Unusual Benefits of Macromolecular Shielding by Polyethylene Glycol for Reactions at the Diffusional Limit: The Case of Factor VIIai and Tissue Factor†

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ABSTRACT: Protein modification with poly(ethylene glycol) (PEG) can prolong circulatory lifetime and lower protein antigenicity in an animal. These benefits may arise from the proposed mechanism of PEG action, molecular shielding of the protein surface, and lowered interaction with other macromolecules. Proteins that depend on macromolecule association for their function would not seem good targets for PEG modification as the benefits may be mitigated by loss of function. Indeed, high loss of function applied to PEG-modified factor VIIa and to active site-blocked blood clotting factors Xa or IXa was studied. A surprising finding was that PEG-modified, active site-blocked factor VIIa (PEG-VIIai, PEG-40 000) retained 40% of its function despite an 18-fold increase in circulatory lifetime. The discrepancy between functional loss and increased circulatory lifetime was consistent with a process that was limited by the diffusion step of assembly rather than the chemical binding step. The impact of PEG-40 000 on diffusion of VIIai is small (about 3-fold) relative to its potential impact on molecular shielding during the chemical binding step of association. These properties extended to a mutant of VIIai (P10Q/K32E, QE-VIIai) that has 25-fold higher function than wild-type factor VIIai. Overall, properties of PEG-modified proteins can suggest features of the kinetic mechanism and may provide enhanced proteins for anticoagulation therapy.

Active site-modified wild-type factor VIIa (WT-VIIai)[1] is an anticoagulant with desirable traits. In vivo, WT-VIIai prevents intravascular platelet deposition at the site of surgery without increasing blood loss (*1*). It also prevents coagulation in an intravascular thrombosis model without increasing bleeding time at a distant site (*2*) and is effective in prolonging survival in a sepsis model (*3*).

Challenges to the use of VIIai include the required dose, 1 mg/kg body weight in several studies (*1*, *2*), and the short circulation halftime, 2−3 h for factor VIIa in the human (*4*). An approach to lower the required dose is the use of factor VIIai variants that have enhanced affinity for the membrane such as P10Q/K32E (QE-VIIa) (*5*). An approach to increase the circulation lifetime of a protein is modification by poly(ethylene glycol) (PEG; see ref *6*). Unfortunately, the proposed mechanism of PEG action does not encourage its application to proteins involved in macromolecular interactions. PEG is thought to provide a diffusion cloud around the modified protein, providing benefit by decreasing accessibility to the macromolecular systems involved in antibody production and protein turnover. In fact, prime targets for PEG modification are enzymes whose low molecular weight substrates (*7*−*9*) diffuse freely through the PEG cloud to the enzyme active site.

Factor VIIa function involves association with the membrane-bound receptor, tissue factor (TF). Receptors are present as multiple copies per particle with the potential to reach the perfectly reactive state where every collision between the ligand (factor VIIai in this case) and the TF-bearing membrane particle results in a binding event. These multisite particle kinetics dominates features of bacterial physiology (*10*) and may underlie resistance of some pathogens to antimicrobial agents (*11*). Previous studies have found that properties of factor VIIa interaction with TF suggest a collision-limited reaction, with major implications for factor VIIa mutants with elevated affinity for the membrane (*5*). If the collisional limit applies to factor VIIai, PEG modification may provide outcomes that are not predicted by the molecular shielding concept.

This study examined the impact of PEG modification on the activity and circulation lifetime of factor VIIai. For most

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[1] Abbreviations: WT-VIIa, wild-type blood clotting factor VIIa; FPRck, phenylalanylprolylarginyl chloromethyl ketone; WT-VIIai, WT-VIIa with active site blocked by reaction with FPRck; PEG, poly(ethylene glycol), with or without methoxylated terminus; PEG-40 000, PEG of 40 000 molecular weight or other molecular, as specified; PEG-40 000−FPRck, PEG-40 000 attached to the amino terminal of FPRck; WT-VIIai(PEG-40 000), WT-VIIai that has PEG-40 000 attached through the FPR covalently linked to its active site or any other PEG that is specified; QE-VIIa, factor VIIa with the mutations P10Q/K32E; QE-VIIai, QE-VIIa with active site blocked by FPRck; QE-VIIai(PEG-40 000), QE-VIIai with PEG-40 000 attached to its active site or any other PEG molecule as specified; TF, tissue factor; WT-VIIa*TF, QE-VIIai*TF, QE-VIIai(PEG-40 000)*TF, etc., the complex of WT-VIIa with TF, QE-VIIai with TF, etc.; Innovin is the trademark name for a purified, reconstituted source of TF provided by the Dade Company.

