Molecular orientation of Factor VIIIa on the phospholipid membrane surface determined by fluorescence resonance energy transfer

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INTRODUCTION

F (Factor) VIII is a plasma protein that is decreased or defective in individuals with haemophilia A. FVIII circulates as a heterodimer consisting of a HC (heavy chain) comprised of A1, A2 and B domains and a LC (light chain) comprised of A3, C1 and C2 domains (see [1] for a review). FVIII is activated following limited proteolysis catalysed by thrombin or FXa to yield FVIIIa, a heterotrimer comprised of subunits designated A1, A2 and A3C1C2, that functions as a cofactor for FIXa in the membrane-dependent activation of FX to FXa (see [1] for a review).

The intermediate resolution X-ray structures of FVIII [2,3] showed that the C1 and C2 domains are aligned such that both domains may interact with the PLV (phospholipid membrane vesicle) surface and with close contact between the A1 and C2 domains. Binding of FVIIIa to PLVs is essential for cofactor function and maximal FXase activity [4]. This binding requires negative charge provided from stereospecific phosphatidyl-L-serine [4,5] and the presence of both C1 and C2 domains for optimal interaction [6].

Membrane-binding regions have been identified in the C2 domain and include Leu2251–Leu2252, Met2199–Phe2200, Lys2227 and Trp2063–Trp2064 [15,16], which correspond to the FVIII sites Lys2092–Phe2093, Arg2159–Arg2163 and Met2199–Phe2200 respectively. Most of these PLV-binding residues are located at spikes near the tips of the C1 and C2 domains, except for Trp2110–His2115, which is on a flanking side of the C2 domain [10].

In the present study we utilized a donor fluorophore labelling a single free thiol on a FVIIIa subunit and an acceptor fluorophore distributed on the PLV. Those donor sites included Cys310, Cys692 or Cys2000 in each FVIII A domain. In addition, Cys1828 and Cys1872 in the A3 domain were utilized following preparation of the double mutants (C2000S/D1828C and C2000S/T1872C). FVIIIa was reconstituted from purified subunits (A1, A2 and A3C1C2) containing a single labelled subunit plus the unlabelled complement. Results from distance calculations using multiple point measurements as determined by FRET (fluorescence resonance energy transfer) identify the molecular orientation of FVIIIa relative to the PLV surface.

EXPERIMENTAL

Materials

Recombinant FVIII (Kogenate™) was a gift from Dr Lisa Regan (Bayer Corporation, Berkeley, CA, U.S.A.). Dioleoyl phospholipids [PC (phosphatidylcholine), PE (phosphatidylethanolamine) and PS (phosphatidylserine)] were purchased from Avanti Polar Lipids. OR (octadecylrhodamine), PyMPO maleimide {1-(2-maleimidylethyl)-4-[5-(4-methoxyphenyl)-oxazol-2-yl]pyridinum methanesulfonate} and fluorescein-5-maleimide were purchased from Life Technologies. The reagents α-thrombin, FIXa, FX and Fxa (Enzyme Research Laboratories), hirudin (DiaPharma), and the chromogenic

Abbreviations used: F, Factor; FRET, fluorescence resonance energy transfer; LC, light chain; OR, octadecylrhodamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLV, phospholipid membrane vesicle; PS, phosphatidylserine; PyMPO maleimide, 1-(2-maleimidylethyl)-4-[5-(4-methoxyphenyl)-oxazol-2-yl]pyridinum methanesulfonate; WT, wild-type.

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Xa substrate, Pefachrome Xa (Pefa-5523, CH₃COO-D-CHA-Gly-Arg-pNA·AcOH; Centerchem) were purchased from the indicated vendors.

Construction and expression of FVIII WT (wild-type) and variants

FVIII variants with the point mutations R1719D and R1721D, and the double mutants C2000S/D1828C and C2000S/T1872C were prepared as B-domainless FVIII (lacking residues Gln924–Ser926 in the B domain) [17] using methods described previously [18]. Protein yields for the variants ranged from >10 to ~100 μg from 0.75 cm² culture flasks, with purity >90 % as judged by SDS/PAGE. The primary contaminant in the FVIII preparations was albumin. FVIII concentrations were measured using an ELISA and FVIII activity was determined by one-stage clotting and two-stage chromogenic FXa generation assays described below.

