Electron Crystallography of Human Blood Coagulation Factor VIII Bound to Phospholipid Monolayers*

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Coagulation factor VIII binds to negatively charged platelets prior to assembly with the serine protease, factor IXa, to form the factor X-activating enzyme (FXase) complex. The macromolecular organization of membrane-bound factor VIII has been studied by electron crystallography for the first time. For this purpose two-dimensional crystals of human factor VIII were grown onto phosphatidylserine-containing phospholipid monolayers, under near to physiological conditions (pH and salt concentration). Electron crystallographic analysis revealed that the factor VIII molecules were organized as monomers onto the lipid layer, with unit cell dimensions: \( a = 81.5 \text{Å}, b = 67.2 \text{Å}, \gamma = 66.5^\circ \), P1 symmetry. Based on a homology-derived molecular model of the factor VIII (FVIII) A domains, the FVIII projection structure solved at 15-Å resolution presents the A1, A2, and A3 domain heterotrimer tilted approximately 65° relative to the membrane plane. The A1 domain is projecting on top of the A3, C1, and C2 domains and with the A2 domain protruding partially between A1 and A3. This organization of factor VIII allows the factor IXa protease and epidermal growth factor-like domain binding sites (localized in the A2 and A3 domains, respectively) to be situated at the appropriate position for the binding of factor IXa. The conformation of the lipid-bound FVIII is therefore very close to that for the activated factor VIIIa predicted in the FX-ase complex.

Factor VIII (FVIII)† is an essential protein in blood coagulation (1). Deficiency in FVIII is responsible for hemophilia A (classic hemophilia), an X-chromosome-linked bleeding disorder (2). During coagulation, FVIII is proteolytically cleaved to an unstable active heterodimeric form (FVIIIa) by trace amounts of thrombin or factor Xa (FXa) (3). FVIIIa functions as a cofactor, responsible for the efficient activation of factor X (FX) by activated factor IX (FIXa) in the presence of negatively charged phospholipids (PL) and Ca\(^{2+}\) ion (4). The assembly of the membrane-bound FX-ase complex (FX/FVIIIa/FIXa) results in the release of FXa, which associates with the cofactor factor Va (FVa) and prothrombin into the membrane-bound prothrombinase complex. The released thrombin further

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1 The abbreviations used are: FVIII, factor VIII; FVIIIa, active heterodimeric form of FVIII; FX, factor X; FXa, factor Xa; FIXa, activated factor IX; FVa, factor Va; FX-ase, factor X-activating enzyme; DOPS, dioleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; PL, phospholipid; LC, light chain; HC, heavy chain; MNS, 4-morpholineethanesulfonic acid; FC, phosphatidylcholine; PS, phosphatidylserine.

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tremely difficult. Previous electron microscopy studies led to a characterization of the size and shape of FVIII molecules in solution (22, 23). A globular core of approximately 12 nm diameter with a thin rod-like protruding portion (suggested to be the B domain) up to 50 nm long, were defined. In addition, fluorescence energy transfer studies further suggested close association of HC and LC (24). Despite these initial structural studies, increasing knowledge of the genetic and molecular basis of FVIII, and more recent structure-function studies, no structural data are available on the macromolecular organization of membrane-bound FVIII (8).

In the present paper we report our success in obtaining two-dimensional crystals of human FVIII grown on a negatively charged phospholipid monolayer. Subsequent electron crystallographic analysis resulted in a two-dimensional projection structure at 15-Å resolution. The domain organization of FVIII, as suggested from our two-dimensional map, is consistent with homology-derived molecular models for the FVIII A domain heterotrimer (25) and membrane-bound C1-C2 domains (26, 27), and with a FVIIIa “docking” model for the FVIIIa/FIXa complex based on the known binding sites of FVIIIa for FIXa and expected orientation to phospholipid membrane surfaces (25, 28).