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studies, PEG was linked to VIIa through an active site-directed inhibitor. Surprisingly, PEG-modified VIIai retained a high level of anticoagulation activity while showing a greatly increased circulation lifetime. This benefit did not extend to PEG-modified factors VIIa, Xai, or IXai, all of which lost most of their activity upon PEG modification. The basis for this differential impact may be the nature of the rate-limiting step. Reactions limited by collision will be impacted only by PEG-based changes in the diffusion coefficient while reactions limited by a chemical binding step will display the classic impact of PEG shielding of the surface. Selective benefits of PEG may assist in detecting kinetic mechanisms of other systems.

MATERIALS AND METHODS

Proteins. Factor VIIa (WT-VIIa) was pure recombinant protein from NOVO Nordisk (Princeton, NJ). The mutant factor VIIa protein (QE-VIIa) was described (*5*). Factor Xa was from Enzyme Research Labs (West Bend, IN), and IXaβ was from Calbiochem (La Jolla, CA). Unless specified, other reagents and methods of assay were as described previously (*5*).

PEG Modification. Most proteins used in this study were modified at their active site by reaction with PEG-modified phenylalanylphenylalanylarginine chloromethyl ketone (FFRck, Bachem) or phenylalanylprolylarginyl chloromethyl ketone (FPRck, Calbiochem). Briefly, FPRck (20 mM) was mixed with an amine-reactive PEG derivative (1 mM of methoxy-PEG−SPA PEG-20 000 or Branched PEG2−NHS ester MW 40 000 from Shearwater Polymers, Inc., Huntsville, AL or with polyoxyethylene bis(*p*-nitrophenyl carbonate) PEG-3350, Sigma) in 100 mM HEPES buffer, pH 8.5. The reactions were allowed to proceed at room temperature for 15 h. The samples with PEG-20 000 and PEG-40 000 were dialyzed for at least 72 h against 0.05 M Tris buffer, pH 7.5, to remove remaining FPRck reagent. The product from the PEG-3400 reaction was separated from unreacted FPRck by column chromatography on Sephadex G-25, eluted with Tris buffer. The PEG-3400−FPRck eluted at the exclusion volume of the column, well-separated from FPRck. During incubation, residual amine-reactive reagent was inactivated by water hydrolysis. The concentration of PEG−FPRck was estimated by reaction with human thrombin (Enzyme Research Labs, Inc.). Thrombin (50 μM) reacted quantitatively with PEG−FPRck reagents (<50 μM) within 1 h at room temperature. The remaining thrombin was quantified by its amidolytic activity toward S2288, and the concentration of PEG−FPRck was determined by the amount of thrombin that was inhibited.

For all reactions, commercial factor VIIa was first purified by ion-exchange chromatography. The protein (1−2 mg) was applied to a column of DEAE−Sephadex (0.5 × 3 cm) and washed with at least 10 column volumes of buffer (0.05 M Tris, pH 7.5). Protein was then eluted with buffer containing 0.5 M NaCl. The resulting factor VIIa was dialyzed to the desired buffer. This procedure removed stabilizing agents in the commercial VIIa, which interfered with subsequent modifications. For FPRck derivatives, purified factor VIIa (0.2−0.5 mg/mL) was mixed with a 50% excess of FPRck, and the reaction was complete within about 2 h at room temperature. The sample was dialyzed to remove excess reagent. Reaction with long-chain PEG−FPRck molecules was less vigorous. A typical preparation of VIIai(PEG-40 kDa) is illustrated. Factor VIIa (0.51 mg in 0.9 mL of Tris buffer containing 5 mM calcium) was made with 16.5 μM in PEG-40 000−FPRck (a 1.5:1 ratio of reagent to protein). After 16 h at room termperature, amidolytic activity, measured in buffer containing 100 nM soluble TF and 0.36 mM S2288 substrate, was 12% of the original. This preparation could be used directly for circulation turnover. Since unreacted factor VIIa has a circulation halftime of 35 min (data not shown), most was removed before the first sample was taken at 90 min.

At a ratio of PEG-40 000−FPRck to VIIa of 4.5:1, 97% of the amidase activity was inhibited after 15 h at room temperature. BSA (1 g/L) was added. The excess reagent was without impact on the standard coagulation assay. Lack of interference with the coagulation reaction may be due in part to the fact that the presence of BSA lowered reactivity of PEG-40 000−FPRck toward enzymes. In some cases, residual factor VIIa was removed by chromatography on Sephadex G-100. The PEG derivative eluted at the exclusion volume of the column, well-separated from free factor VIIa. PEG-modified protein was quantified by absorbance at 280 nm, with WT-VIIa as the standard.