FVIIa subunit purification

A1, A2 and A3C1C2 subunits were purified from Kogenate™ FVIII following activation by thrombin as described previously [19]. A3C1C2 subunits from C2000S/D1828C and C2000S/T1872C FVIII were purified according to the following method. Each FVIII variant (1–3 μM) was reacted with thrombin (20 μM) in 20 mM Hepes, pH 7.2, 0.1 M NaCl and 0.01 % Tween 20 (buffer A) for 30 min followed by an additional 30 min incubation with 20 nM thrombin and the reaction sample was treated with 50 mM EDTA overnight at 4 °C. After a 1:4 dilution with buffer A, the samples were loaded on to a heparin–Sepharose column (1.5 cm × 0.7 cm in diameter, GE Healthcare). After washing with buffer A the bound material (A2 and A3C1C2 subunits) was eluted by 20 mM Hepes, pH 7.2, 0.8 M NaCl and 0.01 % Tween 20 (buffer B). This fraction was then applied to a column of R8B12 antibody (GMA8012; Green Mountain Antibodies) conjugated to Affigel-10 (Bio-Rad Laboratories) by overnight incubation at 4 °C to adsorb the A2 subunit. The unbound fraction was diluted (1:4) with 40 mM Mes, pH 6.0, and 0.01 % Tween 20, and the A3C1C2 subunit was further purified with a Mono S column (GE Healthcare) using a BioLogic DuoFlow system (Bio-Rad Laboratories) employing a 0–0.8 M NaCl gradient at a flow rate of 1 ml/min. The final A3C1C2 product was >95 % pure as judged by SDS/PAGE.

ELISA

A sandwich ELISA was performed as described previously [20] using purified commercial recombinant FVIII (Kogenate™, Bayer Corporation) as a standard. FVIII capture used the anti-C2 monoclonal antibody (GMA8003, Green Mountain Antibodies) and the anti-A2 monoclonal antibody R8B12 was employed for FVIII detection following biotinylation.

One-stage clotting assay

One-stage clotting assays were performed using substrate plasma chemically depleted of FVIII [21] and assayed using a Diagnostica Stago clotting instrument. Plasma was incubated with the APTT (activated partial thromboplastin time) reagent (Trinity Biotech U.S.A.) for 6 min at 37 °C, after which a dilution of FVIII was added to the cuvette. After 1 min the mixture was recalculated, and clotting time was determined and compared with a pooled normal plasma standard.

Two-stage chromogenic FXa generation assay

The rate of conversion of FX into FXa was monitored in a purified system [22] according to methods described previously [23,24]. The specific activity of each FVIII variant was assessed under conditions where FXa was saturated by FVIIIa. FVIII (40 nM), in 20 mM Hepes, pH 7.2, 0.1 M NaCl, 0.01 % Tween 20, 0.01 % BSA and 5 mM CaCl₂ (Hepes buffer) containing 20 μM PS/PC/PE vesicles, was activated with 20 nM α-thrombin for 1 min. The reaction was stopped by adding hirudin (10 units/ml) and the resulting FVIIIa was reacted with FIXa (0.3 nM) for 1 min. FX (300 nM) was added to initiate reactions which were quenched after 1 min by the addition of 50 mM EDTA. The FXa generated was determined following a reaction with the chromogenic substrate Pefachrome Xa (0.46 mM final concentration). All reactions were run at 23 °C.

PLV preparation

PLVs (large unilamellar vesicles) containing 20 % PC, 50 % PE and 30 % PS (or 100 % PC) were prepared using octylglucoside as described previously [25] and phospholipid concentration was determined with an inorganic phosphorus assay [26]. Several PLV materials containing various concentrations of OR were prepared by mixing 10 mg of PC/PE/PS and 0.1–1.2 mg of OR in 1 ml of chloroform and processed as described previously [27]. Phospholipid concentration was determined using the method described in [25] and OR concentration was determined by absorbance at 564 nm (molar extinction coefficient = 95400 M⁻¹·cm⁻¹). The number of OR molecules per unit phospholipid area (σ) was estimated to be 1.25 × 10⁻⁴·1.76 × 10⁻¹² OR molecules/Å² (1 Å = 0.1 nm) on the basis of the criterion that each phospholipid occupies an area of 70 Å² [27].

Fluorophore labelling of FVIII and FVIIa subunits

FVIII (WT, R1719D and R1721D), A1 or A3C1C2 (WT, C2000S/D1828C or C2000S/T1872C) subunits were labelled with PyMPO maleimide (excitation maximum/emission maximum = 417 nm/550 nm) as described previously [11,23] or fluorescein-5-maleimide (excitation maximum/emission maximum = 495 nm/520 nm) using a 20-fold molar excess of PyMPO maleimide or fluorescein-5-maleimide over FVIII (subunit) and incubated for 1 h at 23 °C. Donor fluorophore-PyMPO or fluorescein labelled A2 was purified from labelled WT FVIII as described previously [19]. Labelling efficiency was determined by comparing the fluorescence intensity with the value of 5 μM PyMPO maleimide or fluorescein-5-maleimide saturated with 20 μM FVIII subunit each and the efficiency values were >0.9 in all cases.

