Peptidase Activity Associated with the Tissue Factor of Blood Coagulation*

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ABSTRACT: Particulate and delipidated, solubilized preparations of tissue factor (tissue thromboplastin, factor III) from bovine lung and brain catalyze the hydrolysis of peptide bonds. Incubation of tissue factor with the oxidized B chain of insulin results in the release of a number of free amino acids and three internal peptides. Tissue factor also catalyzes the hydrolysis of a number of synthetic peptide bonds: it is more active against tri- than dipeptides and attacks aminoacyl-β-naphthylamides in the order Ala > Phe > Leu ≫ Gly > Ile ≃ Val. This activity is inhibited by 1,10-phenanthroline; inhibition is reversed by $Co^{2+} \gg Ni^{2+} > Mn^{2+}$. Coagulant and peptidase activities of the soluble apoprotein are coincident following gel filtration, electrofocusing, and disc gel electrophoresis; both activities are recovered isopycnically in the same lipid–protein complex after recombination with phospholipids. These data support the concept that the peptidase and coagulant activities are functions of the same molecule, and that tissue factor may function biologically through a proteolytic mechanism.

The extrinsic pathway of blood coagulation requires the participation of tissue extracts in addition to the components of the blood itself. Lung, brain, and placenta are particularly rich in tissue factor (tissue thromboplastin) which is located in the microsomal fraction (Williams, 1964, 1966; Williams and Norris, 1966). Extraction of phospholipid from particulate tissue factor reduces coagulant activity significantly (Kuhn and Klesse, 1957; Deutsch *et al.*, 1964; Nemerson, 1968; Hvatum and Prydz, 1966), but reassociation of the residual protein with certain phospholipids, which are themselves devoid of activity, restores activity (Nemerson, 1968). The protein component of tissue factor has been solubilized (Nemerson, 1969), and substantially purified (Nemerson and Pitlick, 1970). The soluble protein, like the lipid-depleted particles, has minimal coagulant activity, but is stimulated by reassociation with phospholipids (Nemerson, 1969).

The reaction of tissue factor with factors VII and X is thought to proceed sequentially as shown in reactions 1 and 2.

$$\text{tissue factor} + \text{factor VII} \xrightarrow{Ca^{2+}} \text{tissue factor–factor VII complex} \quad (1)$$

$$\text{factor X} \xrightarrow{\text{tissue factor–factor VII complex, } Ca^{2+}} \text{activated factor X} \quad (2)$$

The mechanisms of these reactions are unknown. Reaction 1 occurs very rapidly and is stoichiometric (Williams and Norris, 1966; Nemerson, 1966). Reaction 2 proceeds more slowly and in a catalytic manner where the rate of the reaction is controlled by the amount of tissue factor–factor VII complex present (Williams and Norris, 1966; Nemerson, 1966). Reaction 2 may be proteolytic since trypsin (Yin, 1964) activates factor X.

In an attempt to clarify the mechanisms of the reactions which initiate extrinsic coagulation, the ability of tissue factor to hydrolyze peptide bonds has been investigated. In the studies reported here it was found that microsomal preparations from lung and brain catalyze the hydrolysis of a number of synthetic peptides. Accordingly, these observations were extended in experiments in which the peptidase and coagulant activities of particulate tissue factor, purified apoprotein, and apoprotein recombined with phospholipids were examined. Data are presented which demonstrate that the peptidase and coagulant activities are coincident following a variety of physical separation techniques. Therefore, tissue factor may function in blood coagulation through a mechanism involving proteolysis.

Materials

All amino acids and aminoacyl derivatives were in the L configuration. Ala-Gly-Gly, Leu-Gly-Gly, Ala-Gly, Leu-Gly, Gly-Gly, Gly-Leu, Ala-Leu, Val-Gly, Leu-Ala, Leu-Leu, Cbz-Gly-Phe-NH_2, Gly-Phe-NH_2, Leu-NH_2, Leu, Val, Ala, Gly, and Ile, purified porcine kidney leucine aminopeptidase (ammonium sulfate suspension), *N*-(1-naphthyl)-ethylenediamine·2-hydrochloride, and diisopropylphosphorofluoridate were products of Mann Research Laboratories, New York, N. Y. The peptides Ala-Nva and Ile-Gly-Gly were purchased from Cyclo Chemical, Los Angeles, Calif. Leucyl-β-naphthylamide was a product of Pierce Chemical Co., Rockford, Ill.; other aminoacyl-β-naphthylamides (as hydrobromides) were purchased from Nutritional Biochemicals, Cleveland, Ohio. Fast violet salt BN was a gift from GAF Corp., New York, N. Y. 1-Chloro-L-3-tosylamido-7-

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amino-2-heptanone (TLCK),[1] 1-chloro-L-3-tosylamido-4-phenyl-2-butanone (TPCK), dithiothreitol, and β-naphthylamine were purchased from Calbiochem, Los Angeles, Calif. Soybean trypsin inhibitor was a product of Sigma Chemical Co., St. Louis, Mo. 1,10-Phenanthroline was purchased from Matheson Coleman & Bell, Norwood, Ohio. Phosphatidylethanolamine was purchased from Supelco, Bellefonte, Pa. Mixed-brain lipids were prepared as previously described (Nemerson, 1968, method 1). Sephadex G-10 and Sepharose 6B were products of Pharmacia, Piscataway, N. J.

Methods

Tissue Factor Preparation. Lung microsomes were prepared by the method of Williams (1964). Tissue factor apoprotein was prepared according to the procedure of Nemerson (1969) as modified by Nemerson and Pitlick (1970), and was recombined with phospholipids according to the method of Nemerson (1969).

Coagulant Activity. Tissue factor activity was determined by a two-stage assay in which factor X was activated in a reaction mixture containing tissue factor, calcium, and saturating quantities of factors VII and X. The rate of activation of factor X is a function of the amount of tissue factor in the reaction. Activated factor X is assayed by its ability to accelerate the coagulation of recalcified normal bovine plasma containing added phospholipid (Nemerson, 1968). When microsomes or relipidated apoprotein were tested, the first stage was incubated for 1 min; when apoprotein was tested the incubation was continued for 4 min.