Factor IXai-PEG and Xai-PEG were produced by similar methods. The PEG-modified proteins were separated from unmodified protein by gel filtration on Sephadex G-100. The PEG-40 000 proteins eluted at the exclusion volume of the column, well-separated from free protein.

Random modification of lysine side chains on VIIai or factor VIIa was achieved in HEPES buffer (0.075 M, pH 8.5). For example, VIIai(PEG-40 000, 0.4 mg/mL) was mixed with PEG(40 000-SPA) (10 mg/mL) and allowed to react for 2 h at room temperature. The inhibitory activity of this VIIai preparation was reduced by about 50% from the VIIai(PEG-40 000). As in other reactions, excess reagent was spontaneously hydrolyzed in water. This modified protein preparation was used for circulation turnover in the mouse and is designated VIIai(PEG-80 000). On a protein basis, it retained 20% the activity of WT-VIIai that contained no PEG.

Protein Turnover Studies. Animal experiments were conducted by procedures approved by the University of Minnesota Research Subjects Protection Program. Female BalbC mice (20 ± 1 g Harlan) were placed in a cage warmed by a heating pad for 10 min to cause tail vein dilation. They were anesthetized by intrapertoneal injection of Avertin (0.017 mL of a 1.25% solution of tribromoethanol/g of body weight). Proteins were injected into the tail vein in 0.20 mL of buffer. At appropriate times, animals were anesthetized, and blood (20 μL) was obtained from a small tail injury. The blood was anticoagulated with 0.14 volume of 0.1 M sodium citrate. Plasma was obtained after centrifugation and diluted 50-fold with 50 mM Tris buffer (pH 7.5) containing 5 mM $CaCl_2$, 100 mM NaCl, and bovine serum albumin (BSA, 1 mg/mL). A commercial source of TF (8.8 μL of Innovin, Dade/mL of diluted sample) was added to initiate the coagulation cascade. After 2 h at 37°, a small fibrin clot was broken by mixing the sample, and remaining proteases were inactivated by addition of diisopropylfluorophosphate (DIFP, 2 mM). Excess DIFP was hydrolyzed by further incubation at room temperature for 4−16 h.

The concentration of VIIai in the diluted, treated plasma sample was determined by a coagulation test. The sample was further diluted 25−600-fold, and 0−4 μL was added to a coagulation assay (112.5 μL of Tris buffer containing 6.7 mM calcium chloride, BSA (1 g/L), and 1 μL of Innovin as a TF source). The reaction was incubated at 37° for 30 min to form the VIIai-TF complex. Normal human plasma (37.5 μL) was added, and the time to form a clot was determined. Samples without VIIai gave clotting times of 28 ± 2 s. The amount of VIIai in the plasma was determined by comparison of clotting time with that of the standards containing known amounts of factor VIIai. Precision was enhanced by dilution of all samples to give similar coagulation times (40 ± 5 s). The high sensitivity of this assay arose from the high affinity of VIIai for TF and the slow dissociation rate, which was insignificant during the time required for coagulation to occur. Initial levels of VIIai in the plasma were 600 nM. Background inhibitor levels in control mouse plasma were often not detectable but were always less than 5 nM VIIai.

Factor Xai and IXai Activities. Factor Xai was examined by a diluted thromboplastin assay. In addition to factor Xai, the assay contained 112.5 μL of standard Tris−BSA buffer with 6.7 mM calcium and 2.5 μL of Innovin. Coagulation was initiated with 37.5 μL of normal human plasma. Factor IXai was assayed by an activated partial thromboplastin time (APTT). APTT reagent (50 μL, Sigma) was mixed with normal human plasma (50 μL) and incubated at 37° for 3 min. Factor IXai and calcium solution (50 μL of 20 mM) were added to start the reaction. Uninhibited assays gave clotting times of 29 ± 2 s.

Factor VIIai Function. In the case of active site-blocked proteins, function refers to a protein's ability to block coagulation or factor X activation. This inhibitor function was measured in two coagulation conditions described previously (*5*). The first was limited by the rate of factor assembly and was generated by adding TF (37.5 μL of 20 mM calcium containing 2.5 μL of Innovin) as the last component to 112.5 μL of Tris buffer containing plasma (37.5 μL of factor VII-deficient plasma) plus VIIa (0.48 nM) and VIIai. The second assay was at equilibrium and involved incubation of factor VIIai, factor VIIa (20 nM), and TF (2.5 μL of Innovin) for 1 h at 37° in 112.5 μL of Tris−BSA buffer containing 6.7 mM calcium. Factor VII-deficient human plasma (37.5 μL) was then added to start the reaction.