Reconstitution of FVIIa

FVIIa reconstitution using donor fluorophore-labelled A3C1C2 purified from WT, C2000S/D1828C or C2000S/T1872C plus unlabelled A1 and A2 was performed by mixing 20 or 40 nM donor fluorophore-labelled A3C1C2 with 1 μM A1 subunit at 37 °C for 2 h followed by an incubation with A2 subunit (400 nM) for 1 h at 23 °C in Hepes buffer containing 300 μM PC vesicles to prevent non-specific binding. FVIIa reconstitution using donor fluorophore-labelled A1 plus unlabelled A2 and A3C1C2 was performed by mixing 40 nM donor fluorophore-labelled A1 with 1 μM A3C1C2 subunit at 37 °C for 2 h followed by an incubation with A2 subunit (400 nM) for 1 h at 23 °C in Hepes buffer.
Factor VIII orientation on phospholipid membranes

containing 300 μM PC vesicles. FVIIIa reconstitution using donor fluorophore-labelled A2 plus unlabelled A1 and A3C1C2 was done by mixing 1 μM A1 with 2 μM A3C1C2 subunit at 37°C for 2 h followed by an incubation with donor fluorophore-labelled A2 subunit (40 nM) for 1 h at 23°C in Hepes buffer containing 300 μM PC vesicles.

FVIIIa binding to PLVs as measured by FRET

Binding of reconstituted FVIIIa or FVIII (R1719D or R1721D) to PLVs was monitored by donor (PyMPO or fluorescein) emission quenching resulting from energy transfer to the acceptor (OR) as described previously [27]. Briefly, three titrations were performed including one where labelled FVIIIa/FVIII was titrated with PLVs and without OR (sample-0), a labelled FVIIIa/FVIII was titrated with PLVs containing OR (sample-1) and an unlabelled FVIIIa/FVIII was titrated with PLVs containing OR (sample-2). After the addition of PLVs, samples were incubated for 10 min prior to determining fluorescence using an Amino-Bowman Series 2 Luminescence Spectrometer (Thermo Spectronic). Wavelength values for PyMPO were 417 nm (excitation, bandwidth: 4 nm) and 540–546 nm (scanned emission, bandwidth 8 nm) and values for fluorescein were 495 nm (excitation, bandwidth: 4 nm) and 523 nm (emission, bandwidth 8 nm). After background fluorescence correction, the actual fluorescence after quenching by OR was calculated by subtracting the sample-2 fluorescence (F2) from the sample-1 fluorescence (F1). Relative fluorescence (F/F0), which is the ratio of F to control sample-0 fluorescence (F0), was plotted against phospholipid concentration.

Calculation of energy transfer parameters

Energy transfer parameters as originally described by Marsh and Lowey [28] were calculated according to previously described methods [29]. Transfer efficiency was determined using the equation:

\[
R_0^6 = (8.79 \times 10^{-5})k^2n^4Q_DJ_{DA}
\]

where \(R_0^6\) is the distance at which the efficiency of transfer is 50%, \(Q_D\) is the quantum yield of the donor fluorophore (PyMPO or fluorescein) in the absence of acceptor fluorophore (OR) that was determined using a quantum yield of quinine sulfate (0.7) in 0.1 M H2SO4 as a standard. In the case of fluorescein, a quantum yield value of disodium fluorescein (0.925) in 0.1 M NaOH [30] was used as a standard. The parameter \(k^2\) is a geometric factor (2/3), \(n\) is the refractive index of the medium (1.33) and \(J_{DA}\) is the spectral overlap in M⁻¹ cm⁻¹ nm⁴ calculated by:

\[
J_{DA} = \frac{\int_0^\infty F(\lambda)e_\lambda(\lambda)\lambda^4d\lambda}{\int_0^\infty F(\lambda)d\lambda}
\]

where \(F(\lambda)\) is the corrected net emission intensity at a given wavelength and \(e_\lambda(\lambda)\) is the molar extinction coefficient of OR at the wavelength in the phospholipid vesicle in the presence of excess unlabelled FVIII.

