Elevated Function of Blood Clotting Factor VIIa Mutants That Have Enhanced Affinity for Membranes

BEHAVIOR IN A DIFFUSION-LIMITED REACTION*

Received for publication, May 29, 2001, and in revised form, July 27, 2001
Published, JBC Papers in Press, August 21, 2001, DOI 10.1074/jbc.M104896200

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Blood clotting factor VIIa is involved in the first step of the blood coagulation cascade, as a membrane-associated enzyme in complex with tissue factor (TF). Factor VIIa is also an important therapeutic agent for hemophilia, where its function may include TF-independent as well as TF-dependent mechanisms. This study compared the activity of wild type factor VIIa (WT-VIIa) with that of a mutant with elevated affinity for membrane (P10Q/Q32E, QE-VIIa). Phospholipid and cell-based assays showed the mutant to have up to 40-fold higher function than WT-VIIa in both TF-dependent and TF-independent reactions. Tissue factor-dependent reactions displayed the maximum enhancement when binding had reached equilibrium in competition with another TF-binding protein. In liposome-based assays, the association rate of WT-VIIa with TF occurred at a physical maximum and could not be improved by site-directed mutagenesis. A practical consequence was identical function of WT-VIIa and QE-VIIa in assays that depended entirely on assembly kinetics. Thus, factor VIIa mutants provided unique reagents for probing the mechanism of factor VIIa action. They may also offer superior agents for therapy.

Factor VII is dependent on vitamin K for biosynthesis and contains 10 γ-carboxyglutamic acids (Gla) in residues 1–45. This Gla domain provides the membrane binding site of factor VII. The activated form of factor VII, factor VIIa, in combination with tissue factor (TF), an integral membrane protein, provides the initial step of the blood coagulation cascade. Assembly of VIIa with membrane-associated factor VIIa may consist of VIIa-TF plus VIIa-membrane contacts, generating a ternary complex with very high affinity. The VIIa-TF complex can activate factor IX to IXa and factor X to Xa and can autocatalyze VII to VIIa (reviewed in Ref. 1).

The two major types of hemophilia disease, A and B, are associated with deficiency of blood clotting factors VIII or IX, respectively. Factor VIII serves as a cofactor during factor IXa activation of factor X. Thus, the disease of hemophilia establishes that activation of factor X by TF-VIIa is insufficient for normal coagulation. A significant fraction of hemophilia patients develop inhibitors that prevent therapy with VIII or IX. Recently, factor VIIa has been found to be an alternative treatment (2, 3).

Under normal conditions, only about 1% of the 10 nM factor VII in plasma is present as WT-VIIa (4). The therapeutic dose of factor VIIa (90 μg/kg or about 50 nM in plasma (3)) is far above this level, and its mechanism of action is not understood. It is possible that factor VIIa displaces zymogen VII from TF, thereby increasing coagulation activity. Alternatively, high levels may act in a tissue factor-independent manner. That is, factor VIIa has a low enzyme activity in the absence of TF and also binds to activated platelets with low affinity. This weak activity may be the basis for therapy by high dose (5).

Specific mutations of the Gla domain of factor VII (P10Q/Q32E) increase its membrane-binding affinity (6). Although the mutant (QE-VIIa) showed higher activity than wild type factor VIIa (WT-VIIa) in several assays, the magnitude differed with assay type. Furthermore, the behavior of the therapeutic agent, factor VIIa, was not thoroughly investigated.

As a membrane-associated enzyme-catalyzed process, blood coagulation offers the prospect of large particles with many enzymes or receptors, conditions that favor reaction at the rate of diffusion (or particle transport) through solution. In vitro reproduction of particle-based enzyme behavior offers many challenges. These include replication of large particle size and large numbers of sites per particle. Of the few in vivo models available, dramatic outcomes of diffusion-limited events dominate such diverse biological phenomena as the regulation of neurotransmission (7) and several aspects of bacterial physiology (8). Although discussed and illustrated with in vitro models (9–12), practical impacts of this behavior on blood coagulation are not fully developed.

The present study was designed to improve the understanding of the mechanism of enhanced function by QE-VIIa. This mutant expressed 1- to 40-fold higher function than WT-VIIa. Maximum enhancement was observed in TF-dependent as well as TF-independent reactions. In liposome-based assays, WT-VIIa assembled with TF at a physical maximum, eliminating.
any benefit of the mutant in those assays that were limited by assembly rate. Enhanced function of QE-VIIa was found in circumstances where dissociation of the complex contributed to function. These results imply important features of normal blood coagulation and the conditions under which QE-VIIa may offer improved approaches to therapy.

