

Crystal Structure of Human Factor VIII: Implications for the Formation of the Factor IXa-Factor VIIIa Complex

Jacky Chi Ki Ngo,¹ Mingdong Huang,¹ David A. Roth,² Barbara C. Furie,¹ and Bruce Furie^{1,*}

¹Marine Biological Laboratory, Woods Hole, MA 02543, USA

²Wyeth Research, Cambridge, MA 02140, USA

*Correspondence: bfurie@mbl.edu

DOI 10.1016/j.str.2008.03.001

SUMMARY

Factor VIII is a procofactor that plays a critical role in blood coagulation, and is missing or defective in hemophilia A. We determined the X-ray crystal structure of B domain-deleted human factor VIII. This protein is composed of five globular domains and contains one Ca²⁺ and two Cu²⁺ ions. The three homologous A domains form a triangular heterotrimer where the A1 and A3 domains serve as the base and interact with the C2 and C1 domains, respectively. The structurally homologous C1 and C2 domains reveal membrane binding features. Based on biochemical studies, a model of the factor IXa-factor VIIIa complex was constructed by *in silico* docking. Factor IXa wraps across the side of factor VIII, and an extended interface spans the factor VIII heavy and light chains. This model provides insight into the activation of factor VIII and the interaction of factor VIIIa with factor IXa on the membrane surface.

INTRODUCTION

Factor VIII is a protein cofactor that, when activated, forms a complex with factor IXa on membrane surfaces to activate factor X during blood coagulation (Furie and Furie, 1988). This glycoprotein is encoded by a gene of 186 kb that is divided into 26 exons (Gitschier et al., 1984; Toole et al., 1984). Factor VIII is synthesized as a single polypeptide chain, including a 19 residue signal peptide. The mature factor VIII contains 2332 amino acid residues arranged within six domains organized as A1-A2-B-A3-C1-C2 (Gitschier et al., 1984; Toole et al., 1984; Figure 1A). Factor VIII circulates in the blood as a heterodimer composed of two polypeptide chains: a light chain with a molecular weight of 80,000 and a heterogeneous heavy chain with a molecular weight varying between 90,000 and 200,000, both derived from the single peptide chain. A region of the C2 domain contains a membrane binding site of factor VIII and the site of interaction with von Willebrand factor (Pratt et al., 1999). Factor VIII is inactive or minimally active as a cofactor in blood coagulation, but is converted into its active cofactor form by proteolytic cleavage. Although active factor VIII can be formed from cleavage at Arg372 and Arg1689, and these are essential for cofactor activa-

tion, fully active factor VIII is generated by three cleavage events involving Arg372, Arg740, and Arg1689 (Pittman and Kaufman, 1988).

Hemophilia A is caused by a defect in the factor VIII gene that leads to diminished or absent factor VIII activity in blood. A heterogeneous genetic disease, hemophilia A, has been associated with missense mutations, nonsense mutations, gene deletions of varying size, inversions, and splice junction mutations (Furie and Furie, 1990; Graw et al., 2005). The major treatment of the bleeding disorder associated with hemophilia A is the infusion of factor VIII, which leads to the correction of hemostasis (Mannucci and Tuddenham, 2001; Key and Negrier, 2007). The B domain of porcine factor VIII shows no sequence homology to the B domain of human factor VIII, yet porcine and human factor VIII have similar specific coagulant activities when evaluated in human plasma. Based upon this observation, engineered B domain-deleted human factor VIII was generated and shown to have full biological activity, and could be expressed in heterologous cells with improved expression efficiency relative to that of the full-length molecule (Toole et al., 1986). The structural heterogeneity of B domain-deleted factor VIII is significantly less than that for full-length factor VIII, with a heavy chain of 90,000 MW and a light chain of 80,000 MW (Figure 1A).

Electron microscopy of human factor VIII utilizing rotary shadowing revealed a globular structure of ~14 nm diameter, with satellite appendages (Fowler et al., 1990), whereas scanning transmission electron microscopic images of porcine factor VIII indicated a globular core structure of 10–12 nm, also with satellite structures, possibly assigned to the B domain (Mosesson et al., 1990). Crystallization of the human factor VIII C2 domain has revealed a domain of factor VIII at high atomic resolution (Pratt et al., 1999). Recently, the intermediate resolution X-ray crystallographic structure of a B-domainless factor VIII has been reported (Shen et al., 2008), and describes the tertiary structure and the domain organization. Other efforts to better understand the structure of factor VIII in the absence of X-ray crystallographic data have been based on homology modeling with related structures of the A domain (Pan et al., 1995; Pemberton et al., 1997), the C domains (Pellequer et al., 1998), and the structural organization of both ceruloplasmin (Zaitseva et al., 1996) and activated protein C-inactivated factor Va (Adams et al., 2004).

