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# Factor VII Binding to Tissue Factor in Reconstituted Phospholipid Vesicles: Induction of Cooperativity by Phosphatidylserine†

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ABSTRACT: The binding of factor VII and tissue factor produces a membrane-associated proteolytic complex which may be the primary biological initiator of coagulation. Homogeneous tissue factor, a glycoprotein purified from bovine brain, was reconstituted into phospholipid vesicles ranging from neutral (100% phosphatidylcholine) to highly charged (40% phosphatidylserine) with octyl glucoside. The vesicles were characterized with respect to size and tissue factor content and orientation. Employing data from protease digestion, we deduced that tissue factor is randomly oriented; thus, its effective concentration in these vesicles was half its total concentration. In all binding experiments, 1 mol of enzyme was bound per mole of available activator at saturation. This stoichiometry was not affected by the form of the enzyme employed or the phospholipid composition of the vesicles. With tissue factor incorporated into phosphatidylcholine vesicles, the $K_d$ was 13.2 ± 0.72 nM for factor VII and 4.54 ± 1.37 nM for factor VIIa. Thus, the one-chain zymogen binds to the activator with only slightly less affinity than the more active two-chain enzyme. Active-site modification of factor VII and factor VIIa with diisopropyl fluorophosphate resulted in tighter binding of the derivatized molecules. Inclusion of phosphatidylserine in the vesicles altered the binding both quantitatively and qualitatively. With increasing acidic phospholipid, the concentration of enzyme required to occupy half the activator sites was decreased. In addition, positive cooperativity was observed, the degree of which depended on the vesicle charge and the form of the enzyme. An explicit two-site cooperative binding model is presented which fits these complex data. In this model, tissue factor is at least a dimer with two interacting enzyme binding sites.

The initiation of coagulation by tissue factor was first demonstrated more than a century ago when it was shown that contact between damaged tissue and blood promoted rapid clot formation (de Blainville, 1834). Biochemical characterization of this thromboplastic activity began with the experiments of Howell (1912), who separated tissue thromboplastin into lipid and protein components, which by themselves were essentially inactive. Chargaff (1948) further characterized the lipid and protein fractions and showed that recombination under the appropriate conditions reconstituted the procoagulant activity. Thus, tissue factor was correctly identified as a lipoprotein. We have previously purified tissue factor to homogeneity from bovine brain (Bach et al., 1981). The molecule is a 41-kilo-

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dalton (kDa)[1] integral membrane glycoprotein, and when reconstituted into phospholipid vesicles, it is a potent initiator of coagulation.

Tissue factor serves as an essential cofactor for the plasma serine protease factor VII. Together they convert factors IX and X from zymogens to active enzymes, thereby initiating the sequence of proteolytic events resulting ultimately in clot formation. It now appears that the biological activity generated by the formation of this activator–enzyme complex may play an essential role in coagulation. Therefore, we have investigated the molecular details of this interaction. The stoichiometry and energy of interaction which define this association have been quantified, and the mode of binding has been characterized under a variety of conditions. We have compared the binding of the active zymogen factor VII with its more active two-chain derivative factor VIIa to determine whether the activity differences are attributable to defective binding to tissue factor. As it is shown that acidic phospholipids accelerate this system, tissue factor was incorporated into phospholipid vesicles ranging from neutral to highly charged. Employing these various activator preparations, the contribution of acidic phospholipid to the formation of the activator–enzyme complex was investigated.

## Materials and Methods

Scintillation cocktail Formula 963, Enlightening, Triton X-100, tritiated sodium borohydride (either ~8 or ~60 Ci/mmol), and [$^{14}$C]phosphatidylcholine (NEC 648, 150 mCi/mmol) were from New England Nuclear. Citrated bovine plasma was obtained from Irvine Scientific, Santa Ana, CA. Chemicals for polyacrylamide gel electrophoresis were from Bio-Rad. Egg phosphatidylcholine (PC) and bovine phosphatidylserine (PS) were purchased from Supelco, Bellefonte, PA. Sigma was the source of chymotrypsin, subtilisin BPN′ (subtilisin), bovine serum albumin (BSA), and trizma base (Tris). Ultrogel AcA 44 was from LKB; Sepharose CL-2B and Sephacryl S-1000 were from Pharmacia. Polysciences Inc., Warrington, PA, was the source of polystyrene monodisperse latex beads. Octyl glucoside (octyl β-D-glucopyranoside) was purchased from Calbiochem. Diisopropyl fluorophosphate (DFP) was obtained from Aldrich. All other chemicals were of reagent grade and were obtained from standard sources.

*Protein Purification and Modification.* Factors VII and X were purified to homogeneity from bovine plasma as described elsewhere (Bach et al., 1984; Jesty & Nemerson, 1976). Factor VII was converted to the more active two-chain factor VIIa (~$10^5$ factor VII units/mg) by reaction with factor Xa (Radcliffe & Nemerson, 1975). Factor VII was activated 100-fold (Bach et al., 1984), and the conversion to two-chain factor VIIa was essentially quantitative as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After 6 h, the reaction was stopped by the addition of EDTA to a final concentration of 5 mM. The lipids were removed by centrifugation in a Beckman Airfuge at 150000*g* for 10 min. Factor VII and factor VIIa were tritiated as previously described (Bach et al., 1984) and stored at 100–200 μg/mL in 0.1 M NaCl and 0.05 M Tris, pH 7.5 (TBS), containing 0.1% bovine serum albumin (TBSA) at −80 °C. Active-site modification of the tritiated proteins was achieved as described (Radcliffe & Nemerson, 1975). Formation of diisopropylphosphoroenzyme (DIP-enzyme) conjugates was monitored by the loss of coagulant activity, which became undetectable after 1 h. After 3 h, each sample was dialyzed into TBS at 4 °C and stored as described above.

Tissue factor was purified to homogeneity from bovine brain by immunoadsorption (Bach et al., 1981) as modified: the protein was extracted from 200 g of bovine brain acetone powder by using Triton X-100 as described. Tracer amounts of $^3$H-tissue factor (see below) were added to the extract, and the subsequent purification was monitored by liquid scintillation counting. The protein was purified by using a monoclonal antibody column (Carson et al., 1985). The peak of tritiated protein was dialyzed into TBS containing 0.1% Triton X-100. The dialyzed material was concentrated on an Amicon YM-10 membrane; further concentration was achieved by precipitating the protein with 5 volumes of ice-cold acetone. After being stored on ice for 30 min, the sample was centrifuged at 5000*g* for 30 min at 4 °C. The protein pellet was air-dried and then resolubilized in 200 μL of TBS and 0.1% Triton X-100. Chymotrypsin (5 μg) was added to the affinity-purified tissue factor (500 μg), and digestion was continued for 6 h at 37 °C. The reaction was stopped with 5 mM DFP (final concentration). Total tissue factor activity was unchanged as was the apparent molecular weight of the polypeptide as judged by SDS–PAGE (Laemmli, 1970). However, a background of contaminants observed on the SDS gels was eliminated by this proteolytic digestion. The reaction products were removed by gel filtration on Ultrogel AcA 44 in TBS with 0.1% Triton X-100. The purified apoprotein was stored at −80 °C.

Homogeneity of purified tissue factor (41 kDa), factor VII (53 kDa), and factor X (55 kDa) was confirmed by the appearance of a single band on SDS–PAGE (Laemmli, 1970). The concentrations of factor VII and factor X were determined by the absorbance at 280 nm using $A^{1\%}_{1cm}$ = 12.9 and $A^{1\%}_{1cm}$ = 9.6, respectively. The tissue factor concentration was initially determined by using a modified Lowry procedure (Hartree, 1972; Dulley & Grieve, 1975), with bovine serum albumin as the standard. Sialic acid residues on tissue factor were labeled with tritium (Van Lenten & Ashwell, 1971) as modified (Silverberg et al., 1977; Zur & Nemerson, 1981), except that 0.1% Triton X-100 was added to solubilize the apoprotein. The concentration of tissue factor in the reaction was 100 μg/mL. Oxidation was achieved by using 100 mol of periodate per mole of tissue factor. After being labeled, the $^3$H-tissue factor was isolated by gel filtration on Ultrogel AcA 44 as described above.

*Reconstitution and Isolation of the Tissue Factor–Phospholipid Complex.* Incorporation of purified tissue factor apoprotein into phospholipid vesicles was achieved by first combining tissue factor and phospholipid in the presence of a large excess of the dialyzable nonionic detergent octyl glucoside (Mimms et al., 1981). Removal of the detergent by dialysis results in the spontaneous incorporation of tissue factor in large phospholipid vesicles. Phosphatidylserine (PS) and phosphatidylcholine (PC) in $CHCl_3$ were combined in molar ratios varying from 0:100 to 40:60 and dried to a thin film on the wall of a borosilicate glass tube under a stream of $N_2$ and then in vacuo for 2 h. A 15-fold molar excess of octyl glucoside (200 mM) in TBS was added; the mixture was incubated at room temperature with occasional vortexing until it was completely clear. Apoprotein (100–200 μg/mL) in TBS

[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; TBS, 0.1 M NaCl and 0.05 M Tris, pH 7.5; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBSA, 0.1% bovine serum albumin in TBS; DIP, diisopropylphosphoro; DFP, diisopropyl fluorophosphate; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s); BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

containing 0.1% Triton X-100 was added to the phospholipid–octyl glucoside preparation, giving a final solution in which the concentration of Triton X-100 was <0.02% and the tissue factor: phospholipid:octyl glucoside molar ratio was 1:100000:1 500000. Tracer amounts of [$^{14}$C]PC and $^{3}$H-tissue factor were added for precise quantification of protein and phospholipid in the final material. The ratio of $^{3}$H counts to $^{14}$C counts was approximately 10 to 1. Aliquots were taken for liquid scintillation counting, and the remainder was dialyzed against 3 × 1 L of TBS at room temperature for 72–96 h, after which the material was gel filtered at room temperature in TBS on Sepharose CL-2B (1.5 × 55 cm). Most of the vesicles eluted in the void volume; the remaining material either appeared in the included volume or was adsorbed to the column and could be recovered by washing with TBS–0.1% Triton X–100. Fractions were pooled according to the profile of turbidity (absorbance at 280 nm) and radioactivity. Recovery of $^{3}$H-tissue factor and $^{14}$C-phospholipid was determined by liquid scintillation counting. Three milliliters of Formula 963 cocktail was added to aliquots from each gel filtration pool and the starting solution. After at least 2 h in the dark, the counts were measured in a Searle Delta 300 liquid scintillation counter. These values were corrected for background counts and spillover of $^{14}$C counts into the $^{3}$H channel. Tritium counts appearing in the $^{14}$C channel were negligible and were ignored. From the initial concentrations of tissue factor and phospholipid and the recovery of $^{3}$H-tissue factor and $^{14}$C-phospholipid, the final concentrations of tissue factor and phospholipid were calculated. The average molecular weights of PC (787) and PS (788) used in these calculations assume the dioleoyl form of the lipids.