MATERIALS AND METHODS

Proteins and Other Materials—The mutant form of factor VIIa (P10Q/K32E, QE-VIIa) was described earlier, as were sources of soluble tissue factor (sTF), human brain thromboplastin, and the method for preparation of large unilamellar phospholipid vesicles from pure individual phospholipids (6). Mouse brain thromboplastin was generated from mouse brain acetone powder (Sigma Chemical Co.) by standard methods (13).

Factor VIIa was a commercial recombinant protein from Novo Nordisk (Princeton, NJ). Pure, reconstructed tissue factor was a trademark product, Inovin (a purified, reconstituted source of TF provided by the Dade company, Miami, FL). The active form of QE-VII (QE-VIIa) was created by autoactivation during incubation with phospholipid vesicles (1.2 g of lipid per g of protein) at 37 °C in Tris buffer containing 5 mM calcium. Unless indicated, the Tris buffer used throughout this study was 0.05 M (pH 7.5) and contained 0.1 M NaCl. Maximum activation was achieved in about 45 min. QE-VIIa was separated from phospholipid by gel filtration chromatography on Sepharose 4B in buffer containing 1 mM EDTA. The phospholipids eluted at the exclusion volume of the gel filtration chromatography on Sepharose 4B in buffer containing 0.15 M NaCl and 0.1 M NaHCO₃.

Coagulation assays were performed in 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose, and BSA (3.5 g/liter).

Blood Clotting Assays—All coagulation assays were performed in 150 mM of total volume containing 37.5 μM of factor VII-deficient plasma (Sigma). For assays at equilibrium and without added VIIai, VIIa and TF were incubated at saturating levels (100 nM) along with varying concentrations of VIIai. The mixtures were incubated for 15 min at 37 °C followed by 30 min at room temperature. Chromogenic substrate, S-2222 (0.24 mM), was added, and absorbance change at 405 nm after addition of 0.3 mM EDTA (8.5 μl of 0.3 mM EDTA per 250 μl of solution). Factor Xa concentration was determined from absorbance change at 405 nm after addition of 0.3 mM chromogenic substrate (S-2222, Chromogenix). Very low background values in samples containing factor X alone or factor VIIa/TF alone were subtracted, and factor Xa concentration was determined by comparison to the activity of pure factor Xa. For competition assays, VIIa, VIIai, and TF were incubated for 60 min in calcium-containing buffer before factor X was added. Data are plotted by Equation 2.

In one, VIIa was first assembled with TF and VIIai was added as the second reagent. In the other, VIIai was added to TF and assembly completed before VIIa was added. These mixtures reached the same coagulation activity within 30 min at 37 °C.

For coagulation assays without pre-equilibrium of VIIa and TF, plasma, factor VIIa, and buffer were mixed (112.5 μl) and coagulation was initiated by addition of 37.5 μl of solution containing Inovin (1.0 μm/assay) in 20 mM calcium. In all cases, coagulation time was determined by the manual hand-tilt method.

Analysis of Protein Function by Competition at Equilibrium—Evaluation of protein efficacy by competition with another (factors VIIa and VIIai) for the same site (TF), at equilibrium, relies on the ratio of the equilibrium binding constants for the two ligands (Equation 1), where VIIai/TF and VIIai/TF are the respective ligand complexes with TF.

\[
K_{D,\text{eq}}/K_{D,\text{eq}} = ([\text{VIIa}]/[\text{VIIa}]/[\text{TF}])/([\text{VIIai}]/[\text{TF}]) \tag{1}
\]

An important requirement of Equation 1 was the use of [VIIai] and [VIIai] was set to a constant. Total protein concentration was known, and conditions were established where total protein approximated free protein. Generally, this involved use of low binding site concentration (TF) and high competing protein concentrations (VIIa and VIIai). Nonspecific interactions also impact on the comparison so that validity was tested.