To better understand the critical role that factor VIII plays in the tenase complex during blood coagulation, we have determined the three-dimensional structure of B domain-deleted factor VIII

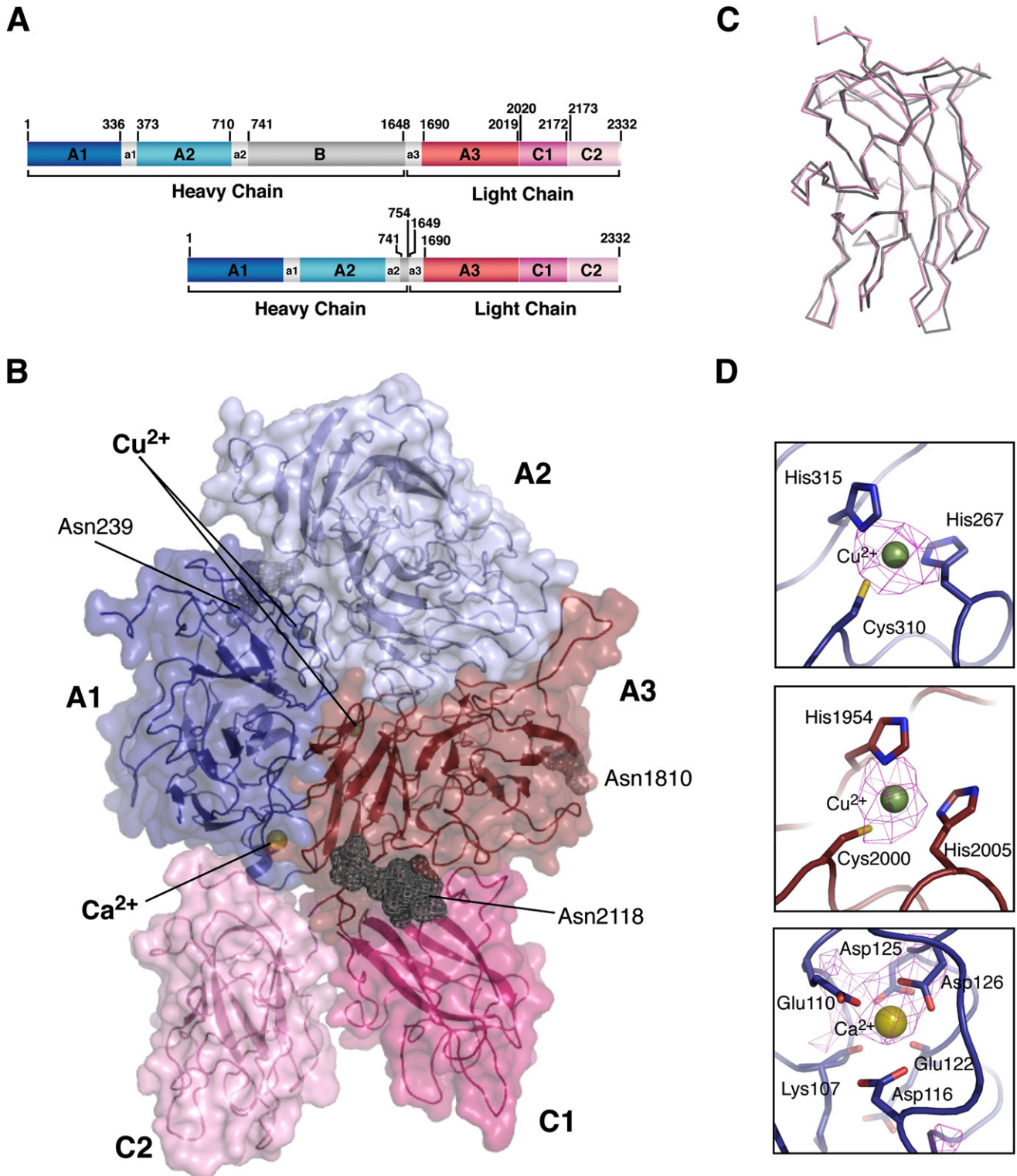


Figure 1. Crystal Structure of B Domain-Deleted Factor VIII

(A) Diagram of the domain organization of human factor VIII and the B domain-deleted factor VIII.

