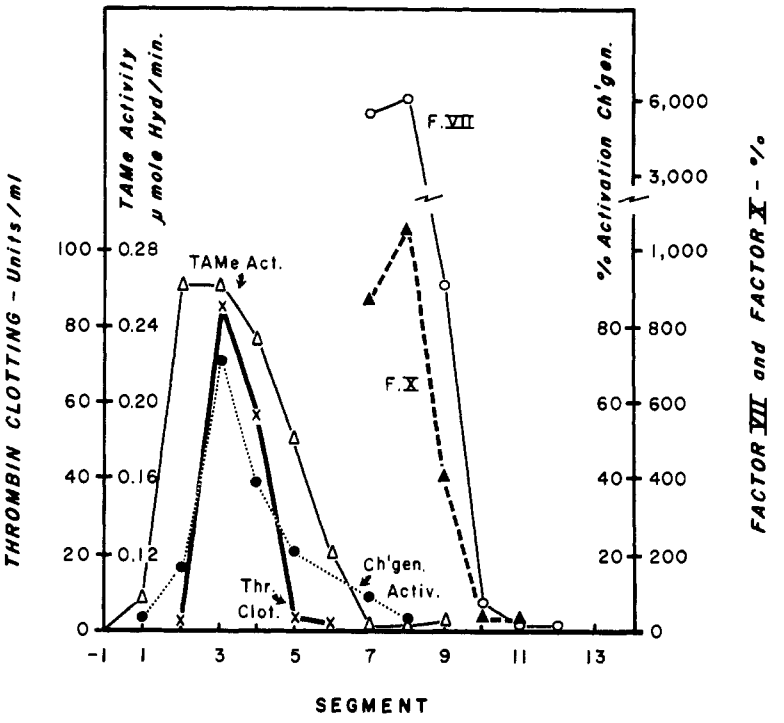

FIGURE 6: Thrombin, factor VII, factor X, TAMe esterase, and chymotrypsinogen activation activities of expressates obtained from starch gel segments of electrophoresis run of a thrombin preparation, assayed as indicated in Methods. Segment designated −1 represents the control, having been taken on the 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%. Chymotrypsinogen activation is expressed as enhancement in per cent of original ATEe esterolytic activity of the zymogen–thrombin mixture, obtained at zero time in the presence of 0.001 N HCl.

ceptible bonds (4 arginyl and 13–15 lysyl) in chymotrypsinogen may underlie its action.

Although thrombin, like trypsin, acted quickly, the final yield of protease was only 40% of that obtainable with trypsin. This discrepancy is attributed to secondary degradation of the chymotrypsinogen either by thrombin or by the evolved chymotrypsin, since a supplement of chymotrypsinogen added after completion of the initial thrombin–zymogen interaction produced substantial additional esterase. Moreover, the lower yield with thrombin could not be due to incomplete zymogen activation since supplements of trypsin or thrombin had no effect. Suboptimal yield of active protease was similarly observed with *S. griseus* protease by Awad and Wilcox (1963). These investigators ascribed it either to elaboration of a chymotrypsin species possessing lower specific activity than that evolved by trypsin, or to activation and simultaneous degradation of the zymogen by concurrent cleavage of other bonds that are not attacked by trypsin, thus producing some inert material. Similar considerations hold for thrombin, especially since it can degrade and digest several diverse proteins[1] as well as polypeptides, a property which has been used to advantage by some investigators (Magnusson, 1965; Mutt *et al.*, 1965). In further support of this possibility is the observation that some, albeit a very small amount of, trichloroacetic acid soluble ninhydrin-staining material still evolves despite the presence of β-phenylpropionate, which suggests that besides hydrolysis of the Arg-Ile bond essential for zymogen activation, thrombin cleaves other arginyl or lysyl bonds, liberating some small trichloroacetic acid soluble fragments. Since theoretically the inhibitor should have prevented peptide production *via* chymotrypsin-catalyzed hydrolysis, the fragments could be attributed to proteolysis by thrombin. This interpretation, however, remains open to question because even with trypsin as activator, in the presence of β-phenylpropionate some Ser-Arg peptide appears (Rovery *et al.*, 1955).

Suboptimal chymotrypsinogen activation (compared with trypsin) was observed also with *A. saitoi* protease by Gabeloteau and Desnuelle (1960), who attributed it to proteolytic degradation of the evolved chymotrypsin. Although this may have occurred also with thrombin since in some experiments there was some decay after peak activity was reached, it is unlikely because under the same conditions we observed that thrombin does not destroy α-, δ-, or γ-chymotrypsin.