The theoretical basis for an appropriate test was provided by rearrangement of Equation 1 to a Hill-type relationship (Equation 2). In this case, the competitor (VIIai) is part of the constant term.

\[
\log([\text{VIIai}]/[\text{TF}]) = \log([\text{VIIai}]/[\text{TF}])/[\text{VIIai}]/[\text{TF}] + \log(K_{D,\text{eq}}/K_{D,\text{eq}}) \tag{2}
\]

A plot of \(\log([\text{VIIai}]/[\text{TF}])/[\text{VIIai}]/[\text{TF}]) vs \log([\text{VIIai}]/[\text{TF}])\) versus \([\text{VIIai}]/[\text{TF}]\) shows a Hill plot \(\log(y) vs \log(\text{titrant})\). In this case, \(y\) is the fraction of TF bound by titrant (VIIa) and \(1 - y\) is the fraction bound by a competitor (VIIai). The concentration of active enzyme (VIIai/TF) was estimated from the level of factor Xa formed by the reaction mixture (see below) and quantitated by comparison to standard factor Xa. Ideal behavior was tested by varying [TF] and [VIIai]. Equation 2 predicts that these plots will have a slope of 1.0, be unchanged due to altered [TF], but will shift in proportion to \(\log([\text{VIIai}]/[\text{TF}])\).

Factor X Activation—The activity of VIIa or VIIai/TF was also detected by factor X activation. Reactions contained VIIai, a source of phospholipid, with or without TF, and factor X (200 nM, Enzyme Research Laboratory, South Bend, IN). These were mixed in 250 μl of Tris buffer containing 5 mM calcium plus BSA (1 g/liter). After a specified incubation time (1–5 min) the reaction was stopped by addition of excess EDTA (8.5 μl of 0.3 mM EDTA per 250 μl of solution). Factor Xa concentration was determined from absorbance change at 405 nm after addition of 0.3 mM chromogenic substrate (S-2222, Chromogenix).

Very low background values in samples containing factor X alone or factor VIIa/TF alone were subtracted, and factor Xa concentration was determined by comparison to the activity of pure factor Xa. For competition assays, VIIa, VIIai, and TF were incubated for 60 min in calcium-containing buffer before factor X was added. Data are plotted by Equation 2.

The fraction of TF as VIIai/TF was obtained from the relationship in Equation 3.

\([\text{Xa activity without VIIai}] = [\text{Xa activity with VIIai}]/[\text{Xa}]/[\text{VIIai}] \tag{3}\)

The fraction of TF as VIIai/TF was equal to 1.0 minus the fraction of TF as VIIai/TF.

Analysis of Competition for Soluble TF—Competitive assembly of VIIai and VIIai with sTF (17.5 nM, described earlier (6)) was measured in 0.25 ml of Tris buffer containing 5 mM calcium and BSA. Factor VIIai was added at saturating levels (100 nM) along with varying concentrations of VIIai. The mixtures were incubated for 15 min at 37 °C followed by 30 min at room temperature. Chromogenic substrate, S-2298 (0.24 mM), was added, and absorbance change at 405 nm was determined. A small background from unbound VIIai was subtracted. For data analysis, Equation 2 was rearranged to Equation 4.

\[\log([\text{VIIai}]/[\text{TF}]) = \log([\text{VIIai}]/[\text{TF}])/[\text{VIIai}]/[\text{TF}] + \log(K_{D,\text{eq}}/K_{D,\text{eq}}) \tag{4}\]

In this case, the sTF concentration was significant so that total VIIai and VIIai concentrations were corrected to their respective free concentrations. Bound protein equaled total sTF (17.5 nM) throughout the
titration but was distributed between VIIai-sTF and VIIai-sTF. The concentration of VIIai-sTF was estimated from absorbance change at 405 nm, compared with that at zero VIIai (where [VIIai-sTF] equaled [sTF]_{total}, 17.5 nM). The plot provided an intercept related to the ratio of VIIai and VIIai affinities, log(K_{DVIIai}/K_{DVIIa}).

Graphs displaying error bars represent the average and standard deviation for at least three measurements. For determinations showing no error bars, S.D. was less than the size of the symbol. Plots that do not show error bars provide individual measurements with duplicate determinations. All linear analysis represented the best-fit result made by the program Kaleidagraph. For titrations with factor VIIa, ideal behaviour with 1–5 µl of Innovin per assay occurred at [VIIai] > 0.5 nM.

RESULTS

Conditions That Show Similarity of WT-VIIa and QE-VIIa—

QE-VIIa has an altered membrane contact site. If the mutations do not impact on interaction with TF, QE-VIIa and WT-VIIa should show identical interaction with soluble TF. This was shown by competition binding of QE-VIIai, WT-VIIai, and WT-VIIai to sTF (Fig. 1A). Displacement of VIIa from sTF by WT-VIIai and QE-VIIai was measured by activity toward chromogenic substrate. The value of log(VIIai/VIIa) at the intercept (~0.87) suggested that WT-VIIai had about 7-fold higher affinity for sTF than did the competing WT-VIIa. The higher affinity of WT-VIIai for TF has been reported previously (14, 16). Although QE-VIIai also displayed higher affinity than WT-VIIa, it was slightly less effective than WT-VIIai. This small difference could arise from impurities or inactive protein in the preparations. The difference could result in underestimation of the advantage of QE-VIIa. Because the difference was small compared with those shown below, this aspect of QE-VIIai was not pursued further. The major observation was the near identity of WT-VIIai and QE-VIIai in a membrane-free state. This suggested that protein-protein interactions were generally unaltered by the mutations in QE-VIIa.