*Determination of Vesicle Size.* A Sephacryl S-1000 column (1.5 × 55 cm) was equlibrated with 0.1% SDS in TBS. Column calibration was performed by utilizing the method of Reynolds et al. (1983) with polystyrene monodisperse latex beads. Fifty microliters of each preparation of beads (2.5% solids) was diluted into 2 mL of column buffer and run over the column. One-milliliter fractions were collected, and the light scattering was measured as the absorbance at 280 nm. The latex beads eluted as narrow symmetrical peaks. The center of each peak was taken as the elution volume for particles of a given diameter: 900 nm (36 mL), 270 nm (37 mL), 205 nm (39.5 mL), 110 nm (56.5 mL). The included volume (85.5 mL) was determined with sucrose by monitoring the refractive index. The calibration data were plotted by the method of Ackers (1967). Vesicle sizing was performed as described above except that SDS was eliminated from the column buffer. To facilitate quantitative recovery, the column was presaturated with 500 mg of bovine brain lipids (Bell & Alton, 1954). A portion of the reconstituted tissue factor was run over the Sephacryl S-1000 column. From the tritium profile, vesicle size and distribution were calculated.

*Protease Digestion.* $^{3}$H-Labeled tissue factor was incorporated into vesicles composed of 30:70 PS/PC as described above, omitting the $^{14}$C-tracer. Protease digestion was performed by combining 4.0 mL of reconstituted $^{3}$H-tissue factor (4.7 nM) and 12 mg of subtilisin. The reaction mixture was incubated at 37 °C, and at each time point indicated in Figure 2, aliquots were removed for analysis. Digestion of the tissue factor polypeptide was monitored by measuring the generation of uranyl acetate soluble tritium (Dirr et al., 1957). Proteolysis was continued for 180 min.

The extent of digestion of the tissue factor polypeptide was quantified by combining 50 μL of the reaction mixture, 20 μL of TBSA, and 100 μL of 0.16% uranyl acetate. Samples were vortexed, incubated at room temperature for 10 min, and centrifuged in a Beckman microfuge B for 5 min. From each supernatant, 100 μL was removed and combined with 3 mL of Formula 963. Tritium counts were measured in duplicate in a Searle Delta 300 liquid scintillation counter. Generation of uranyl acetate soluble tritium was calculated as the percent of tritium counts added to the tube.

Tissue factor procoagulant activity was monitored in parallel with the measurement of protein degradation by combining 20 μL of reaction mixture with 480 μL of TBSA and then inhibiting the protease by adding 1 μL of 2.5 M DFP in 2-propanol. Samples were stored on ice for at least 30 min and then diluted further in TBSA just prior to performing the two-stage tissue factor assay (Bach et al., 1981). In the first stage of the assay, the concentration of factor X was 260 nM, factor VIIa concentration was 1.1 nM, and $CaCl_2$ concentration was 8.3 mM. Factor VII deficient bovine plasma was prepared by immunoadsorption (Bach et al., 1984). A sample of tissue factor reconstituted with 30:70 PS/PC was used as the assay standard. One picogram of tissue factor per milliliter in TBSA was arbitrarily defined as 1 tissue factor unit/mL. A log–log plot of clotting time vs. tissue factor units was linear from 25 tissue factor units/mL (24 s) to 3.2 tissue factor units/mL (60 s). All samples were assayed in duplicate.

To quantify the amount of tissue factor procoagulant activity trapped inside the vesicles and thus protected from the protease, samples of the starting material and the limit digest were treated with DFP as described above, assayed, and then rapidly frozen in a dry ice–acetone bath. After being thawed at 37 °C, the samples were reassayed. The freeze–thaw procedure was repeated 5 times.

A second subtilisin digestion of $^{3}$H-tissue factor–phospholipid was performed exactly as above. Aliquots for analysis were removed at 0 and 180 min. After 180 min, the remaining reaction mixture was subjected to 5 freeze–thaw cycles as described above without inhibiting the protease. The reaction was then continued for another 180 min, and aliquots were again taken for analysis. The reaction mixture was subjected to 5 cycles of digestion, each terminated by repeated freezing and thawing. The samples were analyzed for uranyl acetate precipitable tritium and tissue factor activity as described above.

*Equilibrium Binding Assay.* Initially, vesicle isolation was achieved by pelleting the tissue factor–phospholipid complex at 150000*g* for 10 min at 24 °C in a Beckman Airfuge. Greater than 90% of the vesicles were pelleted under these conditions as monitored by clearing of $^{3}$H-tissue factor and procoagulant activity. However, since all activity measurements are performed at 37 °C, we decided that the binding measurements should likewise be made at this temperature. Thermostatic regulation of the Airfuge was achieved by placing the machine in a 35 °C oven and passing the drive air through a copper coil immersed in a 35 °C water bath, thus maintaining the rotor temperature at 37 °C while the centrifuge was in operation, as determined with a thermistor probe. For runs at a lower temperature, the centrifuge chamber and water bath were appropriately regulated. At 37 °C, the vesicles did not pellet efficiently; in fact, after partial clearing of the suspension, pelleted material reappeared in the supernatant solution, presumably due to vibration. To circumvent this problem, vesicles were isolated by flotation through a discontinuous gradient of 20%, 10%, and 5% sucrose by centrifugation at 150000*g* for 10 min in the Airfuge; 90% recovery was routinely obtained. Recovery was independent of temperature from 4 to 37 °C and vesicle composition from 100%

PC to 40:60 PS/PC. When this strategy was applied to the binding assay, results essentially identical with those obtained by pelleting at 24 °C were observed (data not shown). This indicates that sucrose has no significant effect on the association of enzyme and activator. Therefore, vesicle isolation by flotation was adopted as the standard procedure for all subsequent binding assays.

The recovery of tissue factor–phospholipid vesicles was initially monitored both by liquid scintillation counting using $^3$H-tissue factor as the internal standard and by a coagulation assay. Since the two assays gave equivalent results and liquid scintillation counting was faster, recovery of $^3$H-tissue factor was adopted as the standard procedure. The binding assays were performed as follows: a constant amount of tissue factor was combined with varying concentraitons of $^3$H-enzyme in buffer containing TBSA, 20% sucrose, and 5 mM $CaCl_2$ in a total volume of 75 μL. Each tube was incubated for 30 min at 37 °C, unless otherwise indicated. Following incubation, the samples were carefully overlayed with 50 μL of TBSA containing 10% sucrose and 5 mM $CaCl_2$, and 50 μL of the same buffer containing 5% sucrose. Each of the overlay solutions was prewarmed to 37 °C.

The tubes were then spun in the Airfuge as described above at 37 °C using an A-100 rotor. The concentrations of $^3$H-enzyme remaining free in solution and in the vesicle fraction were determined by one of two sampling procedures: In the first (method 1), the concentrations of the total $^3$H-enzyme and free $^3$H-enzyme were determined separately, and the concentration of bound $^3$H-enzyme was calculated as the difference. Two sets of samples were prepared in duplicate, one with and the other without tissue factor. Following centrifugation, the tip of a Hamilton Digital Dilutor was inserted into the bottom layer (20% sucrose) of each tube, and 50 μL was withdrawn and added to 3 mL of New England Nuclear Formula 963 liquid scintillation cocktail; 150 μL of TBS containing 0.1% Triton X-100 was added to each vial to eliminate the quenching effect of the sucrose. After at least 2 h in the dark, tritium counts were measured as described above. Values for the duplicate samples were averaged and corrected for background. The concentration of total enzyme was calculated from the samples without tissue factor. The measured concentrations of free enzyme were obtained from the samples containing tissue factor. In method 2, the bound and free fractions were recovered directly from each tube, thus eliminating the need to prepare samples without tissue factor. Following centrifugation, the vesicles along with bound $^3$H-enzyme were localized at the air–water interface. This material was recovered by carefully overlaying each tube with 50 μL of TBS containing 0.1% Triton X-100. The buffer along with part of the upper sucrose layer was then withdrawn with an Oxford pipet. This procedure was repeated twice and the material combined for counting. The bound pool included the entire 5% sucrose layer and half the 10% sucrose layer. The remaining material, the free $^3$H-enzyme fraction, was withdrawn and combined with 150 μL of buffer to eliminate sucrose quenching. Liquid scintillation counting and calculation of the data were performed as above, except that duplicate samples were not obtained with this procedure.

Following isolation by flotation, the vesicles along with bound enzyme are localized at the air–water interface, well separated from free enzyme which remains in the bottom layer. Thus, after separation, no further exchange between the bound and free pools can occur. However, during the separation process, a significant fraction of the bound enzyme may dissociate. In the case of method 1, only the bottom layer is sampled, and dissociation of bound enzyme in the 10% and 5% sucrose layers is of no consequence. Method 2 could be measuring a perturbed state because enzyme bound at equilibrium could dissociate during isolation. We have directly compared the two sampling methods, and the results are equivalent (data not shown), implying no significant release of bound enzyme occurs during isolation. The first method has the advantage of requiring less labor, and the second is more conservative of materials; both methods were used.

The range of enzyme concentrations employed in each isotherm was adjusted to meet two criteria. First, the data should be evenly distributed, with nearly equal partitioning of the points above and below the midpoint of the isotherm. Second, at the highest enzyme concentrations, the observed binding should be greater than 75% of saturation. It was not possible to extend the free ligand concentration to several orders of magnitude above the midpoint of the binding isotherm as suggested by Klotz (1982). At these higher concentrations, particularly in the presence of acidic phospholipids, the nonideal properties of the assay begin to dominate and render the data uninterpretable (see below).

*Proof of Equilibrium.* Reversibility of binding was established by combining $^3$H-factor VII with tissue factor in 100% PC vesicles in a buffer composed of TBSA, 20% sucrose, and 5 mM $CaCl_2$. This material was allowed to equilibrate and then diluted 10-fold into buffer containing no enzyme or a 200-fold excess of unlabeled factor VII. Thirty minutes following dilution, the amount of $^3$H-factor VII which remained bound to tissue factor was determined. In the control, the concentration of bound $^3$H-factor VII was 2.10 ± 0.37 nM (SD). The excess cold ligand reduced this value to 0.04 ± 0.16 nM. A second test to establish that the system is at equilibrium was to demonstrate reequilibration following a dilution–jump. The enzyme and activator were combined at high concentration in the presence or absence of calcium as described in the legend for Figure 5. Since the binding of enzyme to activator has an absolute requirement for calcium, in the sample with calcium the activator was essentially saturated with enzyme, whereas in the sample devoid of calcium no activator–enzyme complex was formed. Following incubation, the samples were diluted into calcium-containing buffer, and the concentration of bound enzyme was measured at various times following dilution.

*Data Correction and Curve Fitting.* The measured concentrations of bound and free enzyme were corrected for fractional recovery of tissue factor–phospholipid vesicles, mixing of bound and free pools (method 2 only), and binding of enzyme to phospholipid, i.e., nonspecific binding. Approximately 10% of tissue factor containing vesicles along with bound factor VII remained in the free fraction after centrifugation. By measurement of the fractional recovery of vesicles ($f_1$), a correction factor was obtained. Second, a small fraction (5.2 ± 1.4%) of free enzyme appeared in the bound pool in the absence of added vesicles. This fractional mixing of free enzyme with the bound pool ($f_2$) occurred only when sampling was performed by method 2 and is presumably the result of mixing at the interface of the two pools during handling.