EXPERIMENTAL PROCEDURES

Purification of Human FVIII—Human FVIII (3500–7000 IU/mg) was obtained from immunoaffinity purified FVIII concentrate using an anti-HC antibody (29) and stored at 180 °C under N2. For the lipid-layer crystallization technique experiments, 500 μl of pure FVIII was buffer-exchanged into 20 mM MES, 100 mM NaCl, 2 mM CaCl2 (pH 6.0) by gel filtration on an HR10/10-G25 Sephadex superfine column (Amersham Pharmacia Biotech). To confirm the presence of FVIII as HC-LC heterodimers, high resolution gel filtration of FVIII in the presence and absence of 20 mM EDTA was performed using a Superdex 200 PC3.2/30 column mounted in a SMART system (Amersham Pharmacia Biotech).

Two-dimensional Crystallization—Stock solutions of DOPS (10 mg/ml) and DOPC (50 mg/ml) were prepared in chloroform from lyophilized DOPS and DOPC (Alexis, Laufelingen, Switzerland) and stored at −80 °C. For the lipid-layer crystallization technique experiments, 500 μl of pure DOPS, DOPS/DOPC = 1/4, and DOPS/DOPC = 4/1 in chloroform/n-hexane = 1/1 (v/v) were prepared. For some experiments egg PC and bovine brain PS were used (Avanti Polar Lipids, Alabaster, AL). 16 μl of the protein solution (40 μg/ml) was used in each crystallization trial. Crystallization experiments were conducted as described previously (30–32).

Electron Microscopy and Crystallography—Membrane-bound two-dimensional crystals of FVIII formed at the air/buffer interface were transferred onto carbon-coated grids (hexagonal 400 mesh, copper electron microscopy grids from Agar Sci, Stansted, Essex, UK), negatively stained with 1% uranyl acetate solution, and observed in a Philips CM10 transmission electron microscope operated at 100 kV. All micrographs were recorded at a calibrated magnification of × 37,300. Selected negatives were digitized with a Leascan-45 scanner (Leaf System, Inc) at a 20 μm/pixel increment and further analyzed using the crystallographic image processing software packages CRISP (33) and MRC (34), as described previously (35, 36). After lattice refinement of the best Fourier transforms, the Fourier projection maps were calculated using parts of the CCP4 crystallographic suite including the Pluto (spx) graphic facilities (Refs. 37, 38, for more information also consult Dr. J. Jaeger home page on the WWW).

Molecular Modeling—The protein density contours from the projection map of the FVIII molecule were compared with the overall shape and volume of homology-derived molecular models for the FVIII A1/A2/A3 heterotrimer (25) based on the crystal structure of human ceruloplasmin (39) and the FVIII C1 domain (25) using the molecular graphic software Insight II (MSI, Cambridge, UK). The FVIII C1 model was derived similarly to the FV C1 and C2 domain models (26) based on galactose oxidase (40) and was also used as the guide model for FVIII C2 shape and volume because the C1 and C2 domains of both FVIII and FV are predicted by homology modeling techniques to be very similar (25, 27).

RESULTS

FVIII Characterization—The immuno-purified human FVIII preparation consists of a mixture of polypeptides representing subunits of a series of metal ion-linked HC-LC heterodimers (Fig. 1). SDS-polyacrylamide gel electrophoresis analysis shows a constant size LC doublet (A3-C1-C2) of mass between 76–80 kDa, together with a variable size HC ranging from 90 kDa (A1-A2 alone) up to 230 kDa (A1-A2-B). There is probably less than 1% single chain FVIII (A1-A2-B-A3-C1-C2). A small amount of human albumin is also present in this preparation to stabilize the very low levels of FVIII.