Little difference between QE-VIIai and WT-VIIai was found in two types of TF-dependent reactions. In one, factor VIIa and TF were allowed to reach equilibrium in calcium-containing buffer. Factor VII-deficient plasma was then added to start the reaction (Fig. 1B). Similar outcome was consistent with the high affinity of WT-VIIai for membrane-bound TF. For example, the titrations approached saturation at log([VIIai]) = 1.25 (18 pm factor VIIa). The Kd for WT-VIIai and membrane-bound TF is well below this concentration (e.g. Kd = 3–7 pm (17–19)), providing quantitative binding of WT-VIIai and eliminating a benefit from the higher affinity of QE-VIIai.

The similarity of WT-VIIai and QE-VIIai under equilibrium binding conditions was substantiated by measurement of factor X activation (Fig. 1B). As for the coagulation assay, the proteins gave similar titration curves that approached saturation at 15–20 pm factor VIIai.

A second condition giving similar activity of WT-VIIai and QE-VIIai involved addition of TF and calcium as the last component of the assay (Fig. 1B). These conditions required higher WT-VIIai concentrations than those that involved equilibrium binding, suggesting that the reaction was at least partially limited by the rate of WT-VIIai assembly with TF. Thus, identity of WT-VIIai and QE-VIIai in this assay implied identical association rate constants for these proteins.

Assembly rates were rapid and could not be accurately determined by the methods used. However, approximate assembly rates were suggested by two assays. In a coagulation assay, 20 pm WT-VIIai was incubated with 18 pm TF for 20 s. Factor VIIai (2 nM) and factor VII-deficient plasma were added to start the coagulation reaction. The former blocked further assembly of WT-VIIai-TF and the latter initiated coagulation. The clotting time was 32 s. This compared with a clotting time of 28 s after maximum assembly of factor VIIai and TF and 58 s with no pre-assembly (Fig. 1). Thus, a substantial portion of the factor VIIai-TF complex had assembled in the 20-s preincubation. Factor X activation assays suggested a similar rapid assembly. WT-VIIai (5 pm) plus the TF concentration used in Fig. 1B (18 pm) were incubated in calcium-containing buffer for 0 s, 30 s, or 15 min. Factor X (200 nM) was added. After an additional minute, excess EDTA stopped each reaction. The amount of factor Xa formed in the reaction with no preincubation was 15% of that formed in the reaction with a 15-min preincubation time. The Xa formed in the reaction that was preincubated for 30 s was 58% of that formed in the reaction with a preincubation time of 15 min. This suggested that about half of the factor VIIai-TF complex was assembled in a 30-s preincubation. If the results of either the coagulation experiment or the factor Xa generation experiment were treated as a bimolecular association reaction (ν = k[VIHa][TF]), k would be ~10^9 M^{-1} s^{-1}. As discussed below, this analysis must be modified if the reaction occurred at the diffusion limit and on multisite particles. Nevertheless, the result was sufficient to show that assembly was
Function of Factor VIIa Variants with High Membrane Affinity

A similar outcome for WT-VIIa and QE-VIIa for all conditions in Fig. 1 suggested identical behaviors in many aspects. These included the kinetics of VIIa assembly with TF, the binding of substrate protein (factor X) and the catalytic reaction rate. Similar comparisons and conclusions were obtained when a preparation of crude human brain thromboplastin was used as the tissue factor source (data not shown).

Comparison of WT-VIIa and QE-VIIa on this membrane source (Fig. 3) gave similar outcome to that observed with pure, reconstituted TF (Fig. 2). Thus, although it is likely that the phospholipids of crude brain thromboplastin are of biological origin, the organic solvent extraction may have major impacts on activity. A different source of TF was provided by J82 cell membranes. With intact cells, most TF is encrypted or present in an inactive form (20, 21). Activity becomes de-encrypted by a number of cell treatments, including those leading to cell death. The J82 cells were harvested and stored at 0 °C for 24 h to maximize TF de-encryption. The TF activity in the J82 cell preparations remained associated with large particles that were pelleted in a tabletop centrifuge.