Hydrolysis of Synthetic Peptides. Several di- and tripeptides were tested as possible substrates for tissue factor. The incubation mixture included 67 μg of tissue factor apoprotein, 50 mM imidazole-HCl (pH 6.8), and 1 mM peptide in a final volume of 1.0 ml. Incubation was at 37°, and aliquots were withdrawn at 0 and 3 hr. In experiments in which hydrolysis was determined qualitatively aliquots were immediately withdrawn, spotted on a silica gel plate, and dried. The upper phase of a mixture of butanol–acetic acid–water (70:25:100, v/v) was used for ascending thin-layer chromatography. Quantitation of hydrolysis was determined with a ninhydrin technique (Moore and Stein, 1948) using experimentally determined extinction coefficients of the amino acid and peptide products detected on the chromatograms. In these experiments the reaction was terminated by adding aliquots of the reaction mixture to the ninhydrin reagent.

Hydrolysis of Aminoacyl-β-naphthylamides. Hydrolysis of aminoacyl-β-naphthylamides was detected by measuring the release of β-naphthylamine according to the method of Goldbarg and Rutenberg (1958). Duplicate reaction mixtures containing 0.1 mM alanyl-β-naphthylamide, 50 mM imidazole-HCl (pH 6.8), and enzyme in a final volume of 0.3 ml were incubated at 37°. The reaction was terminated by the addition of 0.3 ml of 40% trichloroacetic acid. For zero-time values the acid was added before substrate.

Hydrolysis of B Chain of Insulin. The oxidized B chain of bovine insulin was prepared chromatographically according to Bailey and Cole (1959), desalted by gel filtration on Sephadex G-10 equilibrated with 0.1 M NH_4HCO_3, lyophilized, redissolved in water, and frozen until use. On occasion, the oxidized B chain was obtained from Mann Biochemical. In each case, the purity of the preparation was assessed by amino acid analysis and thin-layer chromatography.

A suspension of 5 mg of the oxidized B chain was incubated with 6.0 mg of lung microsomes containing 2.4 mg of protein in a final volume of 10 ml of 0.1 M NH_4HCO_3 containing 0.01 mM $CaCl_2$. After incubation for 24 hr at 37°, particulate matter was removed by centrifugation at 10,000 rpm for 15 min. Aliquots of the supernatant were applied to 46 × 57 cm sheets of Whatman No. 3MM chromatography paper; descending chromatography was performed for 16–18 hr in pyridine–butanol–acetic acid–water (90:60:18:72, v/v) with phenol red as a marker. The sheets were dried, developed with dilute ninhydrin in methanol–acetic acid, and eluted with 0.01 N HCl. The eluates from corresponding areas of four to five chromatograms were combined and lyophilized. Half of the lyophilized eluate was dissolved in buffer and analyzed for free amino acids on a Beckman 120C analyzer. The remainder of the eluate was sealed in ampoules with constant-boiling HCl, hydrolyzed at 105° for 24 hr, and analyzed for amino acid content. The cleavage sites were then deduced by subtractive analysis.

If amino acid analysis indicated that an eluate contained more than one peptide, the eluate was further resolved by paper electrophoresis (2000 V for 2 hr) in pyridine–acetic acid–water (100:4:900, v/v; pH 6.4) or by thin-layer chromatography on silica gel as above. After elution of the ninhydrin-positive areas, analysis proceeded as above.

The products of the incubation of apoprotein relipidated with 7.5 mg of mixed-brain phospholipids per mg protein (Nemerson, 1968) and the oxidized B chain of insulin were characterized by peptide maps prepared after 4- and 24-hr incubation. Enzyme protein (0.25 mg) was incubated with oxidized B chain of insulin (1 mg) as above. After the incubation mixtures were lyophilized and resolubilized, chromatography on paper was performed as before, followed by electrophoresis in the second dimension as above. The sheets were dried and developed with ninhydrin–collidine. These results were compared with peptide maps made from the incubation of lung microsomes with the B chain of insulin and with the products of an incubation of relipidated tissue factor which had been treated with DFP (1 mM) prior to incubation.

Separation Techniques. GEL FILTRATION. The purified apoprotein was gel filtered on a 2.5 × 90 cm column of Sepharose 6B equilibrated with 0.05 M imidazole-HCl–0.375 M NaCl (pH 7.0) at 21°. The protein was eluted with an upward flow rate of 12 ml/hr; 4-ml fractions were collected and measured for absorbance at 280 mμ. Individual fractions were assayed for coagulant activity after relipidation with 7.5 mg of mixed-brain phospholipids/mg of protein. Peptidase activity was measured without relipidation using the standard assay system with alanyl-β-naphthylamide and 0.05-ml aliquots of the fraction as the source of enzyme. The incubation was continued for 10 or 20 min at 37° depending on the amount of protein in the fractions.

ISOPYCNIC CENTRIFUGATION. Apoprotein was relipidated with 2.35 mg of phosphatidylethanolamine/mg of protein and centrifuged for 39 hr in a Spinco SW50.1 rotor at 50,000 rpm in a 5–30% sucrose gradient containing 0.05 M imidazole-HCl–0.375 M NaCl (pH 7.2) at 4°. Fractions of ≈0.2 ml were collected from the bottom of the tube. Aliquots were assayed for peptidase and coagulant activities. Apoprotein was centrifuged in the same manner. Coagulant activity was located after relipidation of the fractions with 5 mg of mixed-brain phospholipids/mg of protein; the relipidated samples

[1] Abbreviations used are: TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, 1-chloro-L-3-tosylamido-4-phenyl-2-butanone; *p*-CMB, *p*-chloromercuribenzoate; Cbz-, carbobenzoxy; Nva, norvaline.