Of paramount importance is the question whether chymotrypsinogen was activated by the thrombin, or by contaminants of trypsin, of trypsinlike enzymes, of

other proteases or arginyl esterases, all of which have been described in blood (Gullick, 1963; Hudemann, 1940; Brakman *et al.*, 1964; Mehl *et al.*, 1964; Floch and Groisser, 1960), as well as in commercial and partially purified thrombin preparations (Hudemann, 1940; Brakman *et al.*, 1964). Every effort has been made to resolve this question by the use of appropriate differentiating inhibitors and experimental conditions. Strongly supporting our thesis that chymotrypsinogen was activated by thrombin *per se* are the experiments with the highly purified material provided by Dr. Magnusson. Nevertheless, final proof must await studies with thrombin of ultimate purity.

Finally, by virtue of its ready removal from the interacting mixtures, the water-insoluble thrombin derivative is admirably suited for detailed studies of the activation sequence and products without continued presence of activator or added inhibitors. The explanation of its high specific activity compared with native or acetylated material remains obscure, and raises intriguing questions regarding enzyme–substrate interaction.

References

Abrams, A., and Jacobsen, C. F. (1951), *Compt. Rend. Trav. Lab. Carlsberg 27*, 447.

Alexander, B., Rimon, A., and Katchalski, E. (1965), *Federation Proc. 24*, 804.

Awad, W., and Wilcox, P. (1963), *Biochim. Biophys. Acta 73*, 285.

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

Bailey, K., and Bettelheim, F. R. (1955), *Brit. Med. Bull. 11*, 50.

Bailey, K., Bettelheim, F. R., Lorand, L., and Middlebrook, W. R. (1951), *Nature 167*, 233.

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 Neurath, H. (1955), *J. Biol. Chem. 212*, 241.

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

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

Cunningham, L. W. (1954), *J. Biol. Chem. 207*, 443.

Desnuelle, P. (1960), *Enzymes 4*, 93.

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

Dreyer, W., and Neurath, H. (1955), *J. Biol. Chem. 217*, 527.

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

Elmore, D. T., and Curragh, E. F. (1963), *Biochem. J. 89*, 9P.

Engel, A., and Alexander, B. (1964), *Federation Proc. 23*, 229.

Engel, A., Alexander, B., and Pechet, L. (1966), *Biochemistry 5*, 1543.

Floch, M. W., and Groisser, V. W. (1960), *New Eng. J. Med. 263*, 1129.

Gabeloteau, C., and Desnuelle, P. (1960), *Biochim. Biophys. Acta 42*, 230.

Gladner, J. A., Folk, J. E., Laki, K., and Carroll, W. R. (1959), *J. Biol. Chem. 234*, 62.

Gladner, J. A., and Laki, K. (1956), *Arch. Biochem. Biophys. 62*, 501.

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

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

Hashizume, H., and Imahori, K. (1964), *J. Biochem. (Tokyo) 56*, 128.

Huang, H. T., and Niemann, C. (1953), *J. Am. Chem. Soc. 75*, 1395.

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

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

Jacobsen, C. F. (1947), *Compt. Rend. Trav. Lab. Carlsberg, 25*, 325.

Kézdy, F. J., Lorand, L., and Miller, K. D. (1965), *Biochemistry 4*, 2302.

Köbele, B. (1962), Ph.D. Thesis, University of Tubingen, Tubingen, Germany.

Kunitz, M. (1939), *J. Gen. Physiol. 21*, 601.

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

Laki, K., Gladner, J. A., 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., Brannen, W. T., Jr., and Rule, N. G. (1962), *Arch. Biochem. Biophys. 96*, 147.

Lorand, L., and Middlebrook, W. R. (1952), *Biochem. J. 52*, 196.

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.

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

Mehl, J. W., O'Connell, W., and DeGroot, J. (1964), *Science 145*, 821.

Miller, K. D. (1958), Abstracts, 7th Annual Symposium on Blood, Wayne State University, Detroit, Mich.

Miller, K. D., and Van Vunakis, H. (1956), *J. Biol. Chem. 233*, 227.

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

Mutt, V., Magnusson, S., Jorpes, J. E., and Dahl, E. (1965), *Biochemistry 4*, 2358.

Neurath, H. (1957), *Advan. Protein Chem. 12*, 319.

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.