Comparison of WT-VIIa and QE-VIIa on this membrane source (Fig. 3) gave similar outcome to that observed with pure, reconstituted TF (Fig. 2). Thus, although it is likely that the various membranes used in this study had substantially different affinities for the vitamin K-dependent proteins, the impact of the membrane may be similar for each protein so that the difference between WT-VIIa and QE-VIIa remained the same in all assays.

**TF-independent Activity**—Factor VIIa has a low enzyme activity without TF. This has been observed on vesicles and on activated platelets (5, 22). Vesicles of several compositions were tested, and typical results are shown in Fig. 4A. In this assay, the QE-VIIa mutant gave ~16-fold higher activity than WT-VIIa. As pointed out previously (6), the difference between WT-VIIa and QE-VIIa will depend on the phospholipid concentration in the reaction. High phospholipid, with a corresponding high binding site concentration, will shift more WT-VIIa to the bound state, lowering the benefit derived from the higher affinity of QE-VIIa. Thus, the 16-fold difference in Fig. 4A may underestimate the difference in binding constants.

The biological milieu contains other proteins that bind to

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**FIG. 2.** Competition binding to TF. Competitive binding was established by incubation of VIIai (2 nM), tissue factor (1 μl of Innovin/0.1125 ml, ~18 pm TF) and either WT-VIIa (○) or QE-VIIa (●) in Tris buffer containing 6.7 mM calcium and BSA. After 1 h at 37 °C, the reactions were assayed. A, coagulation assays. Coagulation was initiated by addition of 0.0375 ml of factor VII-deficient plasma to 0.1125 ml of equilibrium mixture. Data are plotted as log(clotting time) versus log(VIIai). The data represent three different experiments run on separate days with triplicate determinations each time (nine determinations for each data point shown). Clotting time zero for the different titrations ranged from 28–31 s. B, factor X activation. After equilibration, factor X (200 nM) was added and the reaction was incubated at 37 °C for 3 min. Excess EDTA was added to stop the reaction, and the factor Xa concentration was determined by chromogenic substrate assay. The results are plotted by Equation 2. Additional titrations at 8 nM factor VIIai are shown for WT-VIIa (□) and QE-VIIa (●).

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**FIG. 3.** Competition for TF provided on J82 cell membranes. Factor VIIai (2 nM), factor VIIai (○) or QE-VIIai (●), and J82 cell membranes (9000 cells) were incubated in 0.25 ml of buffer containing 5 mM calcium for 1 h at 37 °C, with mixing every 20 min to maintain cell suspension. Factor X (200 nM) was added, and the incubation was continued for 5 min. The reaction was stopped with 8.3 μl of 0.3 M EDTA, and Xa was measured by absorbance change at 405 nm in 0.32 mM S-2222. Averages and standard deviation are shown of four samples at each concentration. Maximum velocity, expressed as ΔA405/min was 0.137. The data are plotted according to Equation 2. Furthermore, the slopes of the lines were identical and ~1.0 (Fig. 2B). Similar shifts in coagulation titrations were obtained as factor VIIai was altered. Overall, ideal behavior predicted by Equation 2 was observed at factor VIIai > 0.5 nM and low TF concentrations (<20 pm TF).

**Competition Binding on J82 Cell Membranes**—Membrane composition and concentration are important variables that influence the outcome of coagulation. The TF used in Figs. 1 and 2 was reconstituted in liposomes of purified phospholipids. A second source of TF, crude human brain thromboplastin, provided outcomes that were similar to those in Fig. 2 (data not shown). Although the phospholipids of crude brain thromboplastin are of biological origin, the organic solvent extraction may have major impacts on activity. A different source of TF was provided by J82 cell membranes. With intact cells, most TF is encrypted or present in an inactive form (20, 21). Activity becomes de-encrypted by a number of cell treatments, including those leading to cell death. The J82 cells were harvested and stored at 0 °C for 24 h to maximize TF de-encryption. The TF activity in the J82 cell preparations remained associated with large particles that were pelleted in a tabletop centrifuge.
The supernatant was estimated from H9004 activated with calcium ionophore (A23187, 5)
buffer used for platelet isolation containing 5 mM calcium were acti-
11003 centrifugation at 13,000
9262 were incubated at 37
9262 (50 nM,
263) lipid vesicles (PS/PC/PE, 20/40/40, 2.5
262 conducted in the presence of 2
267 generated about 1 nM factor Xa in 60 min with 2 × 10^9 platelets per milliliter (Fig. 4A). This was about half of the Xa
product production observed by others (5). A second platelet prepara-
408 tion gave about 50% of the Xa production shown in Fig. 4B. That
419 reaction rates differed with platelet preparation was expected,
because Xa production depended on the quality of membrane exposed during platelet activation, a property that is likely to
differ with the individual. In any case, QE-VIIa was much more
9262 active than WT-VIIa in all assays involving platelets. The
39829 example in Fig. 4B showed 25-fold higher activity.