(B) Three-dimensional structure of B domain-deleted factor VIII. The A1 domain (residues 1–336) and the a1 acidic region (residues 337–372) are colored dark blue. The A2 domain (residues 373–710) and the a2 acidic region (residues 711–740) are colored light blue. These regions (A1, a1, A2, a2) comprise the heavy chain. The A3 domain (residues 1690–2019), the C1 domain (residues 2020–2172), and the C2 domain (residues 2173–2332) comprise the light chain and are colored red, dark pink, and light pink, respectively. The a3 acidic region is disordered and not included in this structure. The structure contains two Cu^{2+} ions (green), one Ca^{2+} ion (orange), and three carbohydrate moieties (gray).

at atomic resolution by X-ray crystallography and have generated an *in silico* model of the factor IXa-factor VIIIa complex.

RESULTS AND DISCUSSION

Structure of Factor VIII

The mature B domain-deleted human factor VIII used for crystallization contains residues 1–740 that comprise the heavy chain (A1 and A2 domains), a short peptide linker (residues 741–754) and residues 1649–2332 that comprise the light chain (A3, C1, and C2 domains; Figure 1A; Sandberg et al., 2001). The protein crystallized in the tetragonal space group P4₁2₁2 with an unusually high solvent content of 75% (Table 1). The phasing problem was solved by multistep molecular replacement by using the coordinates of the A domains of bovine factor Vai (Adams et al., 2004) and ceruloplasmin (Zaitseva et al., 1996) and the C2 domain of human factor VIII (Pratt et al., 1999) as search models. The crystallized factor VIII is a heterodimer consisting of the heavy chain (A1–A2 domains) and light chain (A3–C1–C2 domains). Several regions within the structure are poorly ordered and were not modeled, including residues 17–43, 211–223, 334–376, and 714–754 within the heavy chain, and residues 1649–1690 and 1714–1724 within the light chain.

The overall structure of factor VIII can be described as a triangular heterotrimer of the A domains stacked on two smaller globular C domains (Figure 1B). This structure closely resembles that of inactivated bovine factor Va, factor Vai (Adams et al., 2004), except that B domain-deleted factor VIII also contains the A2 domain. The A1, A2, and A3 domains each consist of two connected β barrels that resemble the fold of a typical cupredoxin-type domain (Zaitseva et al., 1996). All three A domains share high structural homology with each other (average root-mean-square deviation [rmsd] = 1.40 Å). The C1 and C2 domains are defined by a distorted β barrel and share structural homology (rmsd = 1.09 Å). The factor VIII C1 domain is homologous to the C1 domain of factor Va (rmsd = 1.04 Å), and the factor VIII C2 domain is homologous to the C2 domain of factor Va (rmsd = 0.93 Å), and is nearly identical to the factor VIII C2 domain determined at 1.5 Å resolution (rmsd = 0.73 Å; Pratt et al., 1999; Figure 1C). The final model includes 630 residues of the heavy chain, 631 residues of the light chain, two Cu²⁺ ions, one Ca²⁺, and three carbohydrate moieties. The three N-acetyl glucosamines are linked to Asn239 in the A1 domain, Asn1810 in the A3 domain, and Asn2118 in the C1 domain.

Factor VIII is a copper binding protein (Bihoreau et al., 1994), and we identified two copper ions and their binding sites internally within the A1 and the A3 domain. These are prototypic copper binding sites, with nitrogen and sulhydryl ligands (Mesterschmidt and Huber, 1990). In the A3 domain, the copper ion is liganded by His1954, Cys2000, and His2005. The copper binding site in the A1 domain is defined by His267, Cys310, and His315 (Figure 1D). These copper ions are located near, but

Table 1. Statistics on Diffraction Data and Structure Refinement of B Domain-Deleted Human Factor VIII

Crystal Data Collection	Data
X-ray source	APS code 24
Wavelength (Å)	0.97918
Resolution (Å)	∞ –3.98
Space group	P4 ₁ 2 ₁ 2
Unit cell parameters	a = b = 134.113 Å, c = 349.760 Å, $\alpha = \beta = \gamma = 90^\circ$
Redundancy (outer shell)	13.2 (8.0)
No. of unique reflections (outer shell)	27631 (2505)
Completeness (outer shell) (%)	99.0 (92.3)
I/ σ (overall/outer shell)	27.2 (1.7)
R _{sym} ^a (overall/outer shell) (%)	13.0 (82.7)
Refinement	
Resolution (Å)	50.00–3.98
R _{cryst} ^b (%)	25.56
R _{free} ^c (%)	32.69
Rmsds	
Bond lengths (Å)	0.017
Bond angles (deg)	1.741

APS = Advanced Photon Source; rmsd = root-mean-square deviation.