These correction factors are employed to derive explicit expressions for $[\text{factor VII}]_{\text{free}}$ ($\text{VII}_f$) and $[\text{factor VII}]_{\text{bound}}$ ($\text{VII}_b$) from the measured concentrations of free and bound enzyme ($\text{VII}_{f,m}$ and $\text{VII}_{b,m}$, respectively):

$$\text{VII}_f = [f_1/(f_1 - f_2)]\text{VII}_{f,m} - [(1 - f_1)/(f_1 - f_2)]\text{VII}_{b,m} \quad (1)$$

$$\text{VII}_b = \text{VII}_{b,m}/f_1 - (f_2/f_1)\text{VII}_f \quad (2)$$

All terms on the right side of eq 1 and 2 are experimentally

Table I: Correction Factors for Enzyme Binding to PS/PC Vesicles in the Absence of Tissue Factor[a]

| PS:PC ratio | $N_{PL}/K_{d,PL}$ ($\mu$M) | PS:PC ratio | $N_{PL}/K_{d,PL}$ ($\mu$M) |
|---|---|---|---|
| 0:100 | 0.0048 | 20:80 | 0.061 |
| 5:95 | 0.021 | 30:70 | 0.092 |
| 10:90 | 0.035 | 40:60 | 0.105 |

[a] The coefficients, used to distinguish binding to phospholipid from binding to tissue factor, were measured as described under Materials and Methods. Using the approximation, eq 6, an explicit expression has been derived, eq 8, for calculating the concentration of enzyme bound to tissue factor from the experimentally determined values.

measured or calculated. Equations 1 and 2 were derived as follows: The measured concentration of bound enzyme ($VII_{b,m}$) is

$$VII_{b,m} = f_1 VII_b + f_2 VII_f \quad (3)$$

where $f_1 VII_b$ is the fraction of true bound enzyme recovered in the bound pool and $f_2 VII_f$ is the fraction of free enzyme mixed in with the bound. Solving eq 3 for $VII_b$ yields eq 2. The measured concentration of free enzyme ($VII_{f,m}$) is the true value of free enzyme, ($VII_f$) minus free enzyme appearing in the bound pool ($f_2 VII_f$) plus bound enzyme remaining in the free pool [$(1 - f_1)VII_b$]. Substituting eq 2 into eq 4 for $VII_b$

$$VII_{f,m} = VII_f + (1 - f_1)VII_b - f_2 VII_f \quad (4)$$

and solving for $VII_f$ yield the explicit expression for the true concentration of free enzyme, eq 1.

All vitamin K dependent clotting factors, including factor VII, bind to negatively charged phospholipids in the presence of $Ca^{2+}$ (Nelsestuen et al., 1978). Therefore, when vesicles contain PS, a fraction of the enzyme appearing in the bound pool will be bound "nonspecifically" to phospholipid. This enzyme–phospholipid association is described by

$$VII_{b,PL} = N_{PL} VII_f PL/(K_{d,PL} + VII_f) \quad (5)$$

where $VII_{b,PL}$ is the true concentration of enzyme bound to phospholipid calculated from eq 2, and $VII_f$ is the concentration of free enzyme from eq 1. $N_{PL}$ is the molar ratio of enzyme to phospholipid at saturation, $K_{d,PL}$ is the dissociation constant for the enzyme–phospholipid interaction, and PL is the concentration of phospholipid in the assay.

Factor VII binding to the most acidic vesicles employed in the study, 40:60 PS/PC, yielded a $K_d$ of 9.0 ± 1.2 $\mu$M. This $K_d$ is approximately 1000-fold greater than that calculated for tissue factor–enzyme binding. Since $VII_f$ is much smaller than the dissociation constant for enzyme–phospholipid binding, when measuring the association of enzyme and activator, eq 5 can be simplified to

$$VII_{b,PL} = (N_{PL}/K_{d,PL}) VII_f PL \quad (6)$$

The coefficient $N_{PL}/K_{d,PL}$ was determined for each PS/PC preparation employed (Table I). The concentrations of phospholipid (1.5 mM) and factor VII (10 nM) utilized in these experiments are similar to those used in the tissue factor binding experiments. From these data, it is apparent that the binding of enzyme to phospholipid increased linearly as the percentage of PS in the vesicles increased. With pure PC vesicles, the coefficient was small but reproducibly greater than zero. Thin-layer chromatography of 50 $\mu$g of PC (Irvine & Dawson, 1978) detected no contaminants,[2] in accord with the supplier's analysis of >98% purity. Therefore, contamination with PS has been ruled out as an explanation for this observation. It is important to note that this nonideal property of the assay is distinct from the aforementioned mixing, since it requires the presence of phospholipid vesicles and is observed with both sampling procedures. The binding of enzyme to phospholipid, whether by a specific or a nonspecific association, is subsumed in the term $N_{PL}/K_{d,PL}$. Therefore, these coefficients are valid correction factors for enzyme in the bound pool which is not associated with tissue factor.

A more general expression in which the binding to activator is distinguished from the binding to phospholipid is obtained as follows: The total enzyme bound ($VII_b$) is the sum of enzyme bound to tissue factor ($VII_{b,TF}$) plus enzyme bound to phospholipid ($VII_{b,PL}$):

$$VII_b = VII_{b,TF} + VII_{b,PL} \quad (7)$$

Substituting $VII_b$ from eq 2 and $VII_{b,PL}$ from eq 6 and then solving eq 7 for $VII_{b,TF}$ yield

$$VII_{b,TF} = VII_{b,m}/f_1 - (f_2/f_1)VII_f - (N_{PL}/K_{d,PL})VII_f PL \quad (8)$$

With eq 1 and 8, true concentrations of free enzyme ($VII_f$) and enzyme bound to tissue factor ($VII_{b,TF}$) may be calculated from the measured concentrations of bound and free enzyme. In the case where maximum binding of enzyme to phospholipid is observed, tissue factor in 40:60 PS/PC vesicles, enzyme binding to phospholipid accounts for no more than 30% of the total observed binding under the conditions employed.

The binding parameters were calculated by using Marquardt's nonlinear least-squares method (Tektronix, plot 50, vol. 4). Data are presented ±1 standard error. A nonparametric sign–run test (Hollander & Wolfe, 1973) was used to measure systematic deviations of the data from the respective equations.

## Results

*Tissue Factor Tritiation.* As the determination of the number of available factor VII binding sites on tissue factor depends, in turn, on the precise quantification of the molecule, we tritiated tissue factor to allow its measurement at the low concentrations employed. Specific radioactivity ranging from ~229 000 to ~663 000 cpm/$\mu$g of protein was obtained depending on the tritiated sodium borohydride used. This represents 2–6 mol of tritium incorporated per mole of tissue factor, thus obviating the difficulties engendered by labeling techniques which result in only fractional incorporation of the label, e.g., aberrant behavior of minority populations.

The covalent association of tritium and protein was shown by trichloroacetic acid precipitation of the protein which resulted in concomitant precipitation of 97% of the radioactivity. To demonstrate that the radioactivity was inserted into tissue factor and not into extraneous material, the protein was subjected to SDS–PAGE (Figure 1). This gel indicates that the mobility of the bulk of the tissue factor protein is unchanged by the tritiation and the radioactivity is primarily associated with the 41-kDa tissue factor protein (tissue factor migrates at 43 kDa in a phosphate system; Bach et al., 1981). A small amount, ~5% of the protein, is noted in higher molecular weight bands which were not present prior to tritiation. From their mobility on the gel, we surmise that these bands consist of tissue factor molecules in dimers and higher multimers arising from intermolecular Schiff base formation during the procedure.

The biological activity of the tritiated tissue factor was evaluated following incorporation into phospholipid vesicles

[2] Thin-layer analysis of the PC was kindly performed by Dr. Aaron Marcus.

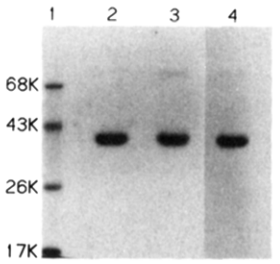


FIGURE 1: SDS–PAGE of tissue factor and $^3$H-tissue factor. The procedure of Laemmli (1970) was employed on a 10% polyacrylamide slab gel. The proteins were reduced with 5% 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and then prepared for fluorography with Enlightening according to the manufacturer's instructions. The X-ray film (Kodak X-Omat AR-5) was exposed for 1 h at −80 °C. Lane 1, molecular weight standards (Coomassie stain): bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen (26 kDa), and myoglobin (17 kDa); lane 2, 10 μg of tissue factor (Coomassie stain); lane 3, 10 μg of $^3$H-tissue factor (Coomassie stain); lane 4, fluorogram of lane 3.

Table II: Composition and Size of Reconstituted Tissue Factor–Phospholipid Vesicles[a]

| PS:PC ratio | PL/TF (mol/mol) | vesicle size distribution <100 nm | 100–150 nm | 150–200 nm | >200 nm |
|---|---|---|---|---|---|
| 0:100 | 75 300 | 16.6 | 17.4 | 65.9 | 0 |
| 5:95 | 73 600 | 12.6 | 30.1 | 32.1 | 24.9 |
| 10:90 | 82 000 | 9.1 | 37.9 | 30.0 | 23.1 |
| 20:80 | 69 000 | 4.0 | 32.9 | 31.5 | 31.5 |
| 30:70 | 110 000 | 21.6 | 70.8 | 6.5 | 1.1 |
| 40:60 | 98 500 | 17.0 | 81.2 | 1.8 | 0 |

[a] The distribution of vesicles in each size range is expressed as the percentage of total recovered $^3$H-tissue factor. The recovery of $^3$H-tissue factor on Sephacryl S-1000 was essentially quantitative when the column was presaturated with lipid. Calibration of the column with latex beads and liquid scintillation counting were performed as described under Materials and Methods.

(PS/PC, 30:70). As judged by a two-stage coagulation assay, the biological activity was unimpaired. Further, the number of factor VII binding sites per tissue factor molecule was unchanged (data not shown). From these data, we conclude that tritiation produces a radiolabeled molecule which remains functionally identical with the unlabeled protein.

*Reconstitution of the Tissue Factor–Phospholipid Complex.* Tissue factor was incorporated into vesicles ranging from 100% PC to 40:60 PS/PC. For all preparations, irrespective of the phospholipid composition, isolation on Sepharose CL-2B resulted in about 65% recovery of $^3$H-tissue factor and 85% recovery of the $^{14}$C-phospholipid. The remaining material could be eluted by washing with 0.1% Triton X-100. For phospholipid mixtures from 100% PC to 20:80 PS/PC, essentially all the isolated vesicles appeared in the void volume. However, for 30:70 and 40:60 PS/PC, 50% of the vesicles were in the included volume; only material from the void volume was employed.

