Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 299–302, January 1986
Biochemistry

Affinity purification of human tissue factor: Interaction of factor VII and tissue factor in detergent micelles

(coagulation/Triton X-100/receptor–ligand binding/proteolytic complex)

ARABINDA GUHA*, RONALD BACH*, WILLIAM KONIGSBERG†, AND YALE NEMERSON*

*Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029; and †Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510

Communicated by Leon E. Rosenberg, September 13, 1985

ABSTRACT **Tissue factor, a known initiator of blood coagulation, was found to be active in Triton X-100. A system consisting of tissue factor, factor VIIa, calcium ions, and coagulation factor X generated activated factor X at an appreciable rate. Based on this observation, we coupled human and bovine factor VII to a solid support. Each column bound tissue factor, solubilized in Triton X-100, in a species-specific manner. These interactions required calcium ions; when the columns were washed with detergent containing calcium ions, no tissue factor was eluted. When calcium ions were omitted from the eluant, tissue factor emerged as a sharp peak. Human tissue factor was extracted from an acetone brain powder into 2% Triton X-100. This extract, made 10 mM in $CaCl_2$, was passed over a factor VII column. Human factor VII (1.2 mg) was coupled to 30 ml of Affi-Gel 15. This column bound ≈15 μg of human tissue factor. The eluted material was ≈25% pure. Final purification was achieved by gel filtration after chymotryptic digestion of contaminants. The tissue factor activity was stable to this treatment. The molecular weight determined by sodium dodecyl sulfate/PAGE (≈46,000) was also unchanged by chymotrypsin. The final material was a single band on PAGE, demonstrated similar resistance to tryptic and chymotryptic digestion as bovine tissue factor, and had approximately the same specific coagulant activity as the previously purified bovine material. Tissue factor was also purified from human placenta, yielding a similar protein. A partial 28-residue sequence of the latter has been obtained.**

One way in which coagulation may be initiated is via the interaction of tissue factor, normally associated with phospholipids, with factor VII, a serine protease zymogen (for a review, see ref. 1). Tissue factor, a membrane-bound glycoprotein, is not normally in the circulation or accessible to plasma proteins. When vessels are disrupted, however, plasma coagulation factor VII may complex with tissue factor, forming a catalytically active species (2). Thus, the chemical and biological characterization of tissue factor is clearly important to understanding the mechanism of coagulation.

Bovine tissue factor has been previously purified to homogeneity by using procedures that extracted lipids from this material (3). In this form, the tissue factor was essentially biologically inert. By inserting the protein into phospholipid vesicles, activity was restored. In this study, we show that Triton X-100, a nonionic detergent, also restores activity to tissue factor, albeit less effectively than lipids. This phenomenon has allowed us to utilize factor VII in the presence of Triton X-100 as an affinity ligand for the purification of human tissue factor to apparent homogeneity in a rapid and efficient manner. The availability of pure human tissue factor will allow us to study the details of the initiation of coagulation in humans. Furthermore, it will allow us to investigate the potential role of tissue factor in clinically important pathological states such as thrombosis.

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MATERIALS AND METHODS

Human brains were obtained from autopsy material and stored at −70°C. Frozen bovine citrated plasma was purchased from Irvine Scientific. Fresh frozen human plasma was obtained from New York Blood Center (New York, NY). All chemicals for analytical sodium dodecyl sulfate/PAGE and Affi-Gel 15 were from Bio-Rad. Triton X-100 was purchased from New England Nuclear. Diaflo YM-10 membranes were the product of Amicon. Bovine serum albumin and α-chymotrypsin were from Sigma, and trypsin was from Boehringer Mannheim. Dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone was obtained from Calbiochem–Behring. All other chemicals were of reagent grade or better and were from standard sources.