To confirm that FVIII in crystallization experiments consisted essentially of HC-LC heterodimers with absence of free LC, FVIII was subjected to high resolution gel filtration in crystallization buffer in the presence or absence of added 20 mM EDTA (to encourage dissociation of the HC-LC heterodimer). Fig. 2 shows the results of silver-stained SDS-polyacrylamide gel electrophoresis gel electrophoresis gel analysis of column fractions. In the presence of Ca2+ (crystallization buffer) virtually all LC and HC were associated in a high molecular weight complex, eluting very early in the column profile (Fig. 2A, lanes 1–2): very little free LC was found, eluting late in the profile (probably less than 1% of the total). As expected, in the presence of EDTA, LC and HC were largely separated. Some undissociated HC-LC may be seen in the first fractions (Fig. 2B, lanes 2–3), but most free HC polypeptides elute later (Fig. 2B, lanes 4–8), roughly in order of molecular size, whereas free LC elutes much later (Fig. 2B, lanes 10–14). Isolated LC elutes even later than albumin, which may be due to a more globular folding than albumin or to nonspecific interaction with the gel-filtration matrix.

Two-dimensional Crystallization—As nonactivated FVIII shows high affinity for negatively charged PL surfaces (41), two-dimensional crystals of FVIII were grown on phosphatidylserine (PS)-containing phosphatidylcholine (PC) monolayers by the lipid layer crystallization method. In this technique, protein concentrates at the buffer/lipid monolayer interface, where specific binding to the DOPS head groups and protein-protein interactions lead to the formation of protein domains.

3 B. Villoutreix, personal communication.
and two-dimensional crystals. In excess of 200 crystallization trials were carried out with human FVIII at room temperature and incubation times varying between 6 and 36 h. 250 negatively stained electron microscopy grids were prepared for observation, and 1000 electron micrographs recorded.

The yield of protein domains formed onto the lipid monolayer increased with lowering of the DOPS content of the lipid mixture. Well defined variations in the shape and size of these domains were clearly observed when changing the lipid composition of the monolayer. With 100% DOPS, few flat membranes were observed, whereas numerous filaments and collapsed vesicular structures were found. Many of these structures presented a one-dimensional order and/or lamellar order, possibly in result of vesicular lipid bi- and multilayer formation, flattened by the staining procedure. The incubations with higher DOPC content presented large, mostly circular protein domains covering more than 80% of the grid surface. These were often heavily stained and thick, resulting from the superposition of more than one lipid monolayer and/or protein aggregates formation. However the domains, even if clearly visible, did not exhibit any tendency for two-dimensional organization on the PL surface. The same ratios were studied with egg PC and bovine brain PS mixtures. Very nice thin, flat protein domains were observed on the monolayers; however, again no two-dimensional crystal order was found.

Only the lipid monolayers formed with DOPS/DOPC = 4/1 (v/v) induced the packing of the bound FVIII molecules in two-dimensional crystals and ordered tubes (Fig. 3). The yield of the two-dimensional areas was relatively low (20%), but their crystalline order was sufficient to conduct further electron crystallographic analysis.

**FVIII Projection Structure**—Ten of the best two-dimensional areas were selected for further crystallographic analysis. The Fourier transforms and resulting projection maps of these two-dimensional crystalline areas exhibited similar features including: (i) similar unit cell dimensions, (ii) the protein density corresponding to one molecule within the unit cell presented similar shape and dimensions, (iii) a well defined density peak with two additional lower density peaks at the same positions within the projection maps were located.

By merging the six best crystalline areas on a common origin, a final Fourier projection map for the membrane-bound FVIII was calculated at 15 Å resolution, showing that the FVIII molecules are organized onto the PL surface in monomers within unit cell dimensions of a = 81.5 ± 1.6 Å, b = 67.2 ± 2.7 Å, γ = 66.5 ± 0.7° (P1 symmetry of the plane group). The surface covered by one FVIII molecule is 81 × 45 ± 3 Å² (3726 Å²), which corresponds to 75% of the unit cell surface. Lipids only and low-protein density contact regions (Fig. 4) fill the remaining 25%.