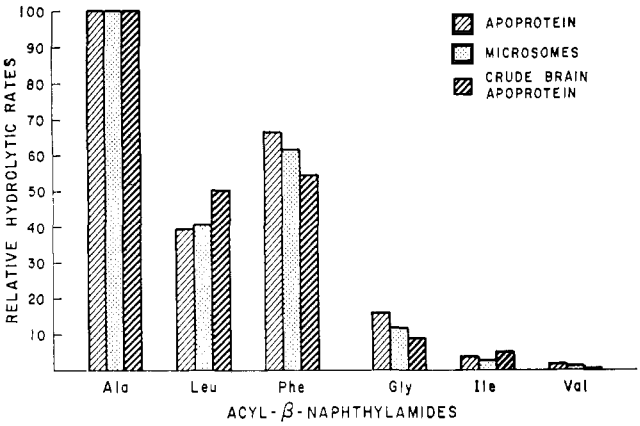

FIGURE 1: Relative rates of hydrolysis of aminoacyl-β-naphthylamides by three tissue factor preparations. The rates are expressed as a percentage of those observed with alanyl-β-naphthylamide for each enzymic preparation. The rates of hydrolysis of alanyl-β-naphthylamide were 41, 51, and 8.5 nmoles per min per mg for lung apoprotein, microsomes, and brain apoprotein, respectively.

were assayed for peptidase activity in the alanyl-β-naphthylamide assay.

ELECTROFOCUSING was performed in a 110-ml column (LKB 8100-10) with carrier ampholytes (Ampholine 8143, pH 5–8) manufactured by LKB, Stockholm, Sweden. Apoprotein (15 ml, 5.6 mg) was incorporated into the 0–50% sucrose gradient (110 ml) which also contained 1% ampholine (pH 5–8) and 2 M urea. Electrofocusing was continued for 3.3 hr at 10°. During this time, the current fell from 6 to 1.8 mA. At the end of the experiment, 2-ml fractions were collected for determination of pH and A_{280}. The fractions were then dialyzed for 24 hr against 0.05 M imidazole-HCl–0.375 M NaCl (pH 7.2) at 4°. Peptidase activity was assayed with 0.05-ml aliquots of the dialyzed fractions; coagulant activity was determined after relipidation with 7.5 mg of mixed-brain phospholipids/mg of protein.

DISC GEL ELECTROPHORESIS. Electrophoresis in 5% acrylamide gels (90 mm) was performed using the pH 9.5 buffer system of Davis (1964). Sample gels and stacking gels were

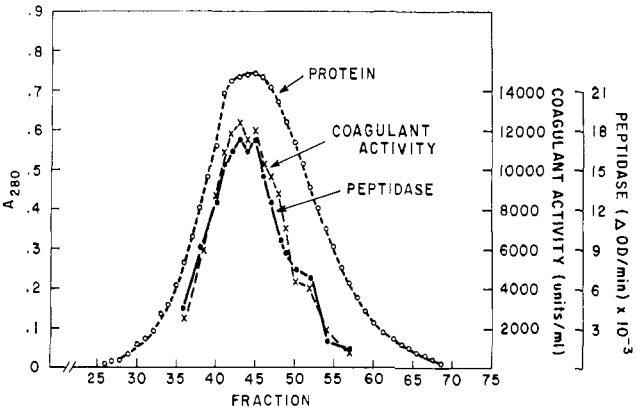

FIGURE 2: Gel filtration of apoprotein on Sepharose 6B. Coagulant activity was assayed after relipidation of individual fractions with 7.5 mg of mixed-brain lipids/mg of protein. Peptidase activity was measured directly in a reaction mixture of 0.3 ml of final volume containing 0.1 mM alanyl-β-naphthylamide, 50 mM imidazole-HCl, (pH 6.8), and 0.05 ml of column fraction. Incubation was continued for 10 or 20 min at 37° depending on protein concentration.

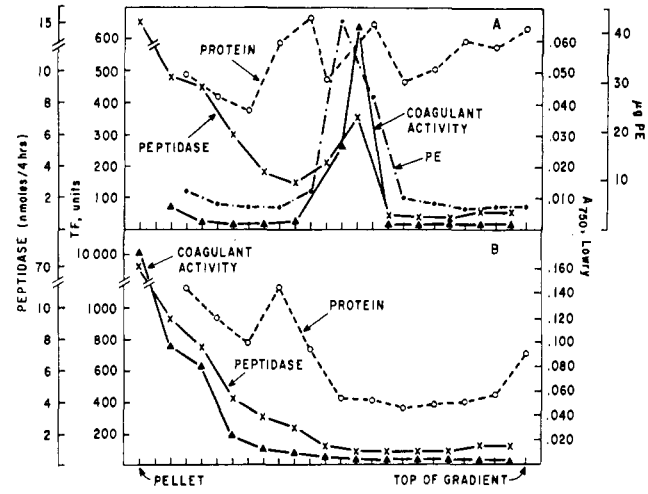

FIGURE 3: Sucrose density gradient centrifugation (39 hr at 50,000 rpm) of apoprotein and apoprotein relipidated with 2.35 mg of phosphatidylethanolamine/mg of protein. Sucrose gradient: 5–30% (w/v) sucrose in 0.05 M imidazole-HCl–0.375 M NaCl (pH 7.2) at 4°. (A) Relipidated apoprotein. Coagulant activity was determined directly on the fractions, peptidase was assayed as described in Figure 2 except incubation was continued for 4 hr. (B) Apoprotein coagulant activity was determined after relipidation of individual fractions with 5 mg of mixed-brain lipids/mg of protein. Peptidase activity determined as for part A, except relipidated fraction used.

omitted. Protein (100 μg) in 100 μl of half-strength reservoir buffer was layered on the gel and Sephadex G-25 was added to minimize convection. The front was within 0.25 in. of the end of the gel after about 1 hr at 2.5 mA/gel. Protein was detected with aniline blue-black stain. Peptidase activity was located by incubation of a separate unfixed gel in 0.6 mM alanyl-β-naphthylamide, 0.5 mM $CoCl_2$, 25 mM imidazole-HCl (pH 6.8), and 12 mg of fast violet salt BN in a volume of 10 ml. After incubation for 2 hr at 37°, the gels were decolorized by immersion overnight in 30% ethanol and then washed with water. Coagulant activity was determined without relipidation after sectioning a companion gel into 2.6-mm slices and triturating the sections in 0.2 ml of 0.05 M imidazole-HCl–0.10 M NaCl (pH 7.2).

CHEMICAL METHODS. Lipid phosphorus was extracted from sucrose gradient fractions as previously described (Pitlick and Nemerson, 1970). Phosphorus was determined by the method of Chen *et al.* (1956). Protein was estimated by the method of Lowry *et al.* (1951).