Comparison with a Low Affinity TF—Rabbit tissue factor is
used in some commercial thromboplastin reagents such as a
product of the Sigma Chemical Co. It is adequate for standard
assays. Indeed, titrations such as those shown in Fig. 2A but
with the commercial source of rabbit brain thromboplastin
gave results similar to those in Fig. 2A (data not shown).

TF from the mouse is much more species-specific and inter-
acts poorly with human factor VIIa (13). This low affinity
presented the opportunity to compare WT-VIIa and QE-VIIa in
the absence of a competing protein. Low affinity was indicated
by the high total VIIa concentrations used. Total VIIa (Fig. 5)
should approximate free VIIa. The curves shown in Fig. 5
provided a 25-fold advantage for QE-VIIa. Thus, in all assays
where Equation 2 applied, the advantage of QE-VIIa was
similar over a wide range of membrane compositions, in the
presence or absence of a competing protein and for different
protein-protein (factor VIIa with TF) affinities.

**DISCUSSION**

In this study, extensive comparison of WT-VIIa and QE-VIIa
showed 0- to 40-fold difference in activity, depending on the
assay method. The apparent explanation for such a wide range
in outcome was that assays were sensitive to different stages of
the coagulation process. This afforded opportunities to examine
the coagulation mechanism and provided insight into several
features of factor VIIa function.

High Affinity Binding—At equilibrium and in the absence of
a competitor for TF, QE-VIIa offered little advantage over
WT-VIIa in a liposome-based TF-dependent assay. Because
QE-VIIa had a higher affinity for membrane-bound TF than
did WT-VIIa, identical function could be explained if all of the
WT-VIIa was bound. This would eliminate the impact of higher
affinity or absence of a competing protein and for different
protein-protein (factor VIIa with TF) affinities.

These were mixed in 112.5
9262 l of buffer containing 6.7 mM calcium and
BSA. They were incubated for 15 min at 37 °C to reach equilibrium, and
coagulation was initiated by addition of 37.5 µl of factor VII-deficient
plasma. Results for WT-VIIa with human TF (data from Fig. 1) are
shown for comparison (□).

exposed membrane. These may displace WT-VIIa and have the
same impact as lowering the membrane concentration. In both
cases, the number of available WT-VIIa binding sites is re-
duced. To test this effect, the experiment of Fig. 4A was con-
ducted in the presence of 2 µM prothrombin fragment 1, approxi-
ately the plasma concentration of prothrombin. Factor Xa production by WT-VIIa was reduced by 75%, probably due
to displacement of VIIa and X from the membrane. QE-VIIa
was inhibited to a lesser degree so the overall difference be-
 tween WT-VIIa and QE-VIIa was 23-fold (data not shown). The
reaction was also conducted at high factor X (2 µM). In this case,
the substrate would force the enzyme from the membrane,
giving lower total activity. QE-VIIa was 35-fold more effective
than WT-VIIa under these conditions (data not shown).

Thus, the enhanced activity of QE-VIIa was most evident un-
der conditions of low phospholipid concentration and/or in
the presence of other membrane-binding proteins. Both of
these conditions should apply to the biological situation.

Factor X activation by factor VIIa was also examined on
activated platelets. As expected, the amount of Xa produced
was dependent on the individual platelet preparation. WT-VIIa
(50 nM) generated about 1 nM factor Xa in 60 min with 2 × 10^8 platelets per milliliter (Fig. 4B). This was about half of the Xa

![Figure 4](http://www.jbc.org/)

**Fig. 4. Tissue factor-independent activity.** A, activity on phospholipid vesicles. Factor VIIa (5 nm, ○) or QE-VIIa (5 nm, ●), phospholipid vesicles (PS/PC/PE, 20/40/40, 2.5 µg/ml), and factor X (200 nm) were incubated at 37 °C for the times shown. Excess EDTA (8 µl of 0.3 M per 0.25 ml of sample) plus 0.32 mM S-2222 was added, and factor Xa concentration was determined by absorbance change at 405 nm. The average and standard deviation of triplicate samples is shown. B, activity on activated platelets. Monomeric platelets (2.0 × 10^9/ml) in the buffer used for platelet isolation containing 5 mM calcium were acti-
vated with calcium ionophore (A23187, 5 µg/ml) for 15 min. Factor VIIa (50 nm, ○) or QE-VIIa (50 nm, ●) plus factor X (200 nm) were added, and the reaction was incubated for the time shown. Excess EDTA (8.3 µl of 0.3 M per 0.25 ml of sample) was added, and platelets were removed by centrifugation at 13,000 × g for 3 min. Factor Xa concentration in the supernatant was estimated from ΔA_{405} in 0.32 mM S-2222 at room