Proteins. Bovine factors VII and X were purified as described elsewhere (4, 5). Human factor VII was purified according to Broze and Majerus (6). Bovine tissue factor was purified to homogeneity as described (3) except that a tissue factor-specific monoclonal antibody column (7) was substituted for the originally described polyclonal immunoaffinity column. All proteins were essentially homogeneous as judged by sodium dodecyl sulfate/PAGE (8). The tissue factor concentration was determined by a modified Lowry assay (9, 10) with bovine serum albumin as the standard. Concentrations of the soluble proteins were determined by absorbance at 280 nm using 12.9 and 9.6 as the extinction coefficients for factors VII and X, respectively.

Assay of Tissue Factor. Tissue factor procoagulant activity was measured by a two-stage coagulation assay (3).

Relipidation of Tissue Factor Apoprotein. Tissue factor apoprotein was reassociated with phospholipids as described (3, 11, 12). Tissue factor samples in 2% Triton X-100 were diluted 1:20 into 0.1 M NaCl/0.05 M Tris·HCl, pH 7.5, prior to relipidation so that the detergent concentration in the relipidation mixtures was below the critical micelle concentration for Triton X-100 (<0.015%) (13). Relipidation was accomplished by combining 1 vol of the tissue factor sample (in 0.1% Triton X-100), 1 vol of lipids (10 mg/ml in 0.25% deoxycholate), 7.5 vol of 0.1% bovine serum albumin in 0.1 M NaCl/0.05 M Tris·HCl, pH 7.5, and 0.5 vol of 100 mM $CdCl_2$. After incubation at 37°C for 2 hr, the samples were diluted at least 1:10 for coagulation assay.

Affinity Adsorbent. Affinity adsorbent was prepared by coupling 1.2 mg of human factor VII in 0.1 M $NaHCO_3$ (pH 8.0) to 30 ml of Affi-Gel 15 in the same buffer at 4°C overnight with gentle stirring. Over 90% of human factor VII bound to Affi-Gel 15, as measured by factor VII coagulation assay (4, 14). Unreacted sites were blocked by addition of 1 ml of 1 M glycine ethyl ester (pH 8.0) for 4 hr at room temperature. The slurry was successively washed with 10 vol of 2 M NaCl, 10

vol of 0.1 M NaCl/0.05 M Tris·HCl, pH 7.5, and, finally, 10 vol of 2% Triton X-100 in the same buffer containing 10 mM $CaCl_2$.

Affinity Purification of Human Tissue Factor Apoprotein. Tissue factor apoprotein was solubilized from human brain acetone powder using 2% Triton X-100 in 0.1 M NaCl/0.05 M Tris·HCl, pH 7.5 (3). The extract was made 10 mM in $CaCl_2$. To 105 ml of tissue factor extract was added 30 ml of human factor VII-Affi-Gel, and it was stirred at room temperature for 2 hr. Approximately 30% of the total tissue factor activity was adsorbed. No further adsorption was observed on prolonged incubation. The slurry was transferred to a column and washed with 300 ml of the 2% Triton X-100 extraction buffer containing 10 mM $CaCl_2$, followed by 200 ml of 0.1% Triton X-100 in the same buffer with 10 mM $CaCl_2$. The bound tissue factor was then eluted with this same buffer without calcium ions. Fifty 3-ml fractions were collected at a flow rate of 60 ml/hr. The activity was eluted in fractions 14–23 (30 ml). A total of 950 ml of extract was processed in similar manner. The pooled tissue factor active peak (275 ml) was concentrated by ultrafiltration using Amicon YM-10 membranes.

α-Chymotrypsin Digestion. To the tissue factor concentrate (5.5 ml, 748 μg of protein) was added 15 μg of α-chymotrypsin, and proteolysis was allowed to progress for 18 hr at 37°C. The reaction was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. The tissue factor apoprotein was precipitated by the addition of 5 vol of ice-cold acetone. The slightly turbid solution was allowed to stand on ice for 30 min and the precipitate was collected by centrifugation at 3000 × *g* for 1 hr. The precipitate was dried *in vacuo*. The pellet was dissolved in 200 μl of 0.1% Triton X-100 in 0.1 M NaCl/0.05 M Tris·HCl, pH 7.5.