Results

Substrate Specificity. Preliminary experiments revealed that microsomes and apoprotein hydrolyzed several peptides and aminoacyl-β-naphthylamides. Accordingly, lung microsomes, lung apoprotein, and crude brain apoprotein, all of which activated the tissue factor pathway, were incubated with six naphthylamides. The results (Figure 1) indicate each preparation had similar substrate specificity with respect to the six compounds studied. Alanyl-β-naphthylamide was the preferred substrate and was therefore used for routine assay.

Coincidence of Peptidase and Coagulant Activities. While these data show that coagulant and peptidase activities coexist, the availability of the soluble protein permitted the application of a variety of physical techniques to characterize the protein and establish with certainty the coincidence of these activities. The apoprotein was gel filtered on 6% Agarose and

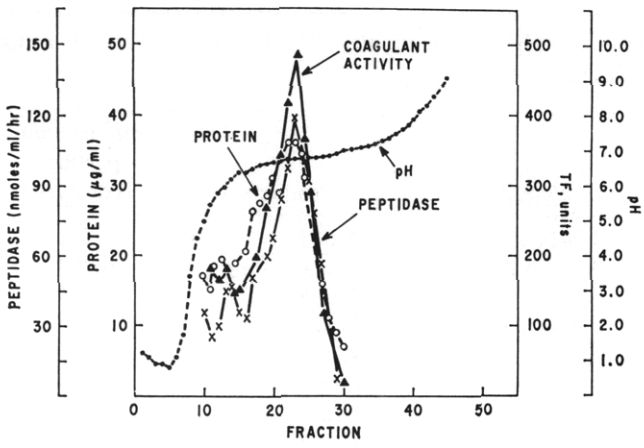

FIGURE 4: Electrofocusing of 5.6 mg of apoprotein which had been incorporated into the sucrose gradient containing pH 5–8 Ampholine and 2 M urea. Fractions (2 ml) were collected and dialyzed. Coagulant activity was determined after relipidation with 7.5 mg of mixed-brain lipids/mg of protein. Peptidase was estimated as described in Figure 2.

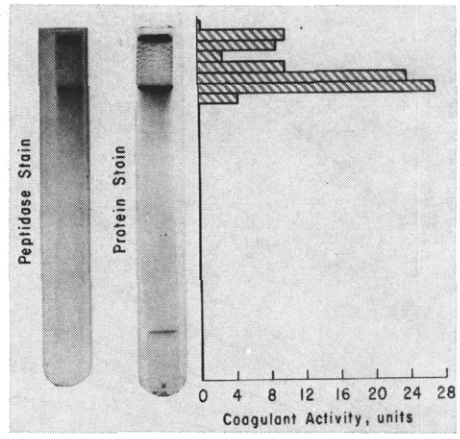

FIGURE 5: Disc gel electrophoresis of apoprotein in 5% acrylamide gels. Protein was located with aniline blue-black. Peptidase was located as described in Methods. Coagulant activity (as apoprotein) was determined after sectioning another gel into 2.6-mm slices and triturating these sections in 0.2 ml of 0.05 M imidazole-HCl–0.10 M NaCl (pH 7.0).

the coagulant activity and peptidase activity of the eluted protein were assayed. The two activities eluted from the column in a nearly identical manner (Figure 2).

Tissue factor apoprotein binds phospholipids forming stable complexes that can be isolated by isopycnic centrifugation in sucrose gradients (Pitlick and Nemerson, 1970). Accordingly, apoprotein was recombined with phosphatidylethanolamine and centrifuged through a sucrose density gradient. After 39 hr, a peak containing phospholipid, coagulant activity, and peptidase was found at a density of 1.080 g/ml (Figure 3A), intermediate to that of phosphatidylehtanolamine alone (which remains at the meniscus (Pitlick and Nemerson, 1970)) and the apoprotein which sedimented to the bottom of the tube (Figure 3B). Some peptidase activity was found near the bottom of the tube in the experiment illustrated in Figure 3A. This was probably due to unbound apoprotein which had not reacted with the phospholipids and therefore had little coagulant activity.

In a further attempt to separate the two activities, purified lung apoprotein was electrofocused in a pH 5–8 ampholyte solution. Because prolonged runs (24 hr) resulted in severe loss of activity, the experiment was terminated at 3.3 hr at which time a sharp refractile band was seen in the column and the current had fallen considerably from 6.0 to 1.8 mA, indicating nearly complete equilibrium. Following dialysis of the fractions, the peptidase activity was determined directly, and the coagulant activity was determined after relipidation. Both activities focused sharply at pH 6.72 (Figure 4).

Previous experiments demonstrated that electrophoresis of apoprotein on 5% acrylamide gels resulted in two bands with coagulant activity (Nemerson and Pitlick, 1970). To determine whether coagulant and peptidase activities could be resolved by this technique, apoprotein was similarly electrophoresed. One gel was stained for protein, one for peptidase activity and one eluted for estimation of coagulant activity. The results (Figure 5) show two distinct protein bands (plus a minor contaminant running near the front). Alanyl-β-naphthylamide hydrolysis was detected in both bands as was coagulant activity. In a control experiment, substrate was omitted from the peptidase stain and the bands stained only very faintly with the diazonium salt used to develop the color.

Characterization of the Enzymatic Activity. The enzyme (apoprotein) was characterized using alanyl-β-naphthylamide as substrate because of the ease and sensitivity of the assay. The activity of the apoprotein was determined between pH 4.0 and 8.5 using several buffers (Figure 6). Maximal activity was obtained with imidazole-HCl at pH 6.8. In phosphate buffers, although considerable activity was observed, there was no clear optimum. The pH optimum was not altered by increases in ionic strength to 100 mM in each instance nor were the rates significantly altered. Accordingly, 50 mM imidazole buffer at pH 6.8 was used routinely.