The size of the isolated vesicles was estimated on Sephacryl S-1000 columns. These data are listed in Table II along with the molar ratio of phospholipid to tissue factor in each preparation. For PS:PC ratios up to 20:80, the majority of the vesicles were greater than 150 nm. For the most acidic vesicles, 30:70 and 40:60, the vesicles were primarily in the 100–150-nm range. In all cases, only a small fraction of the vesicles had diameters less than 100 nm. These data indicate that octyl glucoside reconstitution results in a reasonably uniform population of vesicles irrespective of the phospholipid composition. Since variations in vesicle size and the ratio of tissue factor to phospholipid are small, we judged this material suitable for studying the effects of vesicle charge, per se, on the activity of tissue factor.

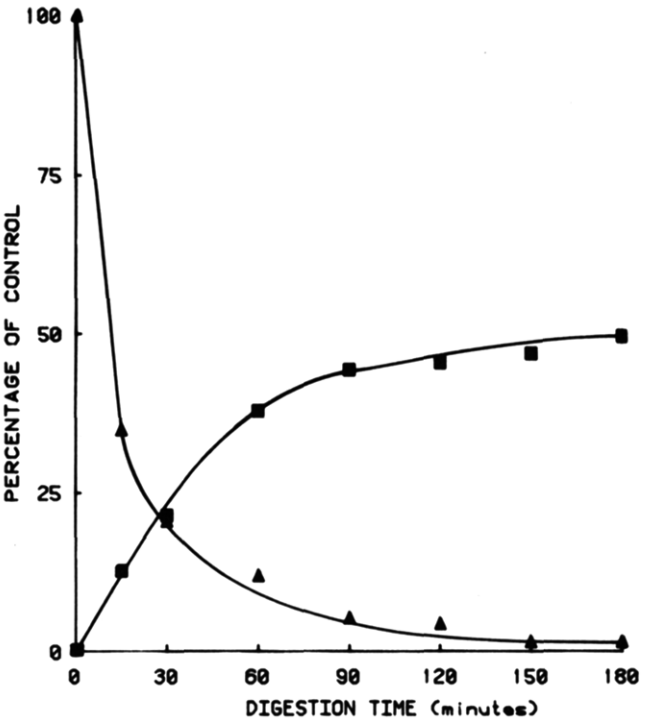


FIGURE 2: Subtilisin digestion of $^3$H-tissue factor incorporated into 30:70 PS/PC vesicles. Hydrolysis of $^3$H-tissue factor was measured as the appearance of uranyl acetate soluble tritium (■) and the disappearance of tissue factor procoagulant activity (▲). Assays were performed as described under Materials and Methods and calculated as the percentage of an undigested control for procoagulant activity, or as the percentage of total tritium in the sample.

*Orientation of Reconstituted Tissue Factor.* Because the phospholipid vesicles are large with respect to the tissue factor molecule, it is clearly possible that some of the incorporated protein is trapped within the vesicle or oriented toward its interior. In either case, the material would be inaccessible to factor VII and therefore biologically inert. To determine the amount of tissue factor actually available on the vesicle surface, we performed a limit digest on the vesicular material with subtilisin, the rationale being that the protease would digest only material present on the outside of the vesicles. In a control experiment where the vesicles were permeabilized with 1% Triton X-100, $^3$H-tissue factor was completely digested by subtilisin to small fragments as judged by SDS–PAGE. Likewise, 100% of the tritium was rendered soluble in uranyl acetate, whereas the intact protein was 99.7% precipitable. All coagulant activity was lost from this material. On the basis of these observations, we reasoned that by determining the loss of total protein, measured as uranyl acetate soluble tritium, and total coagulant activity, we could quantify the fraction of tissue factor actually available on the vesicle surface.

A time course of the subtilisin digestion of $^3$H-tissue factor in 30:70 PS/PC vesicles is illustrated in Figure 2. After 180 min, less than 2% of the coagulant activity remained, but 50% of the tritiated protein was still precipitable by uranyl acetate. SDS–PAGE of the digested material indicated that roughly half of the initial tritium was still associated with the 41-kDa band; no degradation intermediates were noted.

We next determined whether the preserved protein was potentially active. If, as the digestion experiment suggests, half the original protein is intravesicular, reequilibration of the protein in the vesicles would enable a fraction of the re-

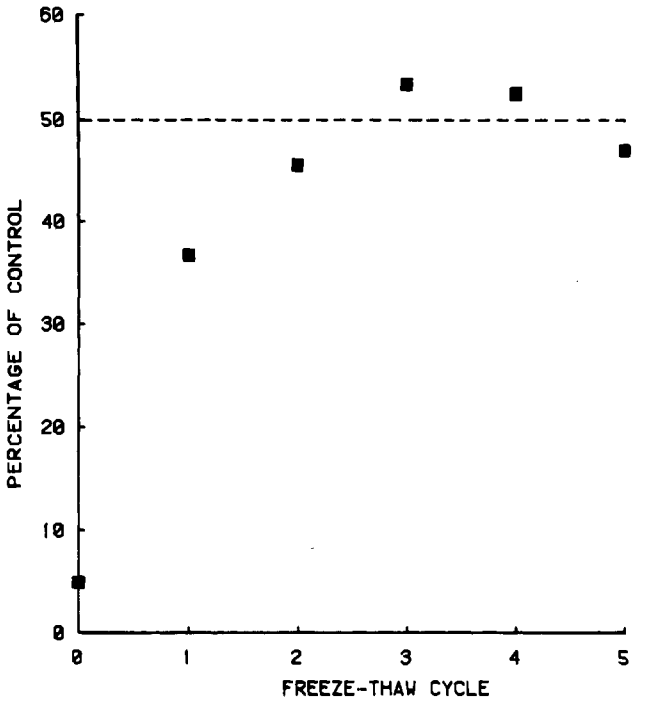


FIGURE 3: Liberation of sequestered tissue factor procoagulant activity by freezing and thawing. A sample of $^3$H-tissue factor in 30:70 PS/PC vesicles was digested with subtilisin for 180 min as described under Materials and Methods. After the protease was inhibited with 5 mM DFP, the procoagulant activity of the sample was measured (cycle 0). The sample was then reassayed after freezing (−120 °C) and thawing (37 °C). This procedure was repeated 5 times (cycles 1–5). The procoagulant activity is expressed as the percentage of activity measured in the control sample which received no protease.

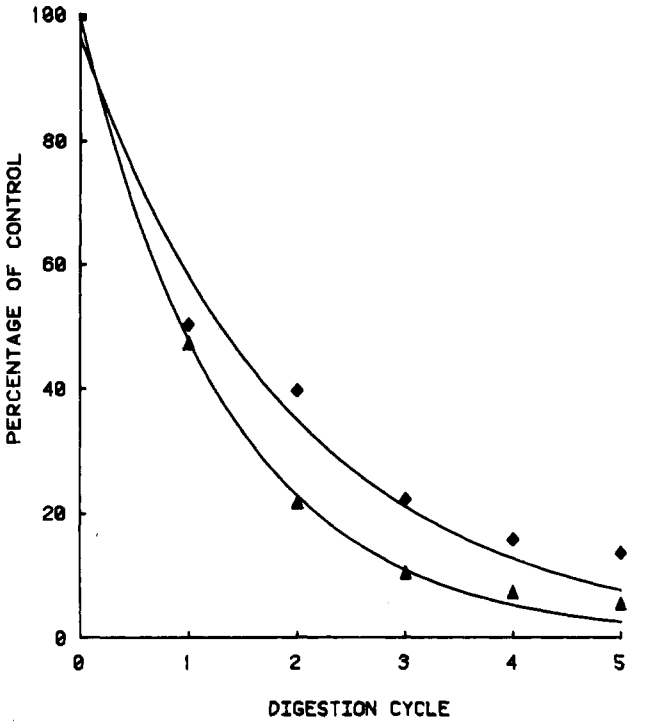


FIGURE 4: Subtilisin digestion of $^3$H-tissue factor in 30:70 PS/PC vesicles with freezing and thawing of the reaction mixture after each 180-min digestion. $^3$H-Tissue factor was hydrolyzed as in Figure 2 and then subjected to freezing and thawing and further digestion as described under Materials and Methods. Proteolysis of tissue factor was monitored as the loss of uranyl acetate precipitable tritium (♦) and the disappearance of sequestered procoagulant activity (▲); i.e., activity was measured after inhibition of the protease and freeze–thawing the sample 5 times. Values are expressed as the percentage of procoagulant activity in the undigested control or the percentage of total tritium in the sample. The equation $y = ae^{-kx}$ was fit to the data as described under Materials and Methods.

sidual protein to reside on the surface and thus be available to subtilisin and the coagulation factors. We found that simply freezing the vesicles had this effect. The digested vesicles were frozen at −120 °C and thawed at 37 °C, following which a sample was removed for measurement of coagulant activity. The freeze–thaw cycles had no effect on the activity of an undigested control sample, but in the limit digest, procoagulant activity appeared which was equal to ~50% of the control value (Figure 3). These results are consistent with a model in which the orientation of tissue factor is random, with about half the molecules being outward facing and with the other half either trapped or inward facing; after digestion and freeze–thaw, the half-in, half-out orientation is reestablished.

To test this hypothesis further, a second protease digestion was performed. After each cycle of proteolytic digestion (3 h), aliquots were removed for estimation of uranyl acetate precipitable tritium and tissue factor activity (before and after freeze–thaw). The remainder was frozen and thawed (5 times), and digestion was continued for a total of 5 cycles. At the end of each 3-h digestion, essentially no procoagulant activity was detected until the samples were frozen and thawed, indicating that the destruction of activity had gone to completion. If each cycle of subtilisin digestion destroys a constant fraction of protein and activity, then the precipitable tritium and procoagulant activity will decay exponentially and may be described by the equation $y = ae^{-kx}$ where $k$ is a first-order rate constant, $x$ is the cycle number, $y$ is the remaining activity or precipitable tritium expressed as a percent of the undigested control, and $a$ is the value of $y$ when $x$ equals 0. For a randomly oriented membrane protein where proteolysis destroys the external-facing molecules and inward-facing molecules are completely protected, and where freeze–thawing randomly reorients the sequestered protein, each digestion cycle will destroy half the remaining protein. Thus, for this ideal case, $a = 100$ and $k = \ln 2$ (~0.693). These experiments (Figure 4) yielded the following results: for the loss of procoagulant activity, $a = 99.8 \pm 1.91$ and $k = 0.715 \pm 0.027$; for the loss of precipitable tritium, $a = 96.3 \pm 6.05$ and $k = 0.508 \pm 0.061$. In other words, the decay of the procoagulant activity proceeded at the rate predicted by the random orientation model. Fifty percent of the remaining procoagulant activity was lost every 0.97 cycle. The rate of decay of precipitable tritium proceeded slightly more slowly, with 50% loss every 1.3 cycles. The slower rate of tritium release compared with the decay of procoagulant activity may be rationalized by the fact that multiple cleavages are required to solubilize the tritium, while destruction of activity may only require the hydrolysis of a single peptide bond. Since the concentration of substrate decreases as the reaction proceeds, the overall rate of the reaction will slow down with each digestion cycle. Thus, while 3 h represented a limit digest of both protein and activity in the first digestion cycle (Figure 2), it appears that longer times may be required for complete release of the accessible tritium in subsequent cycles. From the data presented in Figures 2–4, we conclude that tissue factor is randomly oriented in the reconstituted vesicles; half the active sites are sequestered inside and unavailable for factor VII binding.