RESULTS

Characterization of PEG-VIIai. Upon modification with PEG−FPRck, factor VIIa was converted to a species of higher molecular weight. For example, VIIai(PEG-20 000) migrated as a protein with molecular weight of about 100 000 (Figure 1A, inset). The actual molecular weight should be 70 000, consisting of factor VIIa(MW-50 000) and one PEG-(20 000) molecule. Anomalous migration was expected since PEG is a random, flexible polymer that does not bind SDS. Other derivatives were as follows: VIIai(PEG-3400), which migrated as a protein with molecular weight of 70 000 and VIIai(PEG-40 000), which migrated more slowly than the protein standard with highest molecular weight (not shown).

Activities of the modified proteins were analyzed by two methods. One assay involved addition of TF as the final component. In this case, the level of enzyme (Factor VIIa*TF) and therefore the clotting time was determined by the rate of assembly for TF with either factor VIIa or VIIai. As reported previously for factor VIIa (*5*), reactions limited by assembly rate showed little difference between WT-VIIai and QE-VIIai (Figure 1A). This is expected since mutation does not alter diffusion properties and therefore cannot alter kinetics of a diffusion-limited assembly. Horizontal displacement of plots (Figure 1A) showed that proteins modified with PEG(40 000) showed the same relative change, about 75% reduction in function.

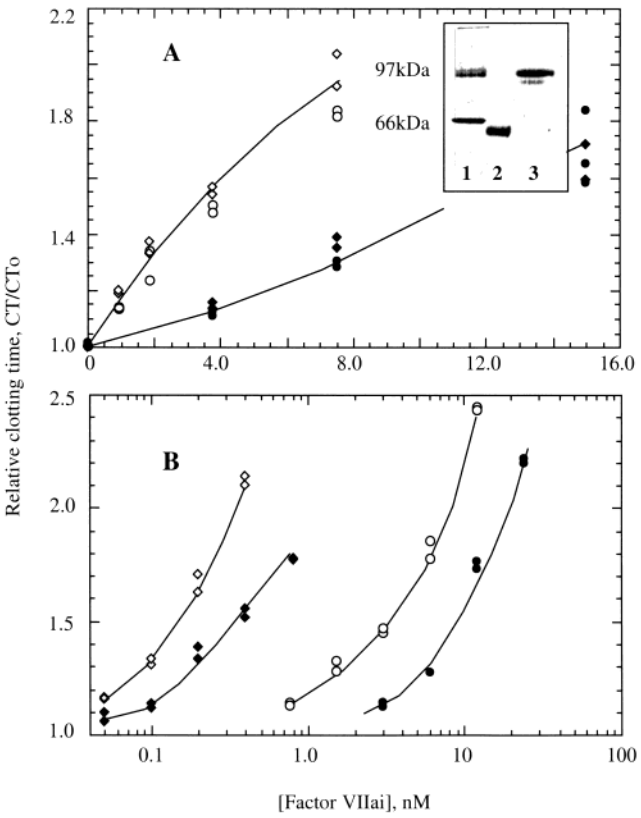


FIGURE 1: Anticoagulation by PEG-modified WT-VIIai and QE-VIIai. Panel A: Assembly-limited anticoagulation. WT-VIIa (0.48 nM), plasma (37.5 μL), and WT-VIIai (○) or QE-VIIai (◊) were mixed in 112.5 μL of buffer-BSA. A solution of calcium chloride (37.5 μL of 20 mM) containing 2.5 μL of Innovin was added to start the coagulation reaction. Also shown are results for WT-VIIai-(PEG-40 000) (●) and QE-VIIai(PEG-40 000) (◆). Data are plotted as the clotting time (CT) relative to the CT without VIIai (CT_o, 28 ± 2 s). Inset: Modification of VIIa by PEG−FPRck. SDS−PAGE is shown of standards (lane 1, MW shown), WT-VIIa (lane 2), and WT-VIIai(PEG-20 000) (lane 3). Panel B: Anticoagulation at equilibrium. WT-VIIa (20 nM), TF (2.5 μL of Innovin), and either WT-VIIai (○) or QE-VIIai (◊) were incubated for 1 h at 37° in 112.5 μL of Tris−BSA buffer containing 6.7 mM calcium. Human plasma (37.5 μL) was added to initiate coagulation. Also shown are results for WT-VIIai(PEG-40 000) (●) and QE-VIIai(PEG-40 000) (◆).