The values of \(Q_{06}/Q_0\), the ratio of donor quantum yield in the sample with OR-labelled and unlabelled PLVs and given by the ratio of the corrected fluorescence intensities of the samples with OR-labelled and unlabelled PLVs (F/F0) under the conditions of an excess concentration of PLVs (60 μM), were determined for several OR/PLV preparations with various \(\sigma\) values. Using these sets of data, the distance of closest approach (L) between the plane of donor fluorophore and the plane of acceptor fluorophore at the outer surface of the phospholipid bilayer was obtained by non-linear least-squares regression by numerically integrating the following equation [31,32]:

\[
\frac{Q_{06}}{Q_0} = \left(1/t_0\right) \int_0^\infty e^{-t/(t_0)} e^{-\sigma S(t)\lambda}dt
\]

with

\[
S(t) = \int_0^\infty \left[1 - e^{-\frac{t}{t_0}}\left(\frac{R_d}{R}\right)^6\right] 2\pi RdR
\]

where \(t_0\) is the fluorescence lifetime of the donor, \(t\) is the time and \(R\) is the distance between the donor and an acceptor.

Calculation of matching planes from distance data

We initially calculated the possible location of the transition dipole of the donor fluorophore (PyMPO or fluorescein). On the basis of the chemical structures, points that were 10–20 Å (PyMPO) or 5–15 Å (fluorescein) away from the sulfur atom of each cysteine residue were selected at 1 Å increments. Among the calculated points, any that would cause a steric clash with any FVIII atoms were eliminated (assuming the centre of the transitional dipole of donor fluorophores needed to be at least 3 Å from any FVIII atom). In the case of the D1828C and T1872C mutants, the minimum energy rotamer co-ordinates for the cysteine mutations were calculated using the Swiss-PDBViewer (DeepView, http://spdbv.vital-it.ch/) and the sulfur atom co-ordinates of the cysteine residues were obtained. Subsequently, all possible plane co-ordinates (\(ax + by + cz + d = 0\)) (obtained by changing polar co-ordinates of a plane normal vector with a change of \(d\) values) were screened and selected by the following criteria. When the plane satisfied the conditions for all donor fluorophore sites (PyMPO and fluorescein at amino acid residues 310, 692, 2000, 1828 and 1872), a calculated distance value between the plane and any possible location of the transition dipole would fall within the range of the distance (L).

Fluorescence anisotropy

Fluorescence anisotropy was measured using an Amino-Bowman Series 2 Luminescence Spectrometer in L-format. A sample FVIIIa preparation was made by mixing 200 nM fluorophore-labelled subunits with 1 μM non-labelled subunits (e.g. 200 nM PyMPO-labelled A3C1C2 with 1 μM A1 and 1 μM A2 subunits) in 60 μM PLVs. Wavelength values for PyMPO were 417 nm (excitation, bandwidth: 4 nm) and 543 nm (emission, bandwidth: 8 nm) and values for fluorescein were 495 nm (excitation, bandwidth: 4 nm) and 523 nm (emission, bandwidth 8 nm). The fluorescence values for all polarizer positions were corrected by subtracting blank values and anisotropy values were calculated as an average of six measurements.

Data analysis

FVIII–phospholipid binding kinetics used the following equation:

\[
\frac{F}{F_0} = 1 - \frac{Q_{06}}{Q_0} \cdot \frac{(A + K_d + \frac{X}{A})^2}{2} \cdot \sqrt{(A + K_d + \frac{X}{A})^2 - \frac{4AX}{\sigma}}
\]

where \(F/F_0\) is relative fluorescence, \(A\) is the concentration of FVIII (25 nM), \(X\) is the concentration of phospholipid vesicles,
the fluorescence intensity of the sample titrated with unlabelled phospholipid. $F$ is the corrected (fluorescein, 495 nm excitation) was monitored as described in the Experimental section. $F_0$ is PLV containing OR and emission at 540–546 nm (PyMPO, 417 nm excitation) or 520–526 ratio (phospholipid/FVIII) and acceptor density was 4.7 fluorescein was chosen for use with the mutants because of improved fluorescence sensitivity. The binding curves yielded hyperbolic patterns that were saturable. The estimated $K_d$ values, 3.4 ± 2.9, 5.0 ± 3.8 and 4.9 ± 1.6 nM respectively, were equivalent to the value (3.2 ± 0.5 nM) reported previously using WT FVIII [11]. These results indicated that neither modification of the A3C1C2 subunit with the fluorophore nor the above mutations and subsequent reconstitution into FVIIIa appreciably affected the affinity of the FVIIIa forms for the membrane. These data also indicated that FVIIIa was nearly saturated at PL > 20 μM. Thus subsequent experiments to determine maximum energy transfer efficiency for distance measurements used 60 μM PL.