^a R_{sym} = $\sum |I - \langle I \rangle| / \sum I$.

^b R_{cryst} = $\sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

^c R_{free} was calculated with 5% of the data excluded from the refinement calculation.

not at, the domain interface, indicating that their role in enhancing light chain-heavy chain interaction is indirect (Wakabayashi et al., 2001). The positions of these copper ions suggest that they may be important for maintaining the structural integrity of the interdomain interface.

A single calcium binding site was located in the A1 domain. This site is defined by carboxyl groups of Glu110, Asp116, Asp126, Asp125, and the backbone carbonyl of Lys107 and Glu122 (Figure 1D). This calcium ion is bound within the A1 domain, but may play a role in the maintenance of the C2–A1 domain interface (Wakabayashi et al., 2001).

The three A domains form a triangular heterotrimer around a pseudo-three-fold symmetry axis, where A1 and A3 domains serve as the base and interact with the C2 and C1 domains, respectively (Figure 2A). The front surface of the triangular heterotrimer of the A domains is planar, whereas the back surface is dominated by three protrusions, formed by large loops from each domain, that create a deep groove at the center of the three domains (Figure 2A). The C1 and C2 domains are adjacent at the base of the triangular heterotrimer. Each C domain projects three β -hairpin loops containing hydrophobic and basic residues

(C) Overlaid polypeptide backbone of the C2 domain of B domain-deleted factor VIII (pink) and that of the 1.5 Å resolution structure of the isolated C2 domain (gray; PDB code: 1D7P; Pratt et al., 1999).

(D) Two Cu²⁺ binding sites and one Ca²⁺ binding site were identified in the B domain-deleted factor VIII. The experimental electron density maps of the metal ions are contoured at 4 σ . Upper panel: Cu²⁺ binding site in the A1 domain. Middle panel: Cu²⁺ binding site in the A3 domain; each Cu²⁺ is liganded by two histidine and one cysteine residue with a trigonal planar coordination geometry. Lower panel: a Ca²⁺ ion is liganded by carboxyl groups of aspartate and glutamate residues as well as two backbone carbonyl oxygens.