The assay at equilibium, when factor VIIai-PEG, factor VIIa, and TF had been incubated for at least 1 h before the start of coagulation, showed a very different result: QE-VIIai(PEG-40 000) had about 25-fold higher function than WT-VIIai(PEG-40 000) (Figure 1B, ref *5*). Horizontal displacement of the plots showed that the function of VIIai-(PEG-40 000) was reduced to about 25% that of WT-VIIai, while function of QE-VIIai(PEG-40 000) was reduced to about 40% that of QE-VIIai. Consequently, at equilibrium,

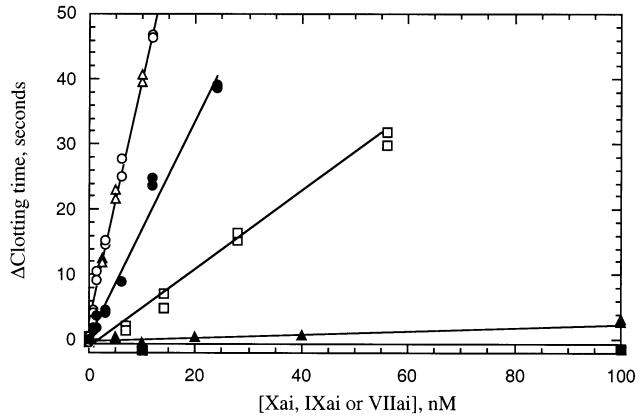


FIGURE 2: Impact of PEG on Xai and IXai. Results for factor VIIai (○) and factor VIIai(PEG-40 000) (●) are from Figure 1. Factor Xai (□) and factor Xai(PEG-40 000) (■) were assayed in a diluted thromboplastin assay. Factor IXai (△) and factor IXai(PEG-40 000) (▲) were analyzed in the standard APTT coagulation test.

QE-VIIai(PEG-40 000) had an 8-fold higher function than WT-VIIai without PEG.

Two other PEG derivatives were formed and assayed by the procedures in Figure 1A,B. As expected, plots for derivatives with PEG-3400 and PEG-20 000 were between those of proteins without PEG and those with PEG-40 000 (Figure 1A,B; data for intermediate PEG sizes are not shown).

Activity of PEG-Modified Factor Xai and IXai. The general impact of PEG modification was tested with factor IXai and Xai. The PEG-40 000 derivative of each protein was made, separated from unmodified protein by gel filtration, and analyzed by an appropriate assay. Activity of WT-VIIai and WT-VIIai(PEG-40 000) are included in Figure 2 for reference (data from Figure 1B) to demonstrate a relatively small loss of function. In contrast, PEG-modified IXai and Xai had lost all detectable activity.

Circulation Lifetime of VIIai Modified with PEG. Female mice (20 ± 1 g) were injected with 0.6−0.9 nmol of the various forms of WT-VIIai. Plasma samples were obtained, and VIIai concentrations were determined by procedures outlined in Materials and Methods. Protein recovery from the circulation at 5 min was high, corresponding to 0.67−1.0 μM. At 70−90 min, recovery was 42 ± 14% ($n = 7$) of injected factor VIIai (Figure 3A) and 54 ± 11% ($n = 4$) for VIIai(PEG-40 000). These recoveries were approximate as they assumed quantitative injection of the protein into the bloodstream at zero time.

Initially, the concentration of an injected protein declines because of turnover plus equilibrium into the extravascular space. Later, protein turnover is the only contributor to protein decline. These mechanisms produced curvature in the first part of the first-order decay curves (Figure 3A). To avoid this region, the level of inhibitor at 70−90 min was used as the first point to obtain first-order decay constants (Figure 3B).