The protein density in projection of a FVIII molecule is similar to the surface covered by one FV molecule, defined by the same type of structural study (31). FV is the closest known protein to FVIII by sequence identity, and FV has the same domain composition (A1/A2/A3-C1-C2) as the FVIII heterodimer lacking the B domain. Furthermore, the main protein density peak (23 Å in diameter) corresponds exactly in size to the main density peak in the FVIIa projection structure, there assigned to the C domains (Fig. 4). The negative contours are shown by *dashed lines*. The positive contours start from 0.1σ above the mean protein density level. The unit cell dimensions are: a = 81.5 Å, b = 67.2 Å, γ = 66.5°. The most pronounced density peak is indicated by *.

Comparison between the FVIII Projection Map and Homology Models for the FVIII A and C Domains—By comparison between our projection map, the known domain organization of FVIII, and homology-derived molecular models for the A (25) and C domains, the best defined and most pronounced density peak in the projection map (23 Å in diameter) corresponds exactly in size to the main density peak in the FVIIa projection structure, there assigned to the C domains (Fig. 4).

**Fig. 2.** High resolution gel filtration of purified FVIII: SDS silver staining of column fractions following SDS-PAGE. A, in crystallization buffer (containing Ca²⁺), HC and LC are eluted as a complex. B, in the presence of EDTA, the majority HC and LC are eluted separately. Some of the nondissociated heterodimeric FVIII elutes very early (Fractions 2 and 3).

**Fig. 3.** Electron micrograph of a FVIII two-dimensional crystal. The FVIII molecules are attached to the negatively charged (DOPS-containing) DOPC monolayer and negatively stained with uranyl acetate. The inset shows the Fourier transform of the best crystalline area, size 1024 × 1024 pixels. The reciprocal lattice vectors are indicated by a* and b*.

**Fig. 4.** Two-dimensional projection map of membrane-bound FVIII. The contour levels are drawn from −0.3σ to 9σ by 0.2σ. The negative contours are shown by *dashed lines*. The positive contours start from 0.1σ above the mean protein density level. The unit cell dimensions are: a = 81.5 Å, b = 67.2 Å, γ = 66.5°. The most pronounced density peak is indicated by *.

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density peaks in the molecular contour result from the superposition of the A1 domain over the A3 and the A2 domains, respectively (Figs. 4 and 5).

Thus, fitting of the homology models for the A1, A2, A3 heterotrimer and the C1-C2 double β-barrel into the FVIII projection structure allowed an assignment of the protein densities to individual domains (Fig. 5). The only clear way to fit the heterotrimer A domain model within the molecular outline from the projection map is by tilting the model such that its pseudo 3-fold axis makes an angle of approximately 25 Å to the membrane surface, which when viewed from above (toward the membrane) presents an almost side-on orientation. In the proposed phospholipid-bound FVIII conformation, the long axis of the membrane-bound C2 domain is almost perpendicular to the membrane plane, whereas the C1 domain is more laterally positioned above the C2 domain, contacting the C terminus of A3. The A1 domain overlaps both the C1-C2 domain pairing and A3, positioned most distal to the membrane and main protein density in projection. The A2 domain is sandwiched between A1 and A3, extending away from the central mass of the molecule. A few loops protrude laterally from the molecular contour of A3 in the heterotrimeric A domain model: these would not be well defined by the negative-staining procedure, because of their low protein density, flexibility, and distance from the PL monolayer (Figs. 5 and 6).

DISCUSSION

To obtain two-dimensional crystals of a soluble protein on a lipid monolayer, high affinity of the protein toward a lipid-like ligand residing within the monolayer is necessary. In the case of FVIII, DOPS serves as the lipid ligand, as the $K_D$ of FVIII binding to a PS-containing monolayer is about 2.2 nM (41). Neither isolated HC (41) nor albumin has any specific binding capacity to the PL surface, thus their presence in the protein solution should not interfere with the two-dimensional crystallization. Further, as the concentrations of single chain FVIII and free LC are each estimated to be less than 1% (Fig. 2), the probability of obtaining two-dimensional crystals from these components is extremely low. The relatively low overall FVIII concentration (~40 μg/ml) in the crystallization experiments and the multiple glycosylation sites of the B domain are likely causes for the low yield of two-dimensional crystals, as crystallization is a co-operative phenomenon ideally requiring a large pool of identically oriented and stable molecules. Obtaining two-dimensional crystals, however, in such conditions demonstrates the aptitude of the lipid layer technique to give structural information from small numbers of molecules if there is a