Northrop, J. H., Kunitz, M., and Herriott, R. (1948), Crystalline Enzymes, 2nd ed, New York, N. Y.,

Columbia University.
Owren, P. A., and Aas, K. (1951), *Scand. J. Clin. Lab. Invest. 3*, 201.
Pantlitschko, M., and Gründig, E. (1957), *Monatsh. Chem. 88*, 253.
Ronwin, E. (1960), *Biochim. Biophys. Acta, 38*, 258.
Rovery, M., Poilroux, M., Curnier, A., and Desnuelle, P. (1955), *Biochim. Biophys. Acta 17*, 565.
Scheraga, H. A. (1961), Protein Structure, New York, N. Y., Academic, pp 129–174.
Seegers, W., McClaughry, R., and Fahey, J. (1950), *Blood 5*, 421.
Seegers, W. H., and Smith, H. P. (1942), *Am. J. Physiol. 137*, 348.
Sherry, S., Alkjaersig, N., and Fletcher, A. P. (1965), *Am. J. Physiol. 209*, 577.
Sherry, S., and Troll, W. (1954), *J. Biol. Chem. 208*, 95.
Tishkoff, G., Pechet, L., and Alexander, B. (1960), *Blood 15*, 778.
Wu, F. C., and Laskowski, M. (1955), *J. Biol. Chem. 213*, 609.

Propylation and Purification of a B_{12} Enzyme Involved in Methane Formation*

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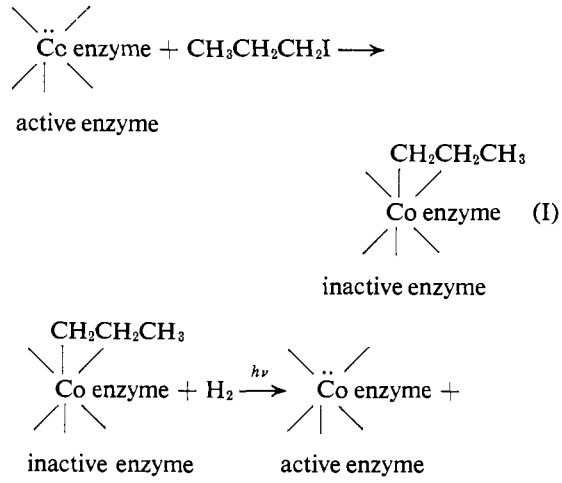
ABSTRACT: A new technique for the isolation of a B_{12} enzyme has been developed by use of a radioactive propyl donor which labels and stabilizes the B_{12} enzyme during purification. [1-^{14}C]Propyl iodide was used to purify a [1-^{14}C]propyl-B_{12} enzyme complex; removal of the [1-^{14}C]propyl group as [^{14}C]propane, by photolysis under hydrogen, yielded a B_{12} enzyme which was active in the final methyl-transfer reaction leading to the formation of CH_4 in *Methanobacillus omelianskii*.

Brot and Weissbach (1965) recently have confirmed that a B_{12} enzyme complex participates in the methyl-transfer reaction leading to the biosynthesis of methionine in *Escherichia coli*. This cobamide enzyme was shown to be strongly inhibited by alkylation with low concentrations of propyl iodide, and this inhibition could be reversed by cleavage of the carbon–cobalt bond with light. In a preliminary report we have presented evidence which supports the existence of a similar enzyme which is involved in the final methyl-transfer reaction leading to the formation of CH_4 in *Methanobacillus omelianskii*. Propylation of this enzyme and its reactivation by light led us to the detection of propane as the anaerobic photolysis product (Wood and Wolfe, 1966). The proposed mechanism of inhibition of this enzyme by propyl iodide, followed by its reactivation by light, is summarized as follows.

Extraction of the [1-^{14}C]propyl-B_{12} compound from the [1-^{14}C]propyl-B_{12} enzyme was accomplished, and this alkyl corrinoid was shown to have similar spectral properties to propyl-Factor III (propyl-Co-5-hydroxybenzimidazolylcobamide). Removal of this [1-^{14}C]propyl group as [1-^{14}C]propane, by photolysis under H_2, followed by oxidation of the resulting reduced B_{12} compound in air, gave a product with identical properties to aquo-Factor III (aquo-Co-5-hydroxybenzimidazolylcobamide).



To obtain the evidence presented below the sensitive propane assay was used to locate B_{12} enzymes during ammonium sulfate fractionation procedures, and [1-

* From the Department of Microbiology, University of Illinois, Urbana, Illinois. *Received August 1, 1966.* This work was supported by National Science Foundation Grant GB-4481.