![Figure 5](http://www.jbc.org/)

**Fig. 5. Factor VIIa activity with murine tissue factor.** The as-
says utilized sufficient mouse brain thromboplastin to give a clotting
time of 28 ± 1 s (clotting time zero) at 10 nm QE-VIIa. The titration was
conducted at the concentrations of VIIa (○) or QE-VIIa (●) shown.
These were mixed in 112.5 µl of buffer containing 6.7 mM calcium and
BSA. They were incubated for 15 min at 37 °C to reach equilibrium, and
coaagulation was initiated by addition of 37.5 µl of factor VII-deficient
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![Diagram](http://www.jbc.org/)

**Diagram of Factor VIIa Variants with High Membrane Affinity**

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**Supplementary Material**

1. Supplementary methods and materials.
2. Supplementary tables.
3. Supplementary figures.
4. Supplementary references.

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measured by kinetic or thermodynamic methods (e.g. Refs. 17–19).

One circumstance of this assay, the use of factor VII-deficient plasma, would not represent the biological situation, which includes high levels of factor VII, the inactive zymogen. At short time intervals, factor VII should act as a competitive inhibitor. Thus, a better comparison of QE-VIIa and WT-VIIa may be obtained in assays containing factor VIIai, a competitor for TF (see below).

Rate of VIIa-TF Assembly—Important properties of the factor VIIa-TF system arose from unusual features of association and dissociation rate kinetics. Equation 5 provides a simple model on which to base discussion.

$$k_r \frac{[VIIa] + [TF]}{[VIIa][TF][K_D]} = \frac{k_r}{k_r + k_d}$$  
(Eq. 5)

For assembly of factor VIIa with TF, $k_r$ and $k_d$ may be complex. For example, a common proposal for assembly of blood-clotting enzymes consists of factor VIIa association with the membrane surface followed by lateral diffusion to complete VIIa-TF assembly. If correct, the higher affinity of QE-VIIa for the membrane should give it longer residence time on the membrane and provide greater lateral diffusion and improved location of TF. Enhanced association rates ($k_r$) should result. This did not occur. QE-VIIa was approximately equal to WT-VIIa in assays dependent on the rate of factor VIIa assembly with TF (Fig. 1B). Lack of enhancement of $k_r$ could be explained if the rate of WT-VIIa assembly with TF occurred at a limiting value. This explanation was consistent with a number of observations.

A logical physical limit for WT-VIIa association with TF is the rate of particle diffusion in solution. Indeed, association of WT-VIIa with TF was very rapid. The approximate site rate constant ($10^8$ M$^{-1}$ s$^{-1}$, see above) was well above the $k_{cat}/K_m$ ratios for diffusion-limited enzymes (about $10^4$ M$^{-1}$ s$^{-1}$; see Ref. 23 for discussion) but was still below the collisional rate constant ($k_{coll}$) for a typical protein-phospholipid vesicle interaction (about $10^{10}$ M$^{-1}$ s$^{-1}$ (24)). As described in detail elsewhere (25–27), diffusion-limited kinetics of multisite particles are complex. Precise mathematical modeling requires knowledge of particle size, the number of sites per particle ($n$), the distribution of sites over all particles (even or biased), as well as their distribution on the surface of each particle (clustered or dispersed). If there are multiple sites per particle, the observed association rate constant is related to $k_{coll}/n$ (25). If sites are clustered, diffusion-limited reaction may apply to a region of the membrane rather than to the entire particle. Often, observation of general kinetic behavior provides the best indication of a diffusion-limited reaction (e.g. Ref. 28). One feature is slow ligand dissociation under conditions of excess receptor but rapid dissociation under conditions of excess ligand (26, 27).

Indeed, work that is in progress shows that this behavior is very pronounced for the factor VIIa-TF interaction with Innovin and other reconstituted liposomes. 2 Rapid exchange at excess ligand was responsible for establishment of equilibrium within only 30 min for all competitive assays used in this study. Thus, association of WT-VIIa with the types of membrane-associated TF that were used in this study appeared to occur at the diffusional limit.