### RESULTS

### Binding of reconstituted FVIIIa to PLVs as determined by FRET

Isolated subunits of FVIIIa labelled at single sites with donor fluorophores were incubated with excess concentrations of the complementary unlabelled subunits to reconstitute FVIIIa. We have previously utilized Cys310 in A1, Cys692 in A2 and Cys2000 in A3 for fluorophore labelling (see [23]), inasmuch as these residues represent the only free cysteine residues in each of the three A domains. Thus modification of these free thiol groups is facile, results in minimal change to the FVIII structure and has little if any effect on biological activity. Two additional A3 domain residues, Asp1828 and Thr1872, were chosen by their location to help distinguish between an upright against a tilted orientation of FVIII relative to the phospholipid membrane. These residues were mutated to cysteine in combination with masking of Cys2000 following its mutation to serine, thereby yielding a single site for modification.

The above approach yielded a series of uniquely labelled FVIIIa molecules to utilize in FRET studies between a donor fluorophore molecule at a single site in the protein and OR molecules distributed on the PLV. Figure 1 shows binding titration results obtained for FVIIIa containing PyMPO–A3C1C2 (WT), fluorescein–A3C1C2 (C2000S/D1828C) and fluorescein–A3C1C2 (C2000S/T1872C) with OR–PLV as detected by FRET. Fluorescein was chosen for use with the mutants because of

**Table 1 Specific activity of FVIII mutants**

<table>
<thead>
<tr>
<th>FVIII variant</th>
<th>One-stage clotting assay (unit/μg)</th>
<th>Two-stage FXa generation assay (nm FIXa generated/min per nM FIXa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.50 ± 0.60 (1.00)</td>
<td>190.7 ± 7.9 (1.00)</td>
</tr>
<tr>
<td>C2000S/D1828C</td>
<td>2.49 ± 0.9 (0.55)</td>
<td>144.2 ± 2.5 (0.73)</td>
</tr>
<tr>
<td>C2000S/T1872C</td>
<td>2.06 ± 0.06 (0.46)</td>
<td>149.8 ± 1.1 (0.76)</td>
</tr>
<tr>
<td>R1719D</td>
<td>3.76 ± 0.42 (0.84)</td>
<td>125.9 ± 1.9 (0.64)</td>
</tr>
<tr>
<td>R1721D</td>
<td>3.15 ± 0.24 (0.70)</td>
<td>99.7 ± 0.2 (0.51)</td>
</tr>
</tbody>
</table>

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$K_d$ is a dissociation constant, $n$ is the binding stoichiometry ratio (phospholipid/FVIII) and $Q_{max}$ is the maximum quenching value. The value of $n$ (100) was estimated as described previously [11]. Computation for non-linear least-squares regression analysis was performed using a standard curve-fitting algorithm (Gauss–Newton algorithm with Levenberg–Marquardt method).

**Distance measurements between donor (PyMPO or fluorescein) and acceptor (OR) fluorophores by FRET**

Reconstituted FVIIIa molecules with PyMPO (or fluorescein) bound to a single cysteine residue at position 310, 692, 2000, 1828 or 1872 were subjected to FRET experiments to obtain $F/F_0$ where FVIIIa was saturated with PLVs (60 μM PL). Asp1828 and Thr1872 in the A3 domain were selected for additional donor fluorophore-labelling sites because these residues are located on the surface of A3 and away from the free cysteine residues (Cys2000). To obtain the A3C1C2 subunit with a single free cysteine residue (at either Asp1828 or Thr1872), Cys2000 in WT A3 domain was mutated to serine. Both FVIII variants with the double mutations (C2000S/D1828C and C2000S/T1872C) were expressed with minimal effects on cofactor activity (46–76 % activity compared with WT FVIII by one-stage and two-stage assay, Table 1). These levels of FVIII activity are within the range for a normal FVIII phenotype. FRET experiments were performed using FVIIIa containing a donor fluorophore in the A3C1C2 subunit at positions 2000, 1828 and 1872, the donor fluorophore A1 subunit at position 310, and donor fluorophore in A2 subunit at position 692.