The rate of protein loss from the circulation was very consistent among animals. The average first-order decay constant from five animals receiving WT-VIIai was −0.0074 ± 0.0003 (SD), corresponding to a circulation halftime of 93 ± 3 min. WT-VIIai(PEG-3400) gave a rate constant of −0.0035 ± 0.00005 (SD, $n = 3$), corresponding to a

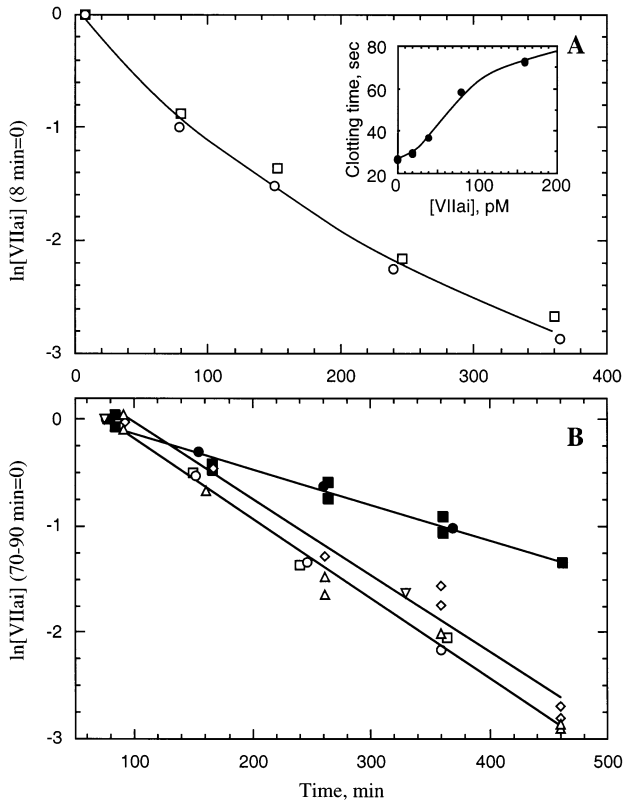


FIGURE 3: Circulation of VIIai in the mouse. Panel A: Loss from zero time. Factor VIIai (30 μg) was injected into the tail vein of two mice at zero time. Samples were removed at the times shown and analyzed for VIIai. Factor VIIai concentrations were standardized to the amount present at the first sample (5−8 min) and are plotted by a first-order decay. Inset: Standard curve for [VIIai]. WT-VIIai (concentrations shown) and TF (1 μL of Innovin) were incubated for 30 min at 37° in 112.5 μL of Tris−BSA buffer containing 6.7 mM calcium. Normal human plasma was added, and clotting time was determined. Panel B: Circulation time of WT-VIIai(PEG-3400). Results for five animals receiving WT-VIIai (open symbols) and three animals receiving WT-VIIai(PEG-3400) (solid symbols) are shown. Loss of WT-VIIai is expressed relative to the amount present at 70−90 min after injection.

circulation halftime of 201 ± 3 min. WT-VIIai(PEG-40 000) gave an average halftime of 28.3 ± 2.3 (SD, $n = 4$) h, 18 times longer than WT-VIIai.

A summary of PEG derivatives is presented in Figure 4. Each derivative was administered to two to five animals. The derivative with PEG-80 000 represented the combined modification with PEG-40 000 at the active site plus PEG-40 000 attached by random modification of lysine side chains. Reaction with activated-PEG(40 000) was allowed to proceed until activity was 50% that of the starting material (WT-VIIai(PEG-40 000) at the active site). While some multiple modifications of lysine side chains may occur, the most active proteins were likely those with a single added PEG. Thus, a PEG mass of 80 000 was assumed for this sample.

A separate study examined WT-VIIai that was modified at amino groups with PEG-20 000. Again, the reaction was allowed to proceed until the function was reduced to about 50% that of the starting material. This derivative showed biphasic behavior in the mouse circulation, suggesting two populations of WT-VIIai(PEG) molecules. Circulation halftimes obtained by linear analysis of early and late regions

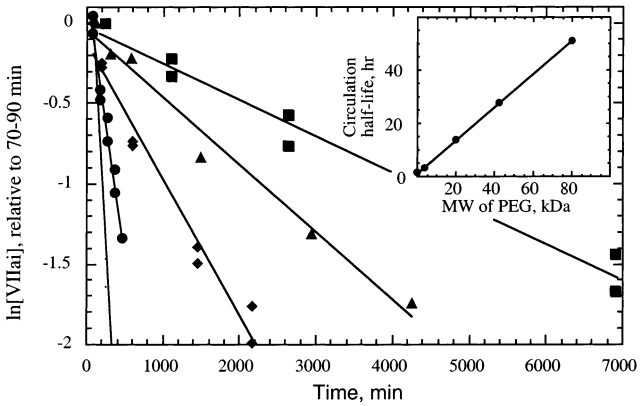


FIGURE 4: Circulation time for VIIai-PEG of different molecular weights. WT-VIIai levels in the circulation are shown relative to the amount present at 70−90 min. From left to right the decay curves and first rate constants for disappearance are as follows: WT-VIIai, 0.00745/min; WT-VIIai(PEG-3400), 0.00345/min; WT-VIIai(PEG-20 000), 8.25×10^{-4}/min; WT-VIIai(PEG-40 000), 4.28×10^{-4}/min; and WT-VIIai(PEG-80 000), 2.26×10^{-4}/min. Inset: Circulation halftime as a function of PEG-MW. Circulation halftimes for the PEG derivatives in Figure 4 are plotted vs MW of the PEG. The intercept is at 93 min, the halftime of WT-VIIai without PEG.