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**Fig. 5.** Top view of membrane-bound FVIII. The first positive protein density contours (drawn in thick black line) correspond to the FVIII molecular contour from Fig. 4. In thinner black lines, the three defined density peaks from the projection map are also delineated. The dimensions of the FVIII molecules, determined by this contour are 81 × 45 Å. The five major FVIII domains are sketched inside this contour in the following order, starting from the farthest from the membrane: A1, red; A2, blue; A3, green; C1, orange; C2, and brown. Outside the contours, few loops can be discerned, which are not visible in the projection map, because of the preferential staining of the bulk of the molecule.

**Fig. 6.** Hypothetical models of FVIIIa/FIXa complex (a) and FVa/FXa complex (b), modified from the cartoon published previously for the FVIIa/FIXa complex (25). PL indicates the negatively charged (PS containing) phospholipid surface. *1 and *2 are the two serine protease binding sites localized in the A2 domain (42) and the EGF-like binding site localized in the A3 domain (43) of FVIII, respectively.
strong and specific binding to the lipid monolayer.

From the electron crystallographic and molecular modeling data, only the heterodimeric HC-LC form of FVIII lacking the B domain, could fit to the calculated projection structure. No protein densities could be assigned to part or all of the B domain, which represents 40% of the mass of the largest species of the protein. The possibility exists that variable lengths of the B domain are located in gaps between the main protein contours, remaining unresolved because of their flexibility and multiple glycosylation state. Alternatively, the absence of the B domain in the two-dimensional crystals could be due to the potential of only the A1-A2/A3-C1-C2 dimers to crystallize. This is in support of the view that two-dimensional crystallization is induced by the concentration of molecules with the same conformation and identically oriented at a gas/liquid, or liquid/solid interface (two-dimensional surface). In addition, it may contribute to the low yield of two-dimensional crystals, as only a small portion of FVIII dimers lack the B domain.

The larger dimensions (10 and 12 nm) previously obtained for the FVIII core (lacking the B domain), by scanning transmission electron microscopy (22) and electron microscopy of rotary shadowed FVIII molecules (23), can be ascribed to the fact that in these latter studies the FVIII molecules assumed different orientations, being further flattened when adsorbed to the carbon support film.

The most successful fitting of an assembly of homology-derived domain models of FVIII to the projection structure resulted in the A domain heterotrimer oriented with the pseudo 3-fold axis at a shallow angle to the membrane (approximately 25°). The longer dimension of the FVIII molecular contour (81.5 Å) matches the heterotrimer dimensions in the A domain homology model (81–83 Å, an agreement which lends support to the validity of this particular model). From this it can be assigned that the C1 and C2 domains are situated directly underneath the A domains. When viewed side-on (perpendicular to the pseudo 3-fold axis), the heterotrimer model has a diameter of 81–83 Å, and its overall maximum "height" is 60 Å, wider than the 45 Å dimension given by the experimental protein density. However it should be noted that, of this, only 40 Å is of high protein density, with a further 20 Å profile of a lower density, mainly isolated loops (25). As pictured in the homology model, these loops would extend into the space between the FVIII molecules in the projection map and could not fall entirely within the molecular contour. This is not surprising, as they are flexible and situated at least 60 Å above the membrane surface. In this case the uranyl acetate stain will accumulate around the membrane binding region, and only the high density part of the heterotrimer will be well outlined (Fig. 5).