*Proof of Equilibrium.* Equilibrium was established by comparing the amount of enzyme bound during a standard incubation to the amount bound following a dilution–jump. As illustrated in Figure 5, after dilution the concentration of the preformed activator–enzyme complex rapidly decayed to the same value observed for the complex formed following

Table III: Enzyme Binding to Tissue Factor in PS/PC Vesicles: Hill Cooperative Model Binding Parameters[a]

| | factor VII | | | factor VIIa | | |
|---|---|---|---|---|---|---|
| PS:PC ratio | $N_{TF}$ | $N_H$ | $K_H$ (nM) | $N_{TF}$ | $N_H$ | $K_H$ (nM) |
| 0:100 | 0.36 ± 0.01 | 1.08 ± 0.07 | 14.9 ± 1.81 | 0.31 ± 0.03 | 1.17 ± 0.40 | 5.47 ± 3.09 |
| 5:95 | 0.30 ± 0.02 | 1.06 ± 0.13 | 6.91 ± 0.13 | 0.54 ± 0.09 | 1.11 ± 0.17 | 5.76 ± 1.02 |
| 10:90 | 0.60 ± 0.45 | 1.01 ± 0.31 | 6.80 ± 5.61 | 0.51 ± 0.06 | 1.13 ± 0.14 | 5.25 ± 0.66 |
| 20:80 | 0.39 ± 0.03 | 1.46 ± 0.19 | 2.99 ± 0.34 | 0.52 ± 0.05 | 1.29 ± 0.16 | 4.92 ± 0.55 |
| 30:70 | 0.44 ± 0.13 | 1.64 ± 0.14 | 1.17 ± 0.11 | 0.59 ± 0.02 | 1.46 ± 0.14 | 3.22 ± 0.34 |
| 40:60 | 0.40 ± 0.02 | 1.31 ± 0.19 | 0.58 ± 0.12 | 0.39 ± 0.01 | 1.55 ± 0.19 | 2.25 ± 0.32 |

[a] The Hill model binding parameters were calculated for the entire set of binding data as described under Materials and Methods.

Table IV: Enzyme Binding to Tissue Factor in PS/PC Vesicles: Hill Cooperative Model Binding Parameters[a]

| | DIP-factor VII | | | DIP-factor VIIa | | |
|---|---|---|---|---|---|---|
| PS:PC ratio | $N_{TF}$ | $N_H$ | $K_H$ (nM) | $N_{TF}$ | $N_H$ | $K_H$ (nM) |
| 0:100 | 0.35 ± 0.06 | 1.14 ± 0.19 | 7.25 ± 1.11 | 0.45 ± 0.03 | 1.10 ± 0.13 | 3.66 ± 0.42 |
| 5:95 | 0.33 ± 0.04 | 1.20 ± 0.23 | 5.36 ± 1.00 | 0.43 ± 0.02 | 1.45 ± 0.17 | 4.33 ± 0.58 |
| 10:90 | 0.33 ± 0.06 | 0.94 ± 0.13 | 4.59 ± 0.89 | 0.42 ± 0.02 | 1.44 ± 0.17 | 2.84 ± 0.37 |
| 20:80 | 0.43 ± 0.05 | 1.02 ± 0.16 | 3.76 ± 0.56 | 0.55 ± 0.06 | 1.43 ± 0.23 | 2.27 ± 0.44 |
| 30:70 | 0.56 ± 0.06 | 0.95 ± 0.16 | 2.08 ± 0.36 | 0.57 ± 0.05 | 1.38 ± 0.20 | 1.67 ± 0.28 |
| 40:60 | 0.31 ± 0.01 | 1.23 ± 0.15 | 0.89 ± 0.13 | 0.55 ± 0.03 | 1.44 ± 0.15 | 2.26 ± 0.24 |

[a] The Hill model binding parameters were calculated for the entire set of binding data as described under Materials and Methods.

dilution. From 20 to 60 min, the measured concentrations of bound enzyme were not significantly different, 2.38 ± 0.053 nM (SD) for the preformed complex and 2.31 ± 0.10 nM for the complex formed following dilution, thus establishing that the system was at equilibrium. Reversibility of the association was also demonstrated, as described under Materials and Methods, by displacing the bound $^3$H-enzyme with unlabeled ligand.

*Enzyme Binding to Tissue Factor in PC Vesicles.* In the first set of binding experiments, tissue factor was incorporated into neutral PC vesicles. Assuming that the association of enzyme and activator is described by a classic Langmuir isotherm, then

$$VII_{b,TF} = N_{TF}VII_fTF/(K_d + VII_f) \quad (9)$$

where $VII_{b,TF}$ is the concentration of enzyme bound to tissue factor, $N_{TF}$ is the molar ratio of enzyme to activator at saturation, TF is the concentration of tissue factor in the assay, $K_d$ is the dissociation constant for the activator–enzyme complex, and $VII_f$ is the concentration of free enzyme.

The $K_d$ for factor VII binding was 13.2 ± 0.72 nM. Factor VIIa was bound more tightly than the one-chain molecule; the $K_d$ was 4.54 ± 1.37 nM. To ensure that we have measured the association of single-chain factor VII with tissue factor, we characterized factor VII on SDS–polyacrylamide gels following exposure to tissue factor. By this method, less than 5% hydrolysis could be detected. As no cleavage was observed (data not shown), we conclude that both factor VII and factor VIIa bind to tissue factor.

Modifying the active site of each form of the enzyme with DFP decreased the $K_d$ for the conjugated one-chain and two-chain molecules. The dissociation constants were 7.70 ± 1.29 and 3.62 ± 0.40 nM for DIP-factor VII and DIP-factor VIIa, respectively.

At saturation, ~0.5 mol of enzyme was bound per mole of tissue factor for the four forms of the enzyme tested; averaging seven isotherms determined by using PC, $N_{TF}$ = 0.44 ± 0.09 (SD). However, as we have shown the effective concentration of tissue factor in the binding assay to be half its total concentration, the true stoichiometry of binding is 1 mol of enzyme per mole of tissue factor.

*Enzyme Binding to Tissue Factor in Acidic Vesicles.* The binding of four forms of the enzyme to tissue factor in vesicles ranging from pure PC to 40:60 PS/PC was measured. The concentration of free enzyme and enzyme bound to tissue factor were calculated from the primary data, and the Langmuir binding parameters were determined. The fit of the data for enzyme binding to tissue factor in acidic phospholipid vesicles yielded nonidealities not noted with tissue factor in PC vesicles. To illustrate this point, we compare an isotherm which fits the model with one which deviates. Scatchard plots of factor VII binding to tissue factor in 100% PC vesicles and 30:70 PS/PC vesicles are shown in Figure 6. The data for factor VII binding to tissue factor in the negatively charged vesicles do not fit a straight line, the deviation suggesting positive cooperativity.

The binding isotherms from Figure 6 are presented as direct plots in Figure 7. The binding of factor VII to tissue factor in 100% PC vesicles is well described by the calculated hyperbolic isotherm. The close correspondence between the data and the calculated best fit to eq 9 is illustrated by the residuals which measure the difference between observed and predicted data values. Factor VII binding to tissue factor in 30:70 PS/PC vesicles appears sigmoidal. As illustrated by the residuals, the first group of data points falls below the line, while the later points are above the calculated line. This sigmoidicity is consistent with positive cooperativity.

In light of this apparent systematic deviation from the Langmuir model, further analysis was performed. The residuals for the Langmuir model for factor VII binding to tissue factor in 30:70 PS/PC vesicles were tested by a nonparametric sign–run test (Holland & Wolfe, 1973), which only assumes that the distribution of each residual has a mean of zero. A normal approximation of the test statistic accepted at the $p$ = 0.05 level the hypothesis of too few runs; i.e., there is a significant deviation of the experimental data from the model.

As an alternative to the Langmuir model, the Hill equation (Hill, 1910) was used to evaluate the binding data:

$$VII_{b,TF} = N_{TF}TF(VII_f)^{N_H}/(K_H + VII_f^{N_H}) \quad (10)$$

In this equation, $N_{TF}$ is the molar ratio of enzyme to activator at saturation, $K_H$ is the Hill constant, and $N_H$ is the Hill coefficient. The calculated Hill parameters for the binding of factor VII, factor VIIa, DIP-factor VII, and DIP-factor VIIa to the various tissue factor preparations are shown in Tables III and IV. For enzyme binding to tissue factor in 100% PC vesicles, the values of $N_H$ were only slightly greater than 1.0, and the difference was not statistically significant

Table V: Enzyme Binding to Tissue Factor in PS/PC Vesicles: Adair Two-Site Cooperative Model Binding Parameters[a]

| PS:PC ratio | factor VII | | | factor VIIa | | |
|---|---|---|---|---|---|---|
| | $N_{TF}$ | $K_1$ (nM) | $K_2$ (nM) | $N_{TF}$ | $K_1$ (nM) | $K_2$ (nM) |
| 0:100 | 0.36 ± 0.01 | 14.9 ± 1.56 | 10.1 ± 2.03 | 0.32 ± 0.03 | 6.32 ± 5.29 | 2.97 ± 2.97 |
| 5:95 | 0.30 ± 0.02 | 7.41 ± 1.40 | 4.89 ± 1.97 | 0.53 ± 0.10 | 6.21 ± 1.02 | 3.60 ± 2.77 |
| 10:90 | 0.62 ± 1.19 | 6.98 ± 11.7 | 7.14 ± 4.31 | 0.49 ± 0.05 | 6.02 ± 0.93 | 2.65 ± 1.41 |
| 20:80 | 0.39 ± 0.03 | 6.27 ± 2.43 | 0.72 ± 0.42 | 0.51 ± 0.05 | 6.79 ± 1.68 | 1.66 ± 0.87 |
| 30:70 | 0.45 ± 0.01 | 5.16 ± 2.19 | 0.24 ± 0.12 | 0.60 ± 0.02 | 6.33 ± 2.06 | 0.82 ± 0.33 |
| 40:60 | 0.41 ± 0.02 | 1.37 ± 0.54 | 0.33 ± 0.17 | 0.40 ± 0.01 | 6.17 ± 3.33 | 0.48 ± 0.29 |

[a] The Adair model binding parameters were calculated for the entire set of binding data as described under Materials and Methods.