In addition to the studies reported with crude thromboplastin and commercially prepared recombinant TF, several other preparations of pure TF, reconstituted in liposomes, were analyzed. These preparations gave results and conclusions that were similar to those described above for Innovin and crude thromboplastin (data not shown). An exception was found in a preliminary study of factor VIIa correction of coagulation time in blood from a factor VII-deficient hemophilia patient. The assay measured the whole blood prothrombin time using the Hemochrom Jr. Signature Coagulation Apparatus (International Technidyne). In that assay, TF is provided in the assay cuvette, in an unspecified manner. Because addition of blood to the cuvette starts the reaction, TF is effectively added as the final component, and the assay should be sensitive to the rate of factor VIIa assembly with TF. This assay showed a 6-fold advantage of QE-VIIa over WT-VIIa (data not shown). Unquestionably, the membrane and TF formulations of the whole blood clotting assay differed from those of commercial, reconstituted TF (Innovin), crude thromboplastin, or other liposome preparations. Thus, QE-VIIa may offer kinetic advantage over WT-VIIa in some situations. The difference could be explained if the TF-lipid combination in the whole blood prothrombin time did not produce diffusion-limited assembly. Overall, the diffusion-limited status of the in vitro reaction will determine whether or not QE-VIIa is superior to WT-VIIa at short time interval.

Maximum Advantage of QE-VIIa from Dissociation Rates—Regardless of the outcome for reactions that are limited by the rate of factor VIIa assembly with TF, the 25- to 40-fold benefit of QE-VIIa was observed in cases where the dissociation rate constant contributed to function. In effect, higher membrane affinity of QE-VIIa was often expressed exclusively in the dissociation rate. The advantage of QE-VIIa was similar for a wide range of membrane compositions (Innovin versus cell membranes versus crude thromboplastin) and protein-protein binding affinities (e.g. human versus murine tissue factor). Maximum advantage was also observed in TF-independent reactions, especially those containing physiological levels of other vitamin K-dependent proteins and that used biological membranes.

Overall, the potential advantage of QE-VIIa over WT-VIIa for therapy will depend on the in vivo conditions important to coagulation. TF distribution on an exposed membrane can be an important parameter. Attainment of the diffusional limit often requires multisite particles. Thus, in short-term reactions limited by assembly rate, QE-VIIa will offer an advantage over WT-VIIa if TF is widely dispersed so that assembly is below the diffusion limit. QE-VIIa will offer a significant advantage in all cases, TF-dependent as well as TF-independent, where product formation is influenced by the rate of VIIa dissociation. These situations include many of the proposed mechanisms of high dose WT-VIIa function (e.g. Ref. 5).

The Nature of the Biological Membrane—The actual biological membrane that is available for blood coagulation is not well characterized. As indicated above, most in vitro tests utilize reconstituted membranes, either pure phospholipids (e.g. Innovin) or crude extracts (e.g. brain thromboplastin). These may differ substantially from their biological counterparts. Although biological membranes contain PE and PS, which are phospholipids that create high affinity for blood clotting proteins (29–31), it is not clear that reconstituted bilayers will actually mimic biological membranes, even if their composition reflects that of the biological membrane. For example, complete biological membranes are asymmetric and contain proteins and other components that may block access to PS and PE. They also show a high level of “immobile” phospholipids and proteins (e.g. Ref. 32). These arise from features that are not found in reconstituted bilayers but may lower protein affinity. In this regard, use of membranes of activated platelets may be important.

The results suggest that activated platelets provide a rela-
tively modest affinity for vitamin K-dependent proteins. The affinity appears sufficient to support normal coagulation but does not appear as substantial as liposomes in support of the TF-independent activity of WT-VIIa. For example, Xa production per each WT-VIIa molecule on PS/PC/PE (20/40/40, 2.5 μg/ml, 5 nM VIIa, Fig. 4A) was more than four times that produced on activated platelets (50 nM VIIa, 2 × 10^8 per ml, Fig. 4B). If VIIa therapy in hemophilia utilizes the TF-independent pathway, QE-VIIa should provide its maximum benefit.

Overall, mutant forms of VIIa with high affinity for membrane provide important tools for studying the reaction mechanism. Their use in vivo may assist in determining mechanisms of coagulation and the nature of the biological membrane, and they may provide benefits of lower doses for therapy.

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J. Biol. Chem. 2001, 276:39825-39831.
doi: 10.1074/jbc.M104896200 originally published online August 21, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104896200

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