In an earlier hypothetical model of the FVIIIa/FIXa complex (25) the A domain heterotrimer was oriented with the pseudo 3-fold axis parallel to the PL surface (25). We have now used the FVIII structural information derived from the projection map to construct a further FVIIIa/FIXa model, shown in a simplified schematic form in Fig. 6A. This model accommodates the following: (i) the suggested orientations of the A and C domains relative to the membrane and to each other, in accord with the projection map and placing C- and N termini closely in space where required; (ii) the known binding sites on FVIII A2 and A3 for the protease and EGF-like domains of FIXa (13, 27, 42, 43); and (iii) the experimentally determined height of the FIXa active site above the membrane surface (44), which is not affected by FVIIIa binding. The FVIII domains, as resolved in projection, are therefore arranged following a geometry consistent with biochemical (binding sites for FIXa), biophysical (height of the FIXa active site) and primary structure data (A1-A2-B-A3-C1-C2 domains sequence). A further implication of this consistency is that following activation by proteolysis, FVIIIa would not show large-scale conformational changes to enable the formation of the FVIIIa/FIXa complex, prior to interaction with FX on a membrane surface.

Previously, the projection structure of FVa by electron crystallography, has been reported (31). It was of interest therefore to compare the two-dimensional maps of FVa and FVIII (Fig. 6, A and B). Although FVa and FVIII are homologous proteins, their projection maps display some striking differences.

First, the more pronounced protein density peak (24 Å in diameter, Fig. 4, *) present in both projection structures and assigned to the C1-C2, is situated differently to the A domains. In the FVIII/FIXa model based on the FVIII projection map, the C1 and C2 domains are localized underneath the heterotrimer contour (Fig. 6A). For FVa, however, the C1 and C2 domains appear to be well defined outside the main contour of the A domains, indicating a cylindrical structure attached with a thin bridge to the A3 domain and housing the double β-barrel structure (Fig. 6B) (26, 27, 31).

Second, the surface covered by the main density of one FVa molecule is 80 × 55 Å which is distinct to that of FVIII (81 × 46 Å). This may indicate a slight difference in the tilt of the A domains heterotrimer to the membrane surface, which is consistent with a different orientation of the C1-C2 domains to the A domains in FVas and FVIII (Fig. 6, A and B).

The proposed side-on views of FVIII and FVa in the FVIIIa/ FIXa and FVas/FXa models (Fig. 6, A and B), are based on the known atomic structures of Fxa and FIXas from x-ray crystallography (45, 46) and the projection structures of FVIII and FVa from EM crystallography (31). The dimensions of the enzyme, FIXa and FXa determine the position of the enzyme-binding regions within the FVas and FVIII, respectively. The active site of FVa has been reported to be localized at 69 Å from the lipid surface (47), whereas the FIXa active site is further away (73 Å) (44). These data support the slightly different FVa and FVIII domain organization (Fig. 6, A and B). It should be also taken in account that the projection map of FVa represents an active conformation, whereas that of FVIIa represents an inactive conformation. It seems likely therefore, that activation of FVIII may result in the reorientation of the C1-C2 domains to the A domains. It has been also reported that the conformation of C2 itself may alter on FVIIa activation and removal of the LC N-terminal acidic sequence (48), resulting in somewhat higher affinity binding of FVIIIa to the PL surface compared with FVIII (41).

The projection map of FVIII also suggests an extended conformation of the membrane-bound LC, not allowing its N- and C-terminal parts to be in close proximity (Fig. 6A). It should be mentioned in this respect that both terminal ends of FVIII LC encompass the binding sites for the FVIII carrier protein, von Willebrand factor, suggesting that both ends are in close proximity. It would be tempting to speculate, therefore, that binding of FVIII LC to the membrane surface is associated with a reorientation of the terminal parts to each other.

The three-dimensional structure of FVIII at 15 Å resolution is currently under analysis and will allow a more precise fitting of the A and C domains models within the molecular contours. We are undertaking further studies to complete the information for the conformational changes during FVIII activation and formation of the FX-ase complex.
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