Table VI: Enzyme Binding to Tissue Factor in PS/PC Vesicles: Adair Two-Site Cooperative Model Binding Parameters[a]

| PS:PC ratio | DIP-factor VII | | | DIP-factor VIIa | | |
|---|---|---|---|---|---|---|
| | $N_{TF}$ | $K_1$ (nM) | $K_2$ (nM) | $N_{TF}$ | $K_1$ (nM) | $K_2$ (nM) |
| 0:100 | 0.33 ± 0.05 | 8.13 ± 1.74 | 3.26 ± 2.29 | 0.45 ± 0.03 | 4.06 ± 0.84 | 2.67 ± 1.08 |
| 5:95 | 0.33 ± 0.04 | 6.52 ± 2.25 | 2.50 ± 1.74 | 0.43 ± 0.02 | 7.86 ± 2.92 | 1.00 ± 0.49 |
| 10:90 | 0.32 ± 0.08 | 4.38 ± 0.76 | 5.62 ± 5.68 | 0.42 ± 0.02 | 5.77 ± 2.31 | 0.76 ± 0.36 |
| 20:80 | 0.43 ± 0.05 | 3.85 ± 0.75 | 3.40 ± 2.22 | 0.54 ± 0.06 | 4.89 ± 2.06 | 0.65 ± 0.46 |
| 30:70 | 0.56 ± 0.05 | 1.93 ± 0.44 | 2.39 ± 1.43 | 0.58 ± 0.04 | 3.49 ± 1.27 | 0.61 ± 0.36 |
| 40:60 | 0.31 ± 0.01 | 1.56 ± 0.49 | 0.56 ± 0.22 | 0.55 ± 0.03 | 4.93 ± 1.49 | 0.64 ± 0.30 |

[a] The Adair model binding parameters were calculated for the entire set of binding data as described under Materials and Methods.

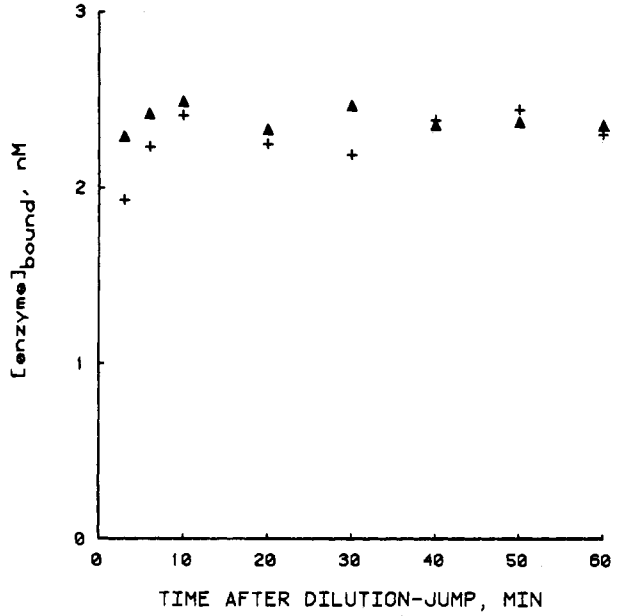


FIGURE 5: Dilution–jump experiment showing approach to equilibrium. The dilution–jump was performed by first combining tissue factor in PC vesicles with $^3$H-factor VII at a concentration approximately 10-fold above the $K_d$ for this association. Two concentrated stock solutions were prepared. In one, the activator–enzyme complex was preformed by mixing tissue factor (140 nM) and $^3$H-factor VII (136 nM) in TBSA containing 20% sucrose and 5 mM $CaCl_2$. The second concentrated stock was identical except that it contained no $CaCl_2$, thus precluding complex formation. The samples were incubated for 30 min at 37 °C and then diluted 10-fold into TBSA and 20% sucrose with either 5 or 5.56 mM $CaCl_2$, giving a final $CaCl_2$ concentration of 5 mM. The samples were then layered as described and incubated at 37 °C for the times indicated in the figure prior to centrifugation. Following centrifugation and sampling, the concentrations of bound enzyme, in the preformed complex (▲) and in the complex formed following dilution (+), were measured as described under Materials and Methods.

in three out of four cases, implying that enzyme binding to tissue factor in the neutral vesicles is not cooperative. However, with the addition of PS, significant changes in $N_H$ were observed, indicating positive cooperativity between enzyme and activator, the magnitude of which depends on the form of the enzyme as well as the vesicle charge.

Plots of the Hill equation fit to the data in Figure 7 showed no systematic deviation of the residuals (Figure 8). To confirm this observation, nonparametric analysis of the residuals was again performed. The pattern of signs of the residuals for the fit to the Hill model was found to have neither too few nor too many runs, $p < 0.01$. Finally, the molar ratio of enzyme to activator at saturation remained constant: $N_{TF} = 0.44 \pm 0.10$ (SD) for the entire data set. Thus, changing the phospholipid composition of the vesicles had no effect on the 1:1 stoichiometry of the activator–enzyme complex.

These observations suggest an explicit model for the binding of factor VII to tissue factor, which takes into account the 1:1 stoichiometry of the activator–enzyme complex and the apparent cooperativity. The minimum number of interacting sites in the cooperative unit, indicated by the Hill coefficient, is 2. Therefore, we hypothesize that tissue factor exists as a dimer in the vesicle bilayer with each subunit of the complex binding one enzyme molecule:

$$A_2 + E \overset{K_1}{\leftrightarrow} A_2E + E \overset{K_2}{\leftrightarrow} A_2E_2$$

where $A_2$ is dimeric tissue factor with two binding sites for enzyme (E), $K_1$ is the dissociation constant describing the initial binding of enzyme to activator, and $K_2$ is the dissociation constant for enzyme binding to the remaining activator site.

The equation describing the two-site cooperative binding model, as originally proposed by Adair (1925), is of the form

$$VII_{b,TF} = N_{TF}TF(K_2 + VII_f)VII_f/(K_1K_2 + 2K_2VII_f + VII_f^2) \quad (11)$$

Equation 11 was fit to the data; the calculated cooperative binding parameters $N_{TF}$, $K_1$, and $K_2$ are listed in Tables V and VI. The differences between $K_1$ and $K_2$ are consistent with positive cooperativity as suggested by the Hill model.

Direct plots of the two isotherms previously compared in Figures 6–8 are shown in Figure 9. Factor VII binding to tissue factor in 30:70 PS/PC vesicles is well described by eq 11, displaying the sigmoidal shape characteristic of positive cooperativity. Visual inspection of the residuals indicates no obvious systematic deviation of the calculated lines. This was confirmed by the previously described nonparametric analysis. Therefore, the binding data appear to be well described by the Adair model.

As with the other binding models, the ratio of enzyme to activator at saturation was ≃0.5 mol of enzyme per mole of activator: $N_{TF} = 0.44 \pm 0.10$ (SD) for the entire set. Thus,

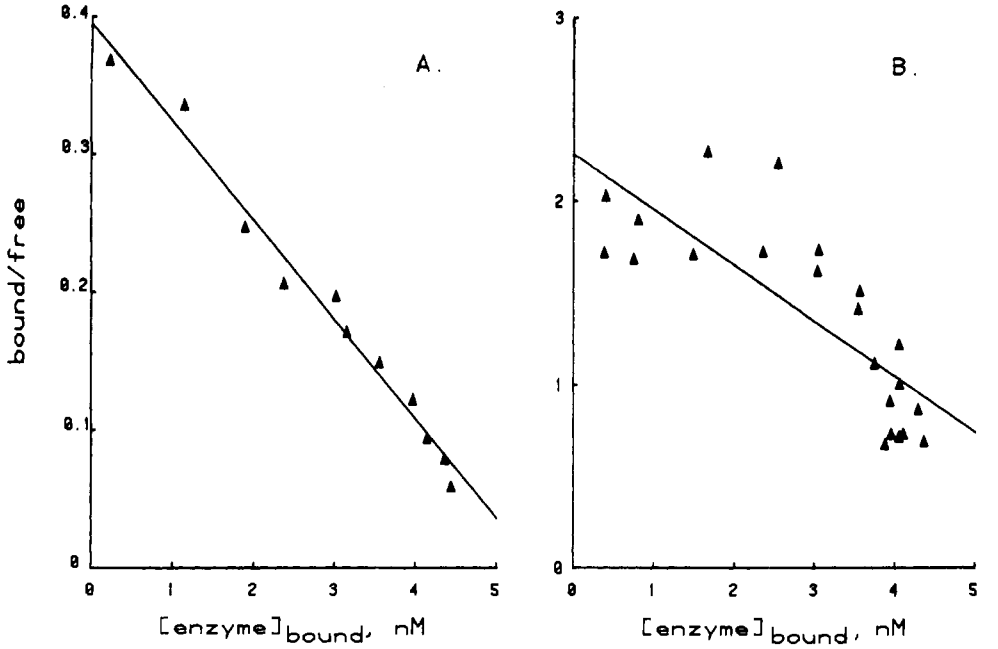


FIGURE 6: Scatchard analysis of $^3$H-factor VII binding to tissue factor performed as described under Materials and Methods and under Results. (A) PC vesicles; (B) 30:70 PS/PC vesicles. The measured concentrations of bound and free enzyme were corrected for the nonideal properties of the assay as described.

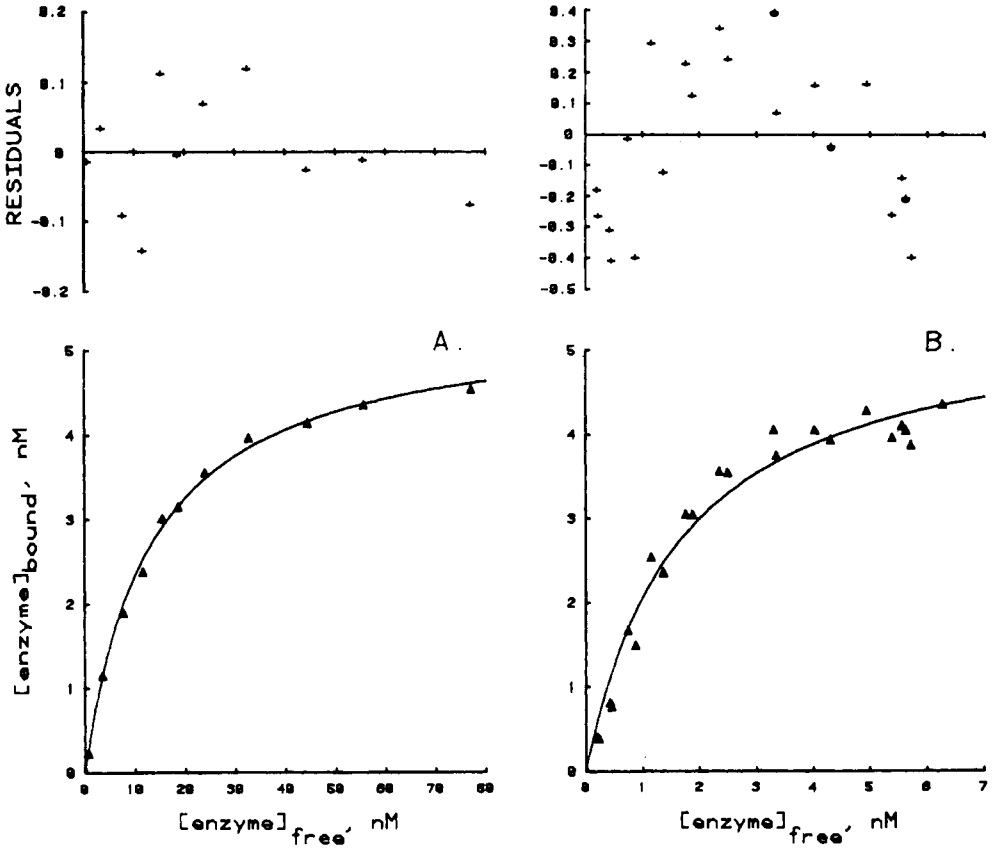


FIGURE 7: Binding isotherms fit to the Langmuir single-site binding model (eq 9). The data for $^3$H-factor VII binding to tissue factor were corrected for the nonideal properties of the assay as described. (A) PC vesicles; (B) 30:70 PS/PC vesicles. The residuals were calculated as the difference between the experimental data and the best fit to the model.

the 1:1 stoichiometry of the proteolytic complex was reconfirmed.