The rate of alanyl-β-naphthylamide hydrolysis was proportional to the amount of apoprotein in the reaction mixture (Figure 7). Michaelis constants were determined for microsomes, apoprotein, and relipidated apoprotein (7.5 mg of mixed lipids/mg of protein) from the data presented in Figure 8. Of note is the substrate inhibition observed above 0.12 mM. The Michaelis constants were obtained from a plot of the least squares of the linear portions of Figure 8 and were 7.5×10^{-5}, 8.2×10^{-5}, and 40×10^{-5} M for apoprotein, micro-

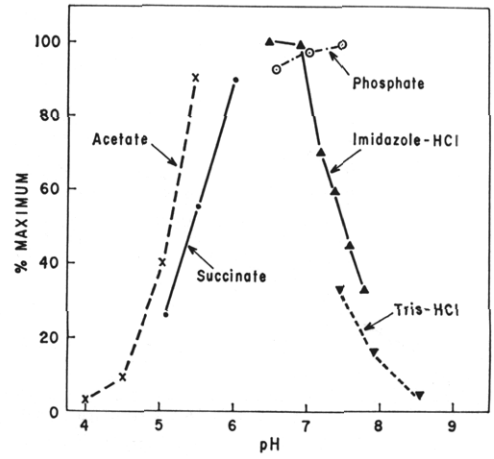

FIGURE 6: Effect of pH on the rate of hydrolysis of alanyl-β-naphthylamide. Standard assay using indicated buffer at 50 mM. Acetate, succinate, and phosphate buffers were prepared as their sodium salts.

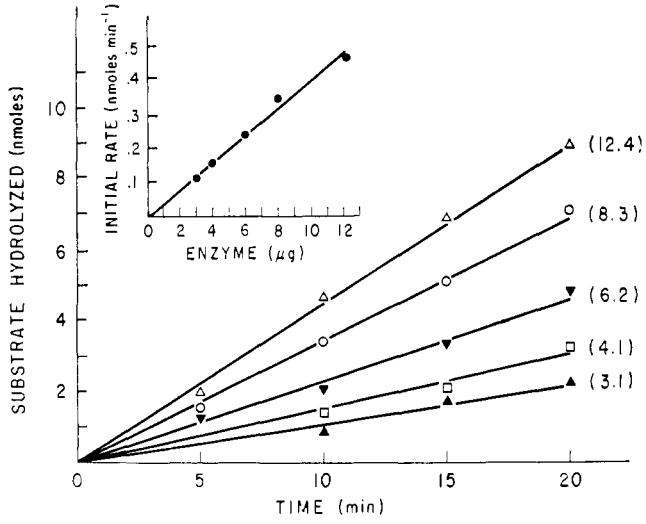

FIGURE 7: Dependence of rate of hydrolysis of alanyl-β-naphthylamide on enzyme concentration. Incubation mixture: 0.1 mM alanyl-β-naphthylamide–0.05 M imidazole-HCl (pH 6.8) with appropriate dilutions of enzyme for final volume of 2.1 ml. At indicated intervals, 0.2-ml aliquots were withdrawn into 40% trichloroacetic acid and the amount of free naphthylamine was determined. The parenthetical numbers refer to micrograms of protein in 0.2 ml; ordinate represents free naphthylamine in 0.2 ml.

somes, and relipidated apoprotein, respectively. Due to the observed substrate inhibition, all routine assays were performed using 0.10 mM alanyl-β-naphthylamide. Under optimal conditions the apoprotein, microsomes, and relipidated apoprotein hydrolyzed 79, 125, and 89 nmoles per mg per min, respectively.

Metal Ion Requirements. Peptidase activity was irreversibly inhibited by 1 mM EDTA. 1,10-Phenanthroline (1 mM) also inhibited the peptidase, but this inhibition was reversible: $CoCl_2$ (1 mM) restored 80% of the original activity, while $NiCl_2$ (1 mM) and $MnCl_2$ (25 mM) restored 20 and 15%, respectively. Other divalent cations (calcium, barium, iron, magnesium, cadmium, and strontium chlorides, and zinc and copper sulfates) were ineffective. In the absence of 1,10-phenanthroline, $CoCl_2$ stimulated 20% at 0.05 mM, but was inhibitory at 0.5 mM. Similar results were obtained with lung microsomes.

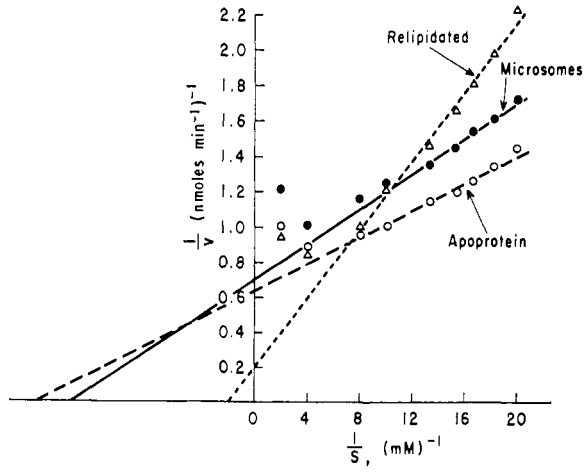

FIGURE 8: Double-reciprocal plot of the effect of substrate concentration on initial rate of hydrolysis of alanyl-β-naphthylamide by microsomes, apoprotein, and relipidated apoprotein. Standard assay with indicated substrate concentrations.

TABLE I: Hydrolysis of Peptides by Apoprotein of Lung Tissue Factor.[a]

Peptide	Products	nmoles/min per mg Hydrolyzed
Ala-Nva	Ala, Nva	35.1
Ala-Gly-Gly	Ala, Gly-Gly	33.3
Ala-Val	Ala, Val	24.3
Leu-Gly-Gly	Leu, Gly-Gly	23.9
Ala-Gly	Ala-Gly[b]	8.6[b]
Ile-Gly-Gly	Ile-Gly-Gly	3.9
Leu-Leu	Leu	3.8
Leu-NH_2	Leu-NH_2	3.7
Leu-Ala	Leu-Ala	3.6
Leu-Gly	Leu-Gly	3.1
Gly-Leu	Gly-Leu	2.1
Gly-Gly	Gly-Gly	1.5
Val-Gly	Val-Gly	0.0

[a] Apoprotein (65 μg) was incubated with 1.0 μmole of peptide in a 1.0-ml final volume for 3 hr at 37°. The products were identified by thin-layer chromatography on silica gel plates. The extent of hydrolysis was calculated from the results of quantitative ninhydrin analysis. [b] At this level of hydrolysis, the products were not detected on the thin-layer chromatograms.