Energy transfer parameter values (quantum yield, spectral overlap between donor fluorophore emission and OR absorption for all fluorophore sites) are listed in Table 2. Table 2 also includes the subsequently calculated $R_0$ values as well as anisotropy values. The geometric factor ($k^2$) used for the calculation of $R_0$ was typically assumed to be 2/3, which reflects the freedom of rotation of donor and acceptor fluorophores. However, the anisotropy values for PyMPO were relatively high and this resulted in an increase in uncertainty of the $k^2$ value. However, because of the significantly low anisotropy (0.056) for the acceptor fluorophore (OR) it appeared reasonable to set the range of $R_0$ values as ± 10 % [29,33]. Each $L$ value was determined after curve-fitting using data with various acceptor density values ($σR_0^2$) and this is illustrated in Figure 2. It is unclear why the deviation from

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Table 2  Energy transfer parameters and the distance (L) of closest approach

FRET analysis using FVIII reconstituted from a PyMPO (or fluorescein (F))-labelled subunit combined with an excess concentration of unlabelled subunits and OR-labelled PLV was performed and the distance of closest approach values (L) was calculated by non-linear least-square regression as described in the Experimental section. The results are the mean of three separate determinations. 1A28, C2000S/D1828C, 1B72, C2000S/T1872C, N/A, not applicable.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Quantum yield</th>
<th>Spectral overlap</th>
<th>Anisotropy</th>
<th>R0 (Å)</th>
<th>L (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyMPO-A1</td>
<td>0.064</td>
<td>1.23 × 10^15</td>
<td>0.34</td>
<td>33.7</td>
<td>33.1–44.9</td>
</tr>
<tr>
<td>PyMPO-A2</td>
<td>0.048</td>
<td>1.22 × 10^15</td>
<td>0.34</td>
<td>32.1</td>
<td>29.7–40.4</td>
</tr>
<tr>
<td>PyMPO-A3C1C2</td>
<td>0.056</td>
<td>1.26 × 10^15</td>
<td>0.33</td>
<td>33.2</td>
<td>16.7–27.5</td>
</tr>
<tr>
<td>PyMPO-1015</td>
<td>0.058</td>
<td>1.20 × 10^15</td>
<td>0.32</td>
<td>33.1</td>
<td>54.0–68.0</td>
</tr>
<tr>
<td>PyMPO-1015</td>
<td>0.053</td>
<td>1.20 × 10^15</td>
<td>0.33</td>
<td>32.5</td>
<td>27.2–37.6</td>
</tr>
<tr>
<td>II-1A</td>
<td>0.22</td>
<td>1.20 × 10^15</td>
<td>0.22</td>
<td>41.3</td>
<td>43.9–57.0</td>
</tr>
<tr>
<td>II-2A</td>
<td>0.24</td>
<td>1.13 × 10^15</td>
<td>0.18</td>
<td>41.5</td>
<td>34.6–47.6</td>
</tr>
<tr>
<td>II-A3C1C2</td>
<td>0.29</td>
<td>1.19 × 10^15</td>
<td>0.19</td>
<td>43.1</td>
<td>33.7–47.0</td>
</tr>
<tr>
<td>II-1015</td>
<td>0.25</td>
<td>1.13 × 10^15</td>
<td>0.19</td>
<td>41.7</td>
<td>56.4–73.5</td>
</tr>
<tr>
<td>II-1015</td>
<td>0.25</td>
<td>1.14 × 10^15</td>
<td>0.19</td>
<td>41.9</td>
<td>39.0–53.1</td>
</tr>
<tr>
<td>OR-PLV</td>
<td>N/A</td>
<td>N/A</td>
<td>0.056</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The fitted curve was more prominent at closer distances between donor and acceptor, and at higher OR density. However, since the correlation coefficient values for all fitted data were >0.94 and S.D. values were <10%, this deviation (if any) would be minor and should not affect final distant range values. The range of each L value was similarly determined by using 10% reduced R0 values or 10% increased R0 values and is listed in Table 2. The S.D. values for curve-fitting were <10% in all cases. Although there were some variations in quantum yield among donor fluorophore sites (up to a ~32% difference), the calculated R0 values varied only by ~5%. The L values tended to be a little higher with the fluorescein-labelled group compared with the PyMPO-labelled group, probably reflecting the difference in status of the transition dipole (location and degree of rotational freedom).