of the first-order decay curve were 14 and 29 h (data not shown). It is possible that these represented one population of WT-VIIai(PEG-20 000) with a single PEG-20 000 and the other a population with two PEG-20 000 derivatives. In fact, the halftime of 29 h was similar to that of WT-VIIai(PEG-40 000) (Figure 4).

These results suggested that, for purposes of extended circulation lifetime, the important parameter was total PEG mass attached to the protein, independent of the manner of attachment, and that circulation halftime was linear with the molecular weight of PEG (Figure 4, inset). There did not appear to be an upper limit to circulation lifetime that could be achieved. The intercept occurred at 93 min, the value for WT-VIIai without PEG.

Continual increase of circulation lifetime with molecular weight of the PEG, independent of single or double site modification, may be expected. The extended PEG polymers were large ($\leq$20 times) when compared with the dimensions of the folded protein. As a result, the density of PEG should be relatively uniform over the surface of the protein, and longer PEG molecules should increase shielding in a uniform manner. Thus, increase in circulation time with polymer size should result, if all methods of protein removal from the circulation involve interaction with macromolecules.

Protein preparations used for animal studies often contained a slight excess of active site-directed reagent (PEG−FPRck). If that reagent modified endogenous coagulation enzymes, it might create inhibitors and contribute to the anticoagulant concentration. Control experiments did not detect this reaction. Two mice received four injections of PEG(20 000)−FPRck (0.2 mL of 50 μM) over an 8 h period. Assay by the methods outlined above showed control levels of inhibitor in the circulation. Consequently, all inhibitors detected by this assay arose from the injected VIIai and its derivatives. Failure of PEG−FPRck to react with enzymes in vivo was expected since dilute BSA (1 g/L) inhibited active site modification (see Materials and Methods).

Antibody Response to WT-VIIai(PEG). While PEG lowers the antigenic character of a protein, observations made in the course of this study suggested that antibody response was not eliminated. For example, proteins modified with PEG-40 000 persisted in the circulation for 5 days (Figure 4) but declined abruptly to an undetectable level (<2% of injected sample) by day 7. This disappearance applied to all animals given WT-VIIai(PEG-40 000). WT-VIIai(PEG-20 000) did not show this abrupt loss during a first administration. However, one animal received a second injection of WT-VIIai(PEG-20 000). This resulted in normal disappearance from the circulation until day two whereupon all detectable inhibitor was removed by day 3. While direct antibody levels were not measured, these observations suggested that PEG modification did not eliminate the antigenic response.

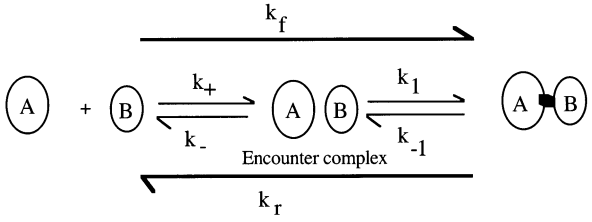
PEG Modification of Factor VIIa. To determine whether PEG would prove beneficial to the enzyme, WT-VIIa (0.57 mg/mL) was modified on lysine side chains with PEG2-40 000−NHS (12 mg/mL) or PEG-10 000−SPA (1.7 mg/mL) in 75 mM HEPES buffer (pH 8.5). Modified protein was separated from free VIIa by chromatography on Sephadex G-100 as described in Materials and Methods. Amidolytic activity toward S2288 substrate was analyzed in buffer containing 5 mM calcium and 100 nM soluble TF. Both preparations of PEG-modified VIIa retained about 50% of the original amidolytic activity toward the low molecular weight substrate. However, coagulation activity was greatly diminished. The specific clotting activity of VIIa(PEG-40 000) was 4% that of WT-VIIa. WT-VIIa(PEG-10 000) had 15% the specific clotting activity of WT-VIIa. Thus, the coagulation function of PEG-modified WT-VIIa was reduced by approximately the extent as the anticipated increase in circulation lifetime.