To determine whether Co^{2+} could substitute for Ca^{2+} in the assay for coagulant activity, the apoprotein was preincubated with 1,10-phenanthroline (1 mM) and then tested in the usual, calcium-containing assay. No inhibition was noted. In a similar experiment, Co^{2+} (25 mM) was substituted for Ca^{2+}. Under these conditions tissue factor had no coagulant action. Thus, the coagulant activity of tissue factor did not appear to require Co^{2+}, nor could Co^{2+} substitute for Ca^{2+}.

Hydrolysis of Peptides. A number of aliphatic and aromatic di- and tripeptides and amides have been tested as possible substrates for tissue factor apoprotein. The results presented in Table I show Ala-Gly-Gly and Ala-Nva were the best substrates. The tripeptides, Leu-Gly-Gly and Ala-Gly-Gly, were hydrolyzed faster than the dipeptides Leu-Gly and Ala-Gly. Using the same enzyme preparation as in Figure 8, the rate of hydrolysis of Ala-Gly-Gly was 199 nmoles/mg per min. The data in Table I were obtained with a less active apoprotein preparation.

The same peptides were used as substrates with microsomes as the source of enzyme. Hydrolysis was estimated from thin-layer chromatograms of the incubation mixtures, and the results were similar to those obtained with apoprotein. Thus microsomes and apoprotein hydrolyzed the same peptides as well as naphthylamides.

In order to compare tissue factor and cathepsin C (an enzyme reported to have procoagulant activity) (Purcell and Barnhart, 1963), the microsomes and apoprotein were incubated with Gly-Phe-NH_2. Glycine was rapidly produced by the action of tissue factor; minimal hydrolysis of Phe-NH_2 was observed. This is in contrast to cathepsin C in which the amide bond is preferentially hydrolyzed (Gutmann and Fruton,

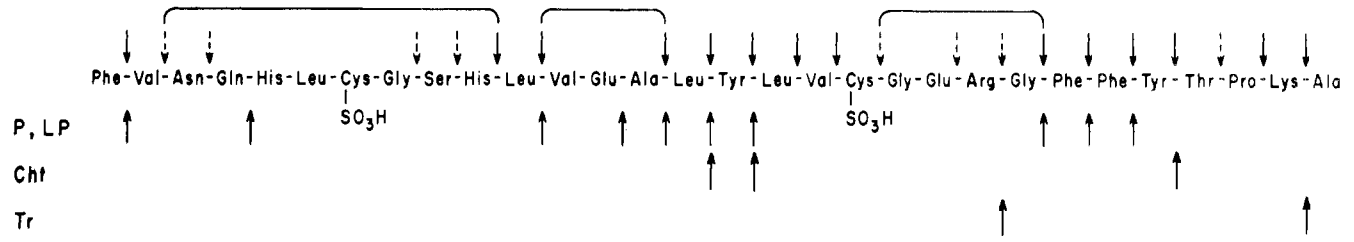

FIGURE 9: Sites of attack of lung microsomes on the oxidized B chain of insulin. The solid arrows above the peptide chain indicate major sites of hydrolysis; the broken arrows indicate minor sites. The brackets enclose the three peptides that were recovered intact. For comparison, the site of attack of several other enzymes are shown below the chain. P = pepsin, Cht = chymotrypsin, Tr = trypsin (Sanger and Tuppy, 1951), L. P. = lung proteinase (Dannenberg and Smith, 1955).

1948). Neither tissue factor preparation hydrolyzed Cbz-Gly-Phe-NH_2.

Hydrolysis of the Oxidized B Chain of Insulin. To investigate the possible endopeptidase activity of lung microsomes and further delineate its substrate specificity, the enzyme was incubated with the oxidized B chain of insulin. Following incubation for 24 hr, particulate matter was removed by centrifugation, and the soluble residue was analyzed for free amino acids and peptides. Both free amino acids and peptides were recovered as shown in Figure 9. The yields of the free amino acids released as a result of the action of the microsomes, expressed as percentages of the amino acids recovered after total acid hydrolysis of the B chain, were: lysine 107.0%, tyrosine 100.1%, phenylalanine 93.7%, leucine 72.3%, alanine 64.9%, valine 58.5%, arginine 34.7%, and cysteic acid 20.2%. Aspartic acid, threonine, serine, and histidine were identified in trace amounts.

In addition, three peptides were recovered from the incubation mixture (Figure 9): Gly-Glu-Arg-Gly, Val-Glu-Ala and Asn-Gln-His-Leu-Cys(SO_3H)-Gly-Ser-His. In some experiments, the N-terminal Asn was not present on this peptide. The recovery of the peptides was not quantified.

Peptide maps were prepared from these products as well as the products that arose from the action of relipidated apoprotein. Both sources of enzyme yielded identical peptide maps. Pretreatment of the enzyme with DFP did not alter the peptide maps, nor did it reduce the ninhydrin color produced by the action of apoprotein on insulin (see below).

Effects of Inhibitors on Enzyme Activity. Valyl- and isoleucyl-β-naphthylamides were not significantly hydrolyzed by tissue factor (see above). Accordingly, they were tested as inhibitors of the hydrolysis of alanyl-β-naphthylamide. Isoleucyl-β-naphthylamide (0.01 mM) inhibited the hydrolysis of 0.10 mM alanyl-β-naphthylamide 47%; valyl-β-naphthylamide inhibited 23% under the same conditions. The inhibition by isoleucyl-β-naphthylamide was studied further by the method of Dixon (1953) ($1/v$ *vs.* [I] at two substrate concentrations). The data (not shown) yielded a K_i of 3×10^{-4} M.

The peptides Ala-Gly-Gly, Ile-Gly-Gly, Ala-Val, and Val-Gly (all at 0.10 mM) were likewise tested for inhibition of the hydrolysis of alanyl-β-naphthylamide. None was observed. Conversely, the hydrolysis of 1 mM Ala-Gly-Gly was inhibited 77% by 0.9 mM isoleucyl-β-naphthylamide and 45% by the same concentration of alanyl-β-naphthylamide.