FVIIIa molecular orientation on a PLV surface

On the basis of the above distance measurements, all possible planes representing a PLV surface orientation which satisfied the distance criteria (L values) for all point data were initially selected. In order to further exclude PLV planes with unlikely orientation, we introduced the condition where both C1 and C2 domains contribute to PLV binding [7–10,12,13]. Following initial screening, PLV planes that were orientated near-orthogonal (90 ± 10°) to the FVIII axis plane were selected (Figure 3). Nearly 9000 possible PLV planes were finally selected and the results are shown in Figure 4. Figure 4(A) shows the distribution of the tilt angle values of the PLV planes against the FVIII axis (a line including the Cα atom of Pro505 and the N atom of Asn2172) shown in a side view of FVIII (Figure 4B). The minimum and maximum angles determined were 31.3° and 48.6° respectively. The intersecting lines of these PLV planes with the FVIII axis plane are shown as red and blue lines respectively (Figure 4B). Thus the possible PLV membrane surface exists within this tilt range relative to the FVIII axis. From this group of selected PLV planes, 94.3% fell within the angle value range of 34–46°, whereas 76.5% fell within the angle value range of 36–42°. A representative PLV membrane plane obtained with an angle value of 38.5° is shown as a PLV membrane surface depicted by white spheres. This orientation shows a reduced distance between the three A domains and the PLV surface, and juxtaposes the A3 domain in close proximity to the surface. Thus residues contained within the latter domain may form direct contacts with the PLV.

Binding of FVIII A3 mutants to PLVs by FRET

The above orientation of FVIII on PLVs revealed that several regions in the A3 domain appeared to be in contact with PLVs. Figure 5 shows the electrostatic potential map of FVIII. Viewing FVIII from the face contacting the PLV surface (bottom view) reveals an area in the A3 domain with significant positive charge (circled). This area is composed of Ser1721–Gly1725 and Arg1690–Pro1692 [3]. Since the positive charges in this region are derived from Arg1719, Arg1721 and Arg1690, we tested the possibility that...
In the present study we present experimental evidence for the orientation of FVIIIa in relation to the PLV surface. Distance measurements obtained by FRET used PyMPO or fluorescein as a donor fluorophore to label existing or created cysteine residues in each isolated FVIIIa subunit. Labelled subunits were subsequently reconstituted with the unlabelled, complementary subunits to form the FVIIIa cofactor. Cysteine residues used for fluorophore modification included Cys310, Cys692, and Cys2000 in the A1, A2, and A3 domains respectively. In addition, FVIII variants with the double mutation of C2000S/D1828C and C2000S/T1872C were generated to utilize Cys1828 and Cys1872 in the A3 domain for FRET.

DISCUSSION

In the present study we describe in the present study is in good agreement with the orientation of FVIII on PLVs previously proposed by Ngo et al. [3] in a model docking FVIII to FxVa on the PLV surface. In that model, the authors performed FVIIIa–FxFa docking calculations.
Figure 4  FVIII tilt angle distribution and orientation of the membrane surface

(A) Histogram of FVIII tilt angle value distribution of selected planes relative to the PLV membrane surface. On the basis of the data from the distance of closest approach (L) for each cysteine residue location, possible planes were selected as described in the Experimental section and the distribution of angle values between selected planes and FVIII axis were calculated. (B) FVIII orientation on the PLV surface. FVIII domain surfaces based on the X-ray crystal structure are calculated and drawn by Swiss-PDBViewer (probe size $= 1.4\, \text{Å}$) as coloured by yellow (A1), blue (A2), red (A3), green (C1) and grey (C2). The intersecting lines of FVIII axis plane with a PLV plane that forms a minimum angle (31.3°) and a PLV plane that forms a maximum angle (48.6°) are shown as red and blue broken lines respectively. An example of a PLV membrane plane obtained with an angle value of 38.5° is shown as white spheres ($0.225x + 0.974y + 0.035z + 6.60 = 0$ in Cartesian co-ordinates in FVIII structure file) [3]. Sulfur atoms for each cysteine residue are depicted as yellow spheres.

on the basis of several known interactive sites in the two proteins. This information was used in conjunction with earlier FRET results [29] that used a donor fluorophore labelled in the active site of FIXa and acceptor fluorophores distributed on the PLV to determine that the FIXa active site was located 75–80 Å above the membrane surface. The resultant model of the membrane-bound FVIII–FIXa complex yielded a tilt to the FVIII protein, which positions the A3 domain close to the membrane surface. Although the co-ordinates of the membrane are not listed in that report, the tilt angle seems to fall in the range of the angle we obtained (31.3–48.6°).