Ultrogel AcA 44 Gel Permeation. The concentrated tissue factor was applied to an Ultrogel AcA 44 column (0.9 × 55 cm) equilibrated in the same buffer used to solubilize the protein. Fractions (0.5 ml) were collected and assayed for activity. The peak tubes were pooled for further analysis.

Kinetic Assays. The rate of activation of factor X was measured by using a radiometric assay as described (15, 16). Briefly, factor X is oxidized with periodate and reductively tritiated by using sodium [^{3}H]borohydride. Upon activation, a peptide containing ≈65% of the radioactivity is cleaved from the zymogen. The peptide is extracted into 5% trichloroacetic acid and the radioactivity is estimated by liquid scintillation counting. At least seven points, in duplicate, were used for each velocity determination. Kinetic parameters were estimated by fitting the Michaelis–Menten equation to the data using Marquardt's nonlinear least-squares algorithm (Tektronix 070-2214-00). No weighting was used. The assay mixture consisted of 10 nM tissue factor and 50 nM factor VIIa in a buffer containing 0.1% Triton X-100, 0.1% albumin, 2 μM dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone, 5 mM $CaCl_2$, 0.1 M NaCl, and 0.05 M Tris·HCl, pH 7.5. Twelve substrate concentrations ranging from 33 nM to 4080 nM were used.

RESULTS

The formation of a proteolytic complex between factor VII and tissue factor in the presence of detergent was demonstrated in an assay system that directly measures the hydrolysis of tritiated factor X (15, 16). The bovine proteins used in this experiment were all purified to homogeneity as described in *Materials and Methods*. The kinetics of factor X cleavage were measured at 37°C in the presence of 10 nM tissue factor and 50 nM factor VIIa in a buffer containing 0.1% Triton X-100. Under these conditions, the K_m app was estimated to be 1058 ± 108 × 10^{-9} M and the V_{max} was 14.0 ± 0.62 nM·min^{-1}. No velocity was observed in the absence of either calcium ions, enzyme, or cofactor. The reaction proceeds much more rapidly if tissue factor is inserted into phosphatidylcholine vesicles, with the k_{cat} being some 160-fold greater (unpublished data). Thus, it is apparent that incorporation of tissue factor into phospholipid vesicles is essential for optimal biological activity. On the other hand, the tissue factor–detergent complex functions, albeit weakly, as a cofactor for factor VIIa.

Table 1. Purification of human tissue factor

Step	Protein, mg	Total units, ×10^{-6}	Units/mg, ×10^{-6}	Yield, %	Purification, -fold
Triton extract	1548	627	0.41	100	1
Affinity pool	0.75	144	192	23	468
Ultrogel pool	0.14	108	800	17	1950

The protein concentrations were determined by the method of Lowry (9, 10) using a bovine serum albumin standard. Triton X-100 extraction from human and bovine brain powders (3) enriched the tissue factor ≈25-fold and ≈50-fold, respectively (data not shown). The standard for the two-stage tissue factor assay was pure bovine tissue factor reconstituted into phosphatidylserine/phosphatidylcholine (30:70) vesicles by the octyl glucoside dialysis procedure of Mimms *et al.* (17). The specific activity of this material was arbitrarily defined as 10^9 units per mg of protein, taking into account the random orientation of bovine tissue factor in these vesicles (unpublished data). No correction for the orientation of deoxycholate reconstituted human protein was used, and since it has not been determined.

Based on these observations, we constructed a factor VII affinity column. Tissue factor solubilized in 2% Triton X-100 containing 10 mM $CaCl_2$, extracted from 35 g of human brain acetone powder, was applied to a human factor VII-Affi-Gel column. Thirty percent of the activity was retained by the column. After extensive washing with 2% and 0.1% Triton X-100, the activity was eluted with 0.1% Triton X-100 without calcium ions. The activity emerged in one column volume and was ≈25% pure (Table 1). The affinity-purified material was digested with chymotrypsin (1:50, wt/wt), for 18 hr at 37°C. After gel filtration on Ultrogel AcA 44, the human tissue factor appeared homogenous as judged by sodium dodecyl sulfate/PAGE (Fig. 1). Upon relipidation with mixed brain phospholipids, the human tissue factor had approxi-