## Discussion

In this study, we show that the apparent stoichiometry of the tissue factor–factor VII complex is 2:1. Three possible explanations for this observation were considered: two tissue factor molecules are required to form one factor VII binding site, our tissue factor preparations are only 50% pure, or tissue factor is randomly oriented in the reconstituted vesicles with half the molecules facing inward and unavailable for interaction with factor VII. For either the second or the third hypothesis, the true stoichiometry of the complex would be 1 mol of factor VII bound per mole of tissue factor. To

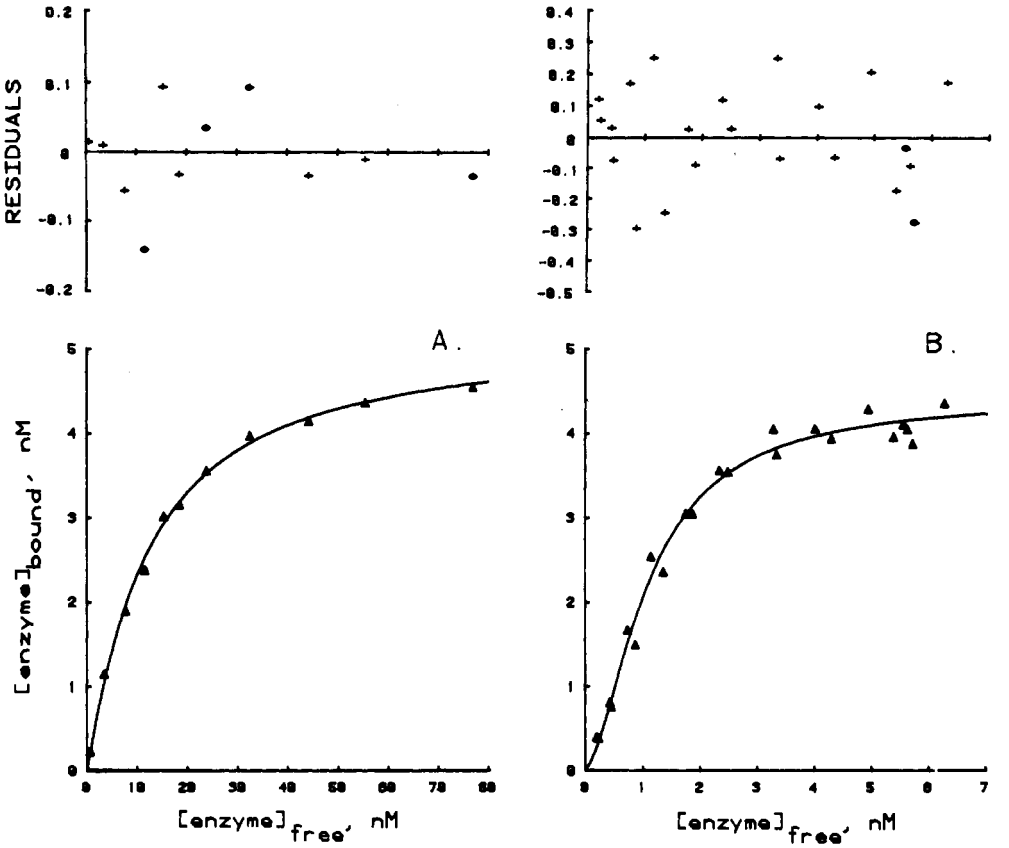


FIGURE 8: Hill cooperative binding model (eq 10) fit to the data for $^3$H-factor VII binding to tissue factor. (A) PC vesicles; (B) 30:70 PS/PC vesicles.

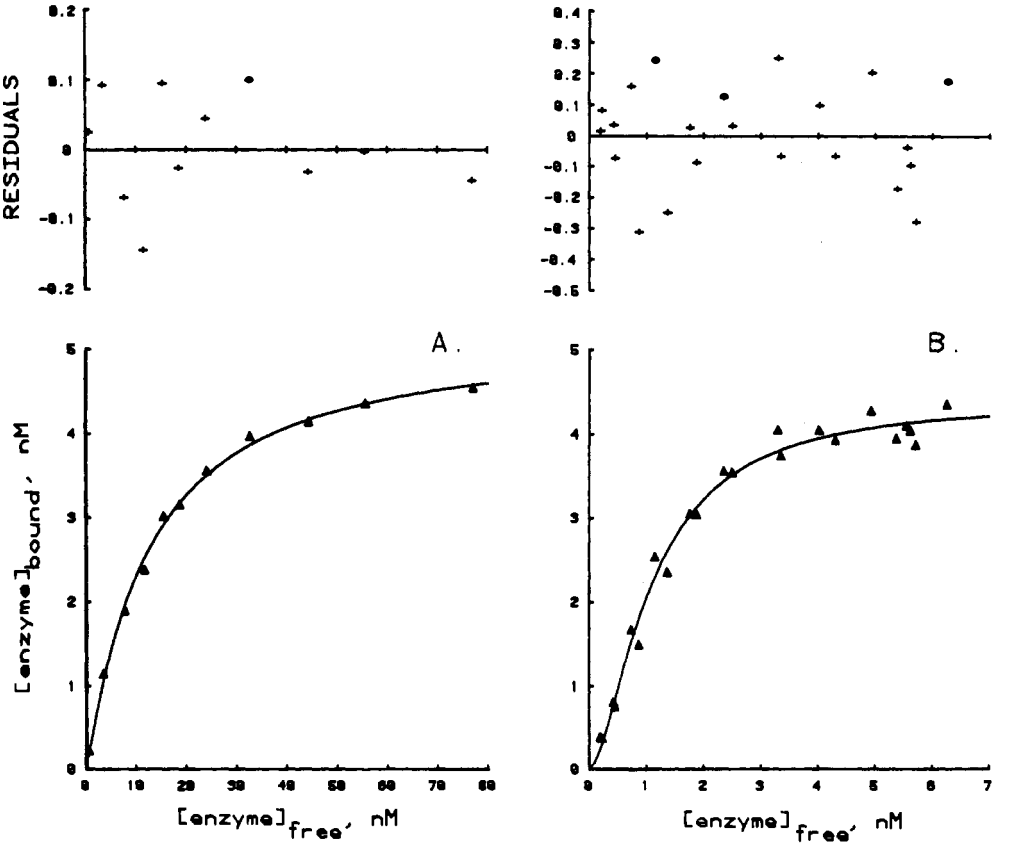


FIGURE 9: These binding isotherms, previously presented in Figures 7 and 8, are the corrected data for $^3$H-factor VII binding to tissue factor. (A) PC vesicles; (B) 30:70 PS/PC vesicles. The Adair two-site cooperative model (eq 11) was fit to the data.

distinguish between these possibilities and resolve the question of stoichiometry, the orientation of tissue factor in the reconstituted membranes was deduced by protease digestion. The results of the experiment illustrated in Figures 2–4 are

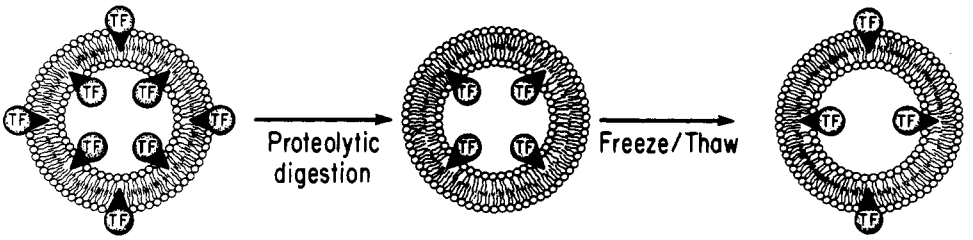


FIGURE 10: Schematic summary of the structure of the tissue factor–phospholipid complex. From the protease digestion experiments (Figures 2–4), the following conclusions are drawn: (1) Tissue factor is randomly oriented in the reconstituted vesicles. (2) The protease-sensitive portion of the protein is exclusively on one face of the bilayer; accordingly, tissue factor may not be a transmembrane protein. (3) Digestion with subtilisin removes tissue factor from the external face of the membrane while molecules facing inward are completely protected from proteolysis. (4) Freeze–thawing of the digested vesicles randomly reorients the sequestered protein.

Table VII: Structural Summary of Tissue Factor Containing Phospholipid Vesicles

| vesicle diameter (nm) | surface area ($nm^2$) | | molecules per vesicle | |
|---|---|---|---|---|
| | outer leaflet | inner leaflet | phospho-lipid | tissue factor |
| 100 | 31 400 | 29 000 | 86 300 | 1.0 |
| 150 | 70 700 | 67 000 | 197 000 | 2.3 |
| 200 | 126 000 | 121 000 | 353 000 | 4.2 |
| 250 | 196 000 | 190 000 | 551 000 | 6.5 |

summarized in Figure 10. We conclude that 50% of the tissue factor protein is on the vesicle surface and susceptible to digestion, with the remaining tissue factor protected inside the vesicles. This sequestered protein is evidently reoriented by freeze–thawing, since half the remaining material becomes available for proteolysis and for expression of procoagulant activity following this treatment. Thus, the effective concentration of tissue factor in our reconstituted preparations, that is, the concentration of available "active sites", is half the protein concentration, and the true stoichiometry of the tissue factor–factor VII complex is 1:1. Since the protein sequestered inside the vesicle is unaltered by subtilisin, as judged by SDS–PAGE, it appears that on the face of membrane opposite the active site no peptide bonds are accessible to the enzyme. This suggests that tissue factor is not a transmembrane protein.