DISCUSSION

This study began with the practical goal of improving factor VIIai function by prolonging circulation lifetime. For theoretical reasons related to the proposed mechanism of PEG function, success was not expected, and the current study was only attempted as a facile extension of a different project. Low expectation arose from the proposed mechanism of PEG function, which is to interfere with macromolecular associations such as those required for blood coagulation reactions. In fact, severe reduction of function was observed for factors VIIa, IXai, and Xai. As expected, modified enzyme (factor VIIa) retained high activity toward a low molecular weight substrate. The surprising outcome was high retention of function for PEG-modified factor VIIai.

Greatly prolonged circulatory lifetime with high retention of function is consistent with novel behaviors of a diffusion-limited reaction. Detailed solution of the reaction mechanism at this limit can be difficult, especially for a reaction containing heterogeneous particles. In these cases, a pattern of kinetic properties may be the only practical basis on which to suggest this reaction type (*12*). Liposomes of reconstituted TF are heterogeneous with respect to both size and number of TF molecules per particle, and the following description is presented as to illustrate that the outcomes for PEG-modified factor VIIai can fit the expectations for a diffusion-limited reaction.

The association rate constant (k_f, eq 3) can be divided into two steps (*13*, *14*). The first (k_+) is particle diffusion to form an encounter complex, and the second (k_1) is chemical binding (eq 3).



The impact of PEG on reactions that are limited by k_+ (eq 3) will equal its impact on diffusion. The diffusion coefficient of the modified protein can be estimated from available information regarding PEG diffusion coefficients and shows a correlation with functional loss of factor VIIai. The diffusion coefficient of a flexible polymer such as PEG is proportional to $MW^{-0.5}$ (*15*), so the known diffusion coefficient for PEG-12 500 (6.5×10^{-7} cm^2/s, ref *16*) predicts a value of 3.6×10^{-7} M^{-1} s^{-1} cm^2/s for PEG-40 000. Addition of this to free VIIai (diffusion coefficient of about 7×10^{-7} cm^2/s) should change protein diffusion by 2−3-fold, similar to functional loss by QE-VIIai(PEG-40 000) (Figure 1B).

While Xai(PEG) and IXai(PEG) had little activity in the assay used in this study, it is possible that they will show diffusion limitation in vivo and that their net functions will be enhanced by PEG modification. This is possible because reactions approach the collision-limited state as particle size and number of receptors per particle increase (*17*). Since the in vivo state may include particles larger than vesicles and may have a larger number of receptors than in vitro assays, factors IXai(PEG) and Xai(PEG) may function at the diffusion limit and retain high activity in vivo. Conversely, diffusion-limited behavior of factor VIIai-PEG may not extend to the in vivo condition where membranes may have a low affinity for VIIai, and TF receptors may be very widely spaced. Only a true physiological experiment can determine the outcome.

While additional experiments in vivo will be needed, the practical implications of a favorable PEG impact can be substantial. Circulation halflife of a protein (Y) is related to body mass (W) by $Y = aW^b$ (*18*) where a is a constant for each protein. For factor VIII, the value of b was 0.18. Consequently, protein turnover in a 60-kg human should be 4.2 times longer than in a 20-g mouse. In approximate agreement, factor VIII showed a circulation halftime of 19.7 h in the human (*4*) versus 4.1 h in the mouse (*18*). Related dependence on body mass was found for factor IX in the rat (5 h), dog (13.3 h) (*19*), and human (22.6 ± 8 h, ref *20*). Extending this relationship to WT-VIIai(PEG-40 000), a 28-h halftime in the mouse suggests a circulation halftime of about 5 days in the human. In some cases, this may exceed the desired anticoagulation time, and shorter PEG polymers may be preferred.

Another practical feature of the PEG-factor VIIai modification was the facile synthesis of homogeneous VIIai(PEG) derivatives with quantitative yield from factor VIIa. In forming the PEG-linked active site-directed inhibitor, the reactive groups of excess PEG reagent are destroyed by spontaneous hydrolysis in water, and excess peptide inhibitor was removed by dialysis. The resulting solution reacted only with the active site of the protease.

Combination of PEG with a high membrane affinity mutant of factor VIIa, QE-VIIai(PEG-40 000), resulted in a protein with an 8-fold higher activity than WT-VIIai and with an 18-fold longer circulation lifetime for a total improvement over WT-VIIai of more than 140-fold. Overall, multiple improvements of factor VIIai may produce efficient reagents that can be widely used in research and therapy.

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