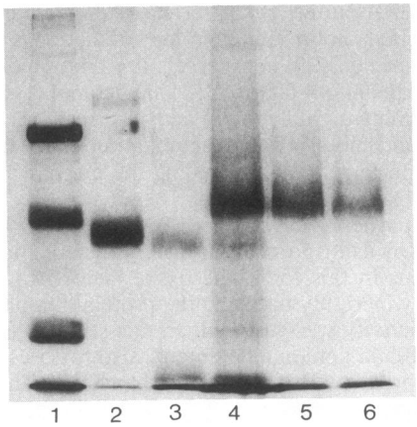

FIG. 1. Sodium dodecyl sulfate/PAGE was performed by the method of Laemmli (8) on a 10% acrylamide gel. Lanes: 1, molecular weight standards (bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; myoglobin, 17,000); 2, bovine tissue factor (3.3 μg); 3, bovine tissue factor tryptic digest (3.3 μg); 4, human tissue factor affinity column eluate (20 μg); 5, human tissue factor (7.2 μg) 6, human tissue factor tryptic digest (7.2 μg). All samples were run reduced in 10% 2-mercaptoethanol.

mately the same coagulant activity, 8×10^8 units/mg (Table 1), as did the homogeneous bovine preparations (10^9 units/mg). The enrichment factors obtained from the human and bovine brain acetone powders were ≈50,000-fold and ≈140,000-fold (3), respectively.

Additional support for the homogeneity of the human protein was obtained from the proteolytic digestions. Bovine tissue factor is known to be resistant to chymotryptic digestion. Similarly, human tissue factor retained 100% of its activity after an 18-hr digestion with chymotrypsin as described above, and there was no change in electrophoretic mobility (Fig. 1, lanes 4 and 5). Bovine tissue factor when digested with trypsin (24 hr; 37°C; 1:100, wt/wt) retains its activity, but a small peptide is released (3); likewise, the human protein is resistant to trypsin (lanes 3 and 6). Both proteins show a reduction in staining intensity of the major band after tryptic digestion. Since the total coagulant activity was unchanged, the most likely explanation for this difference is that some denatured tissue factor or a contaminant was totally digested by the enzyme. We note that the apparent M_r of the bovine protein is lower in the gel shown in Fig. 1 than we previously reported (3). However, the previous results were obtained by using phosphate buffers in a cylindrical gel, whereas the present data were obtained using the Laemmli discontinuous system (8).

Analysis of 697 pmol of tissue factor protein on the Applied Biosystems (Foster City, CA) gas-phase sequenator showed that the protein had a blocked amino terminus. The absence of a free α-amino group could have been the result of a modification during the isolation or it may have been due to a naturally occurring post-translational modification. This issue may be clearly resolved when the structural gene for tissue factor is sequenced. Since the phenylthiohydantoin-derivatized amino acids released after the first round of Edman degradation were only barely detectable after HPLC, we conclude that the level of contamination by material containing free α-amino groups did not exceed 5%. Because of the severely limited amounts of human brain material available to us, we have also begun to purify tissue factor from human placenta. As these extracts have more contaminating proteins than those from brain, a more complicated procedure including concanavalin A chromatography (18) and the use of a polyclonal anti-bovine tissue factor column (3) was used. This material yielded a predominant band at M_r 46,000 when analyzed by $NaDodSO_4$/PAGE as well as very faint contaminants (gel not shown). The material had a specific activity equivalent to the other preparations and yielded a unique unambiguous sequence: X-Gln-X-Tyr-Asn-X-Pro-Asn-Pro-Thr-Ala-Asp-X-Lys-Thr-Ala-Val-X-X-Ser-Ser-Asp-Phe-X-Ala-X-Leu-Ile. The yield of phenylthiohydantoin-derivatized glutamine at cycle two was 487 pmol. This represents a 64% recovery based on 765 pmol of placental tissue factor that was applied. The gas-phase sequencing with the Applied Biosystem machine routinely gives 67% recovery at the second step for sequencing a similar amount of myoglobin, which is used as a standard. The X in the sequence represents cycles in which no phenylthiohydantoin derivatized amino acids were observed. These probably correspond to sites of post-translational modification, perhaps carbohydrate or possibly, in some cases, to cysteine. The latter, which was not carboxamidomethylated, could not be identified. Based on the unique sequence, we conclude that human placental tissue factor as isolated consists of a single polypeptide chain.