Tissue factor was inserted into vesicles ranging from pure PC to 40:60 PS/PC; a summary of the structure of the tissue factor–phospholipid complex is provided in Table VII. For these calculations, we used the following values: the average molar ratio of tissue factor to phospholipid is 1 to 85 000 (Table II), the average surface area occupied by each phospholipid molecule is 0.70 $nm^2$, and the bilayer thickness is 4 nm (Hauser et al., 1973; Mimms et al., 1981). Given these values, only 50% of the 100-nm vesicles will have a tissue factor molecule in an active configuration on the external surface. For 250-nm vesicles, on average, three tissue factor molecules will appear on the vesicle exterior. For each molecule of tissue factor incorporated into either leaflet of the bilayer, there are about 59 000 $nm^2$ of membrane surface. Assuming that tissue factor is a spherical molecule with a radius of 2.5 nm, it will occupy no more than 0.03% of the area on either face of the membrane. These calculations assume a single bilayer, and no direct evidence is presented that the vesicles we have prepared are unilamellar. However, others have shown that vesicles prepared under essentially identical conditions are single bilayers (Mimms et al., 1981).

When tissue factor was incorporated into neutral PC vesicles, the binding of the enzyme was well described by the classic Langmuir model. The dissociation constants for factor VII and factor VIIa, 13.2 ± 0.72 and 4.54 ± 1.37 nM, respectively, indicate that the two-chain molecule binds slightly more tightly to tissue factor than the zymogen. While the difference is significant, the magnitude of the change is small and cannot account for the 100-fold greater proteolytic activity of factor VIIa (Zur et al., 1982; Bach et al., 1984). The defect in the zymogen most likely can be explained by one of three mechanisms: weak binding to the activator, poor substrate recognition by the zymogen, or faulty catalysis. Our data rule out the first hypothesis; we cannot presently distinguish between the remaining possibilities. However, proving the existence of the activator–zymogen complex provides additional support for the concept that one-chain factor VII is proteolytically active.

DIP-factor VII and DIP-factor VIIa bind more tightly than their unconjugated counterparts. This suggests that modification of the active site may induce changes in regions of the molecule which contact tissue factor, thus facilitating tighter binding. It is also interesting to note that the $K_d$ for factor VII binding is very near its plasma concentration, 10–20 nM (Bach et al., 1984), indicating that alteration of either the plasma concentration of the enzyme or the affinity of the activator could have a significant effect on the proposed first step of tissue factor initiated coagulation: the binding of the zymogen to the activator.

Negatively charged phospholipid surfaces accelerate several reactions in the coagulation scheme, including tissue factor mediated reactions (Bangham, 1961; Nemerson, 1968; Pitlick & Nemerson, 1970; Bjorklid & Storm, 1977). While this phenomenon is undisputed, the mechanism by which this acceleration is achieved remains obscure. We have found that phospholipids play a complex role in modulating the association of enzyme and activator. The addition of negatively charged phospholipid to the vesicles altered the binding of enzyme and activator both qualitatively and quantitatively. Since these changes suggest positive cooperativity, the Hill equation was fit to the data. The objectives in employing the Hill model were to determine if the data were well described by a cooperative model and to use the calculated Hill parameters to derive an explicit model for activator–enzyme binding. As shown in Figure 8, the systematic deviations observed with the Langmuir model disappeared when the Hill model was employed. In the absence of PS, the Hill coefficient approaches 1.0, confirming that enzyme binding to tissue factor in PC vesicles is not cooperative. With increasing charge, $N_H$ becomes significantly greater than 1.0 but less than 2.0. This result is consistent with a model where the cooperative unit contains two binding sites, and the interaction between the sites is positive; i.e., the second ligand binds more tightly than the first.

In addition to the qualitative change in binding with increasing vesicle charge, a quantitative change was also observed. For the Hill binding parameters, this is best illustrated by calculating the $K_{1/2}$, the concentration of free ligand required to half-saturate the tissue factor site:

$$K_{1/2} = e^{(\ln K_H)/N_H} \quad (12)$$

Table VIII: $K_{1/2}$ for Activator–Enzyme Binding Calculated from Hill Binding Parameters[a]

| PS:PC ratio | $K_{1/2}$ (nM) factor VII | factor VIIa | DIP-factor VII | DIP-factor VIIa |
|---|---|---|---|---|
| 0:100 | 12.2 | 4.27 | 5.68 | 3.25 |
| 5:95 | 6.19 | 4.84 | 4.05 | 2.75 |
| 10:90 | 6.67 | 4.34 | 5.06 | 2.06 |
| 20:80 | 2.12 | 3.44 | 3.66 | 1.77 |
| 30:70 | 1.10 | 2.23 | 2.16 | 1.45 |
| 40:60 | 0.66 | 1.69 | 0.91 | 1.76 |

[a] The concentrations of enzyme required to half-saturate the tissue factor site ($K_{1/2}$) were calculated from the Hill binding parameters (Tables III and IV) by using eq 12.

As shown in Table VIII, the $K_{1/2}$ decreased with increasing PS for all forms of the enzyme tested. In other words, regardless of the mechanism, half-saturation of the tissue factor was achieved at lower concentrations of enzyme when the vesicles were negatively charged.

One notable exception to the trend of increased apparent cooperativity with increasing PS was the binding of DIP-factor VII. Unlike the other forms of the enzyme, the Hill coefficient for DIP-factor VII binding was not significantly greater than 1.0 in five out of six cases. Only for the most acidic vesicles, 40:60 PS/PC, was there evidence for positive cooperativity. The absence of cooperativity for DIP-factor VII binding indicates that the results obtained with the other forms of the enzyme are probably not an assay artifact. While it has been shown that ligand heterogeneity may produce isotherms which mimic positive cooperativity (Mendel et al., 1985), if this were the reason for the apparent cooperativity we observe, then DIP-factor VII should follow the same trend since all forms of the enzyme were derived from the same preparations of factor VII.

The effects of PS on activator–enzyme binding may be understood by considering ways that phospholipid can interact with and alter the ligand or the receptor. If the enzyme bound to phospholipid contributes significantly to complex formation, then the tighter apparent binding of enzyme to activator observed when the vesicles contain PS could be the result of this "concentrating" effect. We have shown that for the conditions employed in these experiments the concentration of enzyme bound to phospholipid is a linear function of the free enzyme. This means that the binding of factor VII to PS could contribute to the decreaes in $K_{1/2}$ with increased vesicle charge. However, this is not necessarily true, and it cannot explain the apparent cooperativity.

As noted under Results, the two-site cooperative model (eq 11) fits the entire set of binding data, and the trends observed with the Hill model are also apparent in the Adair parameters. In this model, tissue factor is a dimer with two interacting enzyme binding sites. PS could promote dimer formation or enhance the interaction between the subunits of the hypothetical tissue factor dimer. In either case, a direct effect of PS on the structure of the activator could result in cooperative binding that varies with vesicle charge.

Binding of human factor VII and factor VIIa to a monocyte cell surface receptor has been reported (Broze, 1982). Correlating the appearance of biological activity and receptor sites indirectly established that the receptor is tissue factor. The $K_d$ for this system, ~82 pM, is significantly lower than we observe for the bovine proteins. This may represent a species difference, or the affinity of the activator for the enzyme may have been altered by the purification and reconstitution procedures we employed. Measuring the binding of pure human enzyme and activator will resolve this question. It is interesting to note that Broze (1982) saw deviation from single-site binding which could be intepreted as positive cooperativity. Complex binding was observed at 37 °C but was not evident at 0 °C. In the purified bovine system, the apparent cooperativity is not lost when binding is performed at 4 °C (data not shown). Of note is that in the human system the differences in binding between factor VII and VIIa are small and are thus consistent with the data obtained using bovine proteins.

It is clear that negatively charged phospholipid has dramatic effects on the properties of tissue factor. Whether or not the changes observed in vitro play a role in modulating tissue factor initiated coagulation in vivo remains to be established. It has been shown that the external leaflet of the plasma membrane in the eucaryotic cell is composed primarily of uncharged phospholipids, while charged phospholipids, such as PS, are primarily localized on the cytoplasmic face of the membrane (Rothman & Lenard, 1977; Zwaal et al., 1977). This asymmetric distribution of charged phospholipids may be a physiologically important mechanism for modulating the initiation of coagulation by tissue factor in which minor injury might result in a simple, relatively low-affinity system such as we observe when reconstituting with PC. Damage to cells which results in exposure of the acidic lipids could lead to tighter binding of enzyme to tissue factor and a larger burst of coagulant activity. By this mechanism, it is possible to envision the initiation of coagulation being regulated by the extent of tissue injury.

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**Registry No.** Factor VII, 9001-25-6; factor VIIa, 65312-43-8; tissue factor, 9035-58-9.

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# An Ordered Addition, Essential Activation Model of the Tissue Factor Pathway of Coagulation: Evidence for a Conformational Cage†

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ABSTRACT: One way in which coagulation may be initiated is by the action of factor VIIa (a plasma serine protease) and tissue factor (a membrane-bound lipid-dependent glycoprotein). We show that in the absence of either factor VIIa or tissue factor, the activation of the natural coagulation substrates, factors IX and X, is not detectable; i.e., tissue factor is an essential activator. We propose that the reaction is fully ordered; that is, the enzyme–activator complex picks up substrate to form a ternary product forming species. Our model precludes the formation of enzyme–substrate and activator–substrate complexes. We have derived equations for the two possible variations of this model: one in which product formation is accompanied by the release of the enzyme–activator complex and the other in which product, free enzyme, and free activator are formed with each catalytic cycle. Our data support only the former which is consistent with both steady-state and rapid equilibrium assumptions. The model is supported by experiments using a monoclonal anti-tissue factor antibody, which affects only the $K_{m\,app}$, and a modified form of factor VIIa, which, depending on the sequence in which reagents are added to the reaction, either decreases the $V_{max}$ or increases the $K_{m\,app}$. We present equations describing the initial velocity of these reactions. Utilizing dilution–jump experiments, we show that the system is hysteretic and suggest that this phenomenon is due to a slow release of enzyme from activator. However, the kinetically determined dissociation constant of enzyme and activator, previously found to be 4.5 nM under equilibrium conditions, was estimated to be 0.04–0.09 nM. Accordingly, we examined other essential activation models in which the product-forming species consists of a complex of enzyme, activator, and substrate at a molar ratio of 1:1:1; none could account for the apparent tight binding of enzyme and activator. We therefore postulate an ordered addition, essential activation model in which the enzyme undergoes two conformational transformations: one as a consequence of binding to tissue factor, resulting in a species which binds to and hydrolyzes its natural substrates. The other conformational change in the enzyme is induced by substrate, resulting in a species which binds more tightly to its activator. Thus, we hypothesize a "conformational cage" which precludes the dissociation of enzyme from activator while significant concentrations of substrate are present.

We have previously presented evidence that two catalytically active proteins, factor VII (a zymogen) and its two-chain derivative factor VIIa, participate in the initiation of the tissue factor pathway of coagulation (Zur et al., 1982). We also found that both forms of factor VII exhibit a dependence on tissue factor, a membrane-bound glycoprotein: in the absence of tissue factor, no product formation is observed. With respect to coagulant activity, then, tissue factor is an obligatory or "essential" activator.

When factor VII or factor VIIa is reacted with diisopropyl fluorophosphate (DFP),[1] all coagulant activity is lost. Each

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