Other possible enzyme inhibitors were also studied. The inhibitors were preincubated for 1 hr with apoprotein. The enzyme was diluted 50-fold for assay of alanyl-β-naphthylamide hydrolysis. The inhibitors used were: DFP, TLCK, TPCK, iodoacetic acid, *p*CMB, and dithiothreitol (each at 0.278 mM); DFP was also studied at 5 mM and soybean trypsin inhibitor at 1.0 mg/ml. No inhibition was noted. The relipidated enzyme was studied for coagulant activity after incubation with the same inhibitors at the same concentrations. The enzyme was then diluted 32-fold for assay; again, no inhibition was noted.

A similar experiment was performed using the oxidized B chain as substrate. The enzyme with inhibitor was diluted only 10% by the addition of insulin. The extent of hydrolysis was quantified by the ninhydrin technique at 0, 2, 6, and 24 hr. Partial inhibition was observed with TLCK, *p*CMB, and iodoacetic acid (11, 14, and 18%, respectively). The products observed by thin-layer chromatography, however, were identical despite the presence of these inhibitors.

1,10-Phenanthroline, which inhibits the hydrolysis of alanyl-β-naphthylamide (see above) inhibited the hydrolysis of insulin 95%; $CoCl_2$ (2.5 mM) restored 60% of the activity. The thin-layer chromatograms of the incubation mixture with $CoCl_2$ were also identical with the controls.

Comparison to Leucine Aminopeptidase. Apoprotein was compared to authentic porcine leucine aminopeptidase after preincubation of each enzyme with 1 mM $MnCl_2$. The hydrolytic rates of each enzyme with the substrates Ala-Gly-Gly and leucinamide (5 mM) were determined at pH 8.5 in 10 mM Tris-HCl buffer containing 2 mM $MgCl_2$, at 37°, optimal conditions for leucine aminopeptidase. Tissue factor failed to hydrolyze leucinamide, while leucine aminopeptidase hydrolyzed 3.26 μmoles/mg per min. Tissue factor hydrolyzed 0.058 μmole of Ala-Gly-Gly/mg per min while leucine aminopeptidase hydrolyzed this substrate at the rate of 0.905 μmole/mg per min. Thus, leucine aminopeptidase preferentially hydrolyzed leucinamide, while tissue factor hydrolyzed only the tripeptide Ala-Gly-Gly under the same conditions.

Discussion

In this study, data are presented which clearly show that tissue factor prepared from different organs, whether particle bound or solubilized, and having different degrees of purity, catalyzes the hydrolysis of peptide bonds. Lung microsomes and soluble tissue factor prepared from lung and brain hydrolyzed the same naphthylamides and peptides, and had the same metal ion requirement. As it is difficult to determine whether more than one enzyme is present on a particle, apoprotein was used to investigate the identity of coagulant and peptidase activities.

The behavior of tissue factor coagulant and peptidase activities was studied by several methods. Agarose gel filtration, which separates molecules as a function of their shape and size, clearly demonstrated the coincidence of coagulant and peptidase activities. These activities were also not differ-

entiated by disc gel electrophoresis in which separations occur as a function of size and shape as well as charge density, or isoelectric focusing which separates proteins according to their isoelectric points. Moreover, the peptidase activity was bound to lipid when a preparation of apoprotein was relipidated and centrifuged to equilibrium. Thus, the coagulant and peptidase activities were inseparable by a variety of techniques that would be expected to resolve molecules of different size, shape, charge density, isoelectric point, or affinity for phospholipids. While these data do not prove the identity of coagulant and peptidase activities, the likelihood that they are properties of different molecules diminishes as each technique fails to resolve the activities.

Phospholipids enhance the coagulant activity of tissue factor by 500- to 1000-fold, while the V_{max} of the peptidase is increased only by a factor of 2–3. The reaction of tissue factor with factor VII involves the interaction of two large molecules, whereas the peptides and aminoacyl-β-naphthylamides are much smaller. It could be postulated that phospholipid enhances the affinity of tissue factor for factor VII, but that the lipid is remote from the active center and, therefore, does not effect the binding of small substrates. Since the assay used for estimating tissue factor activity couples the reactions involving factors VII and X, it is also possible that part of the observed phospholipid effect on coagulant activity is due to an increased affinity of factor X for the factor VII complex with relipidated tissue factor.

Although Ca^{2+} is required for the coagulant activity of tissue factor, it does not stimulate the peptidase activity of the chelated enzyme. Conversely, Co^{2+} is necessary for the peptidase activity but not for the coagulant activity. There are several possible explanations for this discrepancy. First, there may be two discreet active centers on the tissue factor molecule, one catalyzing the coagulant reaction and requiring Ca^{2+}, and one catalyzing the hydrolysis of peptide bonds and requiring Co^{2+}. Alternatively, a single region whose catalytic function is altered by different metals may catalyze both reactions analogously to the peptidase and esterase functions of carboxypeptidase A (Coleman and Valee, 1961). Finally, Co^{2+} may actually stimulate the reaction between tissue factor and factor VII, but the coupled assay would not detect this if only Ca^{2+} were effective in the activation of factor X.

A number of Co^{2+}-stimulated particle-bound peptidases from human and rat liver, and rat and porcine kidney have been described (Smith *et al.*, 1965; Mahadevan and Tappel, 1967; Felgenhauer and Glenner, 1966; Pfleiderer *et al.*, 1964; Wachsmuth *et al.*, 1966a,b). While the biological functions of these enzymes have not been clarified other than a possible role in the degradation of proteins, their specificity for small substrates is very similar to tissue factor. These enzymes have been solubilized by a variety of techniques: deoxycholate extraction, autolysis, and partial tryptic digestion. An aminopeptidase from autolyzed rat kidney has been studied in detail by Felgenhauer and Glenner (1966). Its apparent molecular weight is similar to that estimated for tissue factor (232,000 *vs.* 330,000 and 220,000 for the two species of tissue factor (Nemerson and Pitlick, 1970)), but when examined on disc gel electrophoresis, the enzyme was disperse suggesting that the autolysis yielded a series of partially degraded proteins which retained peptidase activity. The Michaelis constant derived from the hydrolysis of leucyl-β-naphthylamide was 1.4×10^{-4} M for the kidney peptidase; tissue factor had a K_M of 7.5×10^{-5} M using alanyl-β-naphthylamide as a substrate. Like tissue factor, the kidney peptidase hydrolyzes alanyl-β-naphthylamide more readily than leucyl-β-naphthylamide.