DISCUSSION

We have developed a facile and unique technique for the rapid purification of human tissue factor. To accomplish this, we exploited the observation that tissue factor functions in 0.1% Triton X-100, thus enabling us to use factor VII as an affinity ligand. The human tissue factor protein, which can be obtained from brain in ≈48 hr, is homogeneous by four criteria: a single band on sodium dodecyl sulfate/PAGE, the finding of a unique sequence of 28 residues (placental) and the lack of any contaminants possessing a free amino terminus (brain preparation), the procoagulant activity being resistant to tryptic and chymotryptic digestion, and the specific coagulant activity being about equal to that of the known homogeneous bovine tissue factor. The entire sequence obtained for placental tissue factor was searched against the National Biomedical Research Foundation's Nucleic Acid/Protein Data Bank. No significant homology with any other protein sequence was found. Based on these results, we conclude that we have purified human tissue factor to an essentially homogeneous form, which has an apparent M_r of ≈46,000 as judged by $NaDodSO_4$/PAGE with and without mercaptoethanol (not shown). This material is significantly different from previously described preparations of human brain tissue factor (19, 20) in two important respects. First, it has a lower apparent M_r (46,000 vs. 53,000). Second, the purification required to achieve apparent homogeneity starting from acetone powders is much greater in our preparation (≈50,000 fold vs. ≈2000-fold).

Two reactions, in addition to the tissue factor-dependent activation of factors IX and X, are known to be markedly accelerated by phospholipids: the activation of prothrombin by factors Xa and Va and the activation of factor X catalyzed by factors IXa and VIIIa. The ability of the ionic detergent sodium cholate to function in prothrombin activation was demonstrated by Barthels and Seegers in 1969 (21). Indeed, their data indicate that the bile salt was about as effective as a mixture of brain lipids in this reaction. We now show that tissue factor is active in the nonionic detergent Triton X-100. While the activity of tissue factor–factor VIIa in detergent micelles is not great, the k_{cat} being only 0.6% of that observed with phosphatidylcholine, it is clear nevertheless that tissue factor and factor VIIa interact to form a catalytically active complex. Complex formation is also indicated by the binding of tissue factor to the factor VII affinity columns. The specificity of the complex formation is indicated by the requirement for calcium ions, which are absolutely essential for the binding of bovine factor VII to bovine tissue factor (unpublished data). Indeed, elution from the affinity column is accomplished simply by omitting calcium ions from the buffers.

Although bovine and human tissue factor function in a similar manner, there is little cross-reactivity between the species. Antibodies to bovine tissue factor inhibit the human protein (unpublished data). However, the interaction is such that when used in a column, human tissue factor is retarded but not tightly bound. Thus, the antibody column can facilitate purification of the human protein, but the binding is not sufficiently strong to enable efficient affinity purification of the human material on immunoaffinity columns specific for bovine tissue factor. Two monoclonal antibodies raised against bovine tissue factor (7) neither inhibit nor bind the human protein (data not shown). Thus, the enzyme-affinity techniques we have developed will now allow us to produce the quantities of tissue factor required for a detailed analysis of the initiation of human blood coagulation.

Note Added in Proof. Since our submission of this manuscript, we have become aware of a communication by Broze *et al.* (22) describing a similar method of purification of human brain tissue factor.

We thank Allison Gillespie and Gregory Mayr for their excellent technical assistance. We also acknowledge the expert assistance of Dr. Ken Williams in performing the amino-terminal sequence analysis. This work was supported in part by Grant HL 29019 from the National Institutes of Health.

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