The orientation of FVIIIa on the membrane surface we propose is supported by known PLV-interactive sites in the protein and, importantly, suggests other potential interactive sites. Rigorous structural information on the FVIII–PLV interaction is limited. A previous study [10] reported a high-resolution X-ray crystal structure for a small-molecule inhibitor of the FVIII–PLV interaction bound to the C2 domain. These authors showed the inhibitor bound to residues Trp2313–His2315, which is localized to the PLV-interactive surface in the model described in the present study. Furthermore, we note that there is a relatively large area rich in basic residues contained in the A3 domain that is likely to be in contact with the PLV surface (see Figure 5, bottom view). One of these regions, Ser1713–Gly1725, contains residues Arg1719 and Arg1721 that are not defined in the intermediate resolution X-ray structures. However, we show that charge reversal point mutations (arginine to aspartate) at these residues resulted in marked increases in $K_d$ values consistent with an electrostatic interaction of these basic residues with the polar head groups of PS. Interestingly, FIXa and FXa inefficiently cleave the FVIII LC at Arg1719 or Arg1721 respectively [34,35] as compared with other cleavage sites in the cofactor, e.g. Arg336. One reason for this slow cleavage rate may reflect partial masking of the site(s) by PLVs, which is a necessary component for cleavage of FVIII substrates by these proteases. In addition, regions defined by Arg353–Gln355 in the C1 domain and Gln276–Lys281 in the C2 domain show that high density...
Figure 5  Electrostatic potential distribution on the FVIII surface (probe size = 1.4 Å)

Electrostatic potential was calculated by the Swiss-PDBViewer with a simple coulomb interaction mode using a uniform dielectric constant (80) and shown as red (negative) and blue (positive). A large acidic region in the middle of the ‘back’ of FVIII is marked with a white circle. The area circled in yellow corresponds to a basic region composed of Arg1719, Arg1721 and Arg1900. Several residues on A3, C1 and C2 subunits are drawn in stick representation.

Figure 6  Binding of PyMPO–FVIII mutants to PLV detected by FRET

PyMPO-labelled R1719D FVIII (40 nM; □) and R1719D FVIII (40 nM; ▄) in Hepes buffer containing 300 μM PC vesicles were titrated with phospholipid vesicles containing OR and emission at 540–546 nm was monitored as described in the Experimental section. F0 is the fluorescence intensity of the sample titrated with unlabelled phospholipid. F is the corrected fluorescence intensity of the sample titrated with phospholipid vesicles containing OR. The acceptor density was 4.7 × 10⁻⁴ OR molecules/Å². Data were fitted to an equilibrium binding equation by non-linear least-squares regression as described in the Experimental section and plots were drawn.

basic as well as hydrophobic residues are located at the PLV-binding surface in FVIII (see Figure 5, bottom view). Although no experimental evidence exists for residues within these sequences as contributing to interaction with PLV, their presence warrants further investigation.

The model from the present study showing FVIII associated with a rigid flat plane representing the PLV surface does not perfectly accommodate all regions in the A3, C1 and C2 domains proposed to contact the PLV surface. Additional mechanisms such as conformational changes particularly at inter-domain interfaces (A3–C1, A1–C2 and so on), burial of a relatively large area of FVIII into the phospholipid bilayer (C1 and C2) and/or local bends in the PLV surface may resolve these discrepancies. Immersion of basic or hydrophobic groups into the lipid membrane is not uncommon [36,37]. For example, Majumder et al. [37] experimentally confirmed a deep penetration of Trp2063/Trp2064 indole moieties of FVa into the lipid bilayer (∼9 Å from the centre of lipid bilayer, total length of acyl group is ∼15 Å). Furthermore, residues selected for labelling with donor fluorophores include Cys310, Cys692 and Cys2000, which contain a free thiol for facile labelling, yet appear to be partially buried according to the FVIII structure [3]. Although the labelled FVIII proteins retained high specific activity, labelling at these sites might have some impact on the surrounding structure, possibly affecting orientation. We note that reduced quantum yield and increased anisotropy values were observed when PyMPO was used as a fluorescence donor. These characteristics are undesirable in estimating spatial separations by FRET as they lead to increased uncertainty in the R₀ and k² parameters respectively. Thus further investigations using alternate probe sites with additional fluorophores may be necessary to more rigorously determine the membrane-bound orientation.

In conclusion, the results of the present study provide experimental evidence for the molecular orientation of FVIIIa on a membrane surface. Data from multiple FRET pairings indicate a defined tilt to the protein relative to surface. Further experiments are required to refine this model and to assess new potential interactive sites suggested by the model.

AUTHOR CONTRIBUTION

Hironao Wakabayashi designed and performed the experiments, analysed the data and wrote the paper. Philip Fay contributed to the direction and design of the study, and contributed to the preparation of the paper.

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