The kinetic parameters of the other particle-bound enzymes, as well as their substrate specificity, vary in some details from organ to organ and with the method of solubilization and species of origin, but bear a strong resemblance to tissue factor in a number of respects. It is tempting, therefore, to speculate that these enzymes are all related to tissue factor and experiments are now in progress comparing the molecular, enzymatic, and antigenic properties of these various peptidases prepared from bovine tissues.

The maximum observed hydrolytic rate of alanyl-β-naphthylamide by apoprotein was 79 nmoles/mg per min; under similar conditions the apoprotein hydrolyzed 199 nmoles/mg per min of Ala-Gly-Gly. These numbers are considerably lower than those reported for many other peptidases and proteolytic enzymes; *e.g.*, kidney peptidase hydrolyzes 283 nmoles/mg per min of leucyl-β-naphthylamide (Felgenhauer and Glenner, 1966). It is possible, therefore, that the peptidase is simply a contaminant of tissue factor. This is considered unlikely for several reasons: (1) the purity of the apoprotein; (2) similar peptidase is present in lung microsomes and the solubilized apoprotein of lung and brain; and (3) coagulant and peptidase activities are coincident when analyzed by several techniques. Peptidase activity is also associated with the bulk protein. Disc gel electrophoresis, for example, shows peptidase in the two major protein bands (Figure 5). It would seen unlikely that a trace contaminant would be distributed by chance in a manner exactly corresponding to the bulk protein.

A more attractive possibility is that the low turnover number is a reflection of the substrate employed and that for optimal activity, tissue factor requires a substrate similar to bovine factor VII, a plasma protein with a molecular weight estimated to be 34,000 (Tishkoff *et al.*, 1968). Furthermore, the interaction of tissue factor with factor VII results in the formation of a tightly bound stoichiometric complex. Therefore, if the ability of tissue factor to complex with factor VII depends on its peptidase activity, a rapid turnover of the catalytic site would not be required. Finally, the coagulant function may not involve hydrolysis of peptide bonds, but rather a transpeptidation, and, therefore, the turnover number may be low because it is a measure of the wrong enzymatic function.

Following incubation of the oxidized B chain of insulin with tissue factor three internal peptides were isolated from the insulin chain. These could arise from either endopeptidase action or the combined action of amino- and carboxypeptidases. Experiments with peptide substrates, however, revealed no carboxypeptidase activity. Therefore, the observed hydrolytic products must be the result of an endopeptidase with broad specificity, or the combined action of amino- and endopeptidases. While two enzymes may be working in concert to produce the observed insulin cleavages, this possibility does not seem likely because peptide maps of digestion products were similar at 4 and 24 hr in experiments with both purified lung apo protein and lung microsomes.

The attack by tissue factor on the B chain of insulin is unlike that of several other mammalian endopeptidases (Figure 9) (Sanger and Tuppy, 1951; Dannenberg and Smith, 1955). It also differs from that of several bacterial enzymes, as for example, subtilisin and the neutral protease of *Bacillus subtilis* (Tuppy, 1953; Feder and Lewis, 1967).

Although tissue factor failed to hydrolyze Cbz-Gly-Phe-NH_2, but did hydrolyze substrates with free amino groups, its mode of action is in sharp contrast to that of leucine aminopeptidase and the kidney aminopeptidases of Felgenhauer and Glenner (1966) and Pfleiderer *et al.* (1964). Tissue factor readily hydrolyzes Ala-Gly-Gly, but fails to cleave leucin-

amide, the leucine aminopeptidase substrate. In addition, leucine aminopeptidase readily splits the bonds Asn_3-Gly_4-His_5-Leu_6 in the oxidized B chain of insulin (Hill and Smith, 1957) while Asn and Gln were recovered as amino acids in only trace amounts from tissue factor digests of insulin, and the His_5–Leu_6 bond was not cleaved at all. The kidney peptidase described by Pfleiderer *et al.* (1964) and that described by Felgenhauer and Glenner (1966) are clearly aminopeptidases. Pfleiderer's enzyme completely digests the B chain of insulin while Felgenhauer and Glenner's enzyme systematically digests angiotensin II and its amide and cleaves the His-Pro-Phe peptide which is resistant to leucine aminopeptidase.

A number of the bonds sensitive to tissue factor are also split by the beef lung proteinase described by Dannenberg and Smith (1955). This enzyme, however, is soluble, requires free sulfhydryl groups for activity, and has a pH optimum of 4.0 with hemoglobin as a substrate. Therefore, tissue factor peptidase activity does not appear to be that of any well-known peptidase insofar as attack on insulin is concerned.

It may yet be possible to separate the coagulant and peptidase activities. However, even if the two activities do not represent the same active site, the peptidase may affect coagulation by virtue of its close association with tissue factor. If these two activities represent different enzymes, it must be acknowledged that when blood is exposed to tissue factor and coagulation is initiated, it also would be exposed to peptidase. A number of enzymes in the coagulation system are very sensitive to proteolytic modifications which may lead to increased activity or destruction (Rapaport *et al.*, 1963). Even if the peptidase is not a property of tissue factor *per se*, it may function as an accessory control mechanism once the plasma is exposed to injured tissue.

The coincidence of the coagulant and peptidase activities of tissue factor does not prove that tissue factor initiates blood coagulation *via* proteolysis, although this possibility is enticing and has precedent in the coagulation mechanism; *e.g.*, thrombin is a fragment of prothrombin which is formed by the proteolytic action of activated factor X (Jobin and Esnouf, 1967; Magnusson, 1965; Barton *et al.*, 1967). Moreover, enzymes not usually associated with blood coagulation such as trypsin and the venom of Russell's viper probably activate factor X by proteolysis as the activated factor X so formed is of lower molecular weight than its precursor (Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964). This question will be settled only when direct analysis of the formation of the tissue factor–factor VII complex shows hydrolysis of factor VII proceeding *pari passu* with the development of coagulant activity.

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