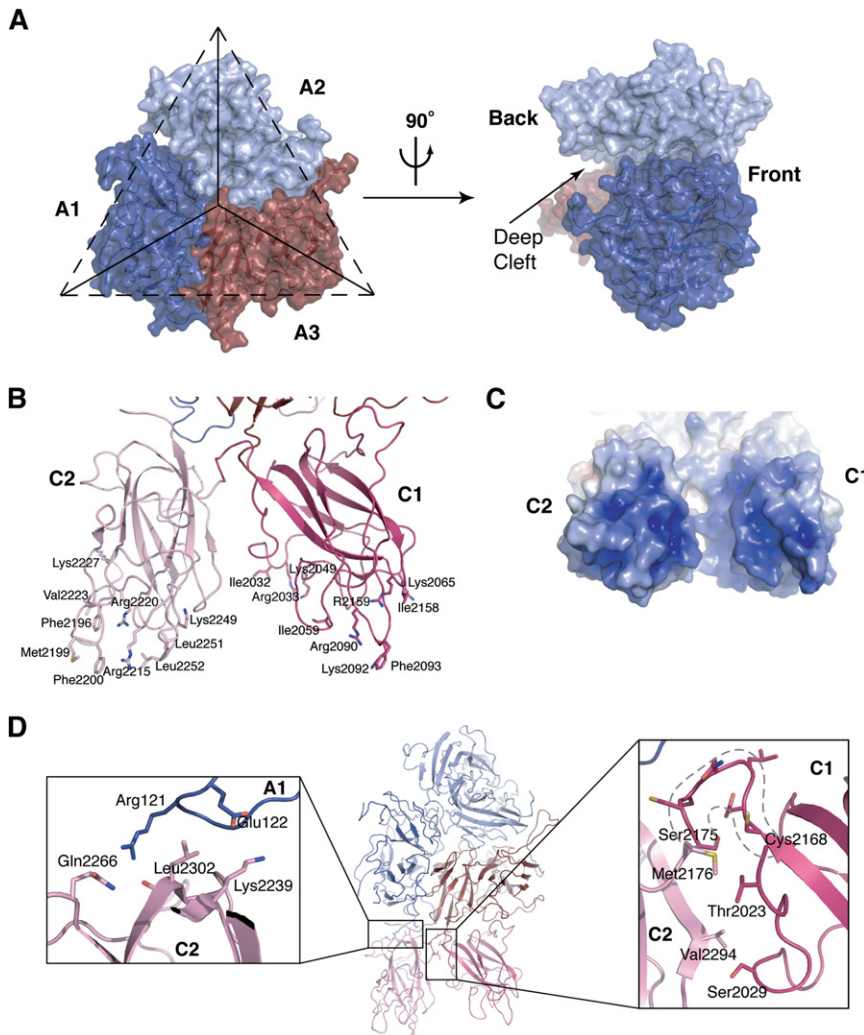


Figure 2. Structural Features of the A and C Domains

(A) The A1, A2, and A3 domains form a triangular heterotrimer around a pseudo-three-fold symmetry. Side view of the A domain heterotrimer shows that the front surface is relatively flat when compared with the back face, which contains a deep cleft formed by three large loops.

(B) The C1 and C2 domains contain numerous basic or hydrophobic residues positioned in the hairpin loops at the base of the domains.

(C) As an indication of the basic and hydrophobic nature of the putative lipid binding surface of the C domains, the solvent-accessible surface at the bottom of both C1 and C2 domains is colored by electrostatic potential (± 8 kT/e) computed by APBS (Baker et al., 2001).

(D) The C2 domain has few interactions with the A1 (left inset) and the C1 (right inset) domains. The key residues involved in direct contact are indicated. The loop that connects the C1 and C2 domains is highlighted with a dashed line in the right inset.

Factor VIII and factor V are procofactors that show approximately 40% sequence similarity and a parallel domain arrangement in their primary structures (Kane and Davie, 1986). Both cofactors are activated to their active cofactor forms, factor VIIIa and factor Va, by thrombin-mediated limited proteolysis (Pittman and Kaufman, 1988; Nesheim and Mann, 1979). Both cofactors are inactivated by activated protein C-mediated limited proteolysis to yield factor VIIIai and factor Vai. The three-dimensional structure of factor V has not been

toward the same plane. These loops likely contribute to the interaction of factor VIII with the phospholipid bilayer (Figures 2B and 2C).

Although the C2 domain is connected to the C1 domain and located adjacent to the A1 domain, there are few direct contacts of the C2 with either domain (Figure 2D). The interface between the C2 and A1 domains only buries 371 Å² of solvent-accessible surface area. Only Arg121 from the A1 domain is in sufficient proximity to form hydrogen bonds with the backbone carbonyl of Leu2302 and the side chain of Gln2266, and only Glu122 is capable of an electrostatic interaction with Lys2239 (Figure 2D, left inset). The only close interactions between the C1 and C2 domains are observed within the loop that connects the two domains (residues 2168–2175), and between Met2176 and Thr2023, Val2294s and Ser2029 (Figure 2D, right inset). These contacts cover a limited accessible surface area (344 Å²). Together, these observations strongly suggest the potential flexibility of the C2 domain, and are in agreement with the concept that the C2 domain undergoes conformational changes upon proteolysis within the light chain, resulting in enhanced affinity of factor VIIIa for anionic phospholipid surfaces (Saenko et al., 1998).

solved, thus precluding direct comparison with our current structure of human B domain-deleted factor VIII. However, a high-resolution X-ray crystal structure of activated protein C-inactivated bovine factor Va, factor Vai, allows partial comparison, since activation and subsequent inactivation of factor V is associated with the removal of the B domain and the A2 domain (Adams et al., 2004). The spatial arrangement of the A1 and A3 domains in B domain-deleted factor VIII are nearly identical with that of factor Vai, while the C1 and C2 domains show slightly different conformations when comparing our factor VIII structure with that of factor Vai. Despite the differences in the C1 and C2 domains, structures of factor VIII and factor Vai can be superimposed with an rmsd of 1.54 Å for the α carbons of the 524 residues that span the A1, A3, C1, and C2 domains (Figure 3A).

The activation of factor VIII by thrombin requires cleavage of a peptide bond in the A2 domain after Arg372, and the removal of the B domain linked to the 41 residue N-terminal region of the A3 domain, typically referred to as the α 3 acidic region (residues 1649–1689) by thrombin cleavage after Arg1689 (Pittman and Kaufman, 1988). This exposes the factor VIII surfaces that are important for factor IXa binding. Activation is also associated with, but does not require, cleavage after Arg740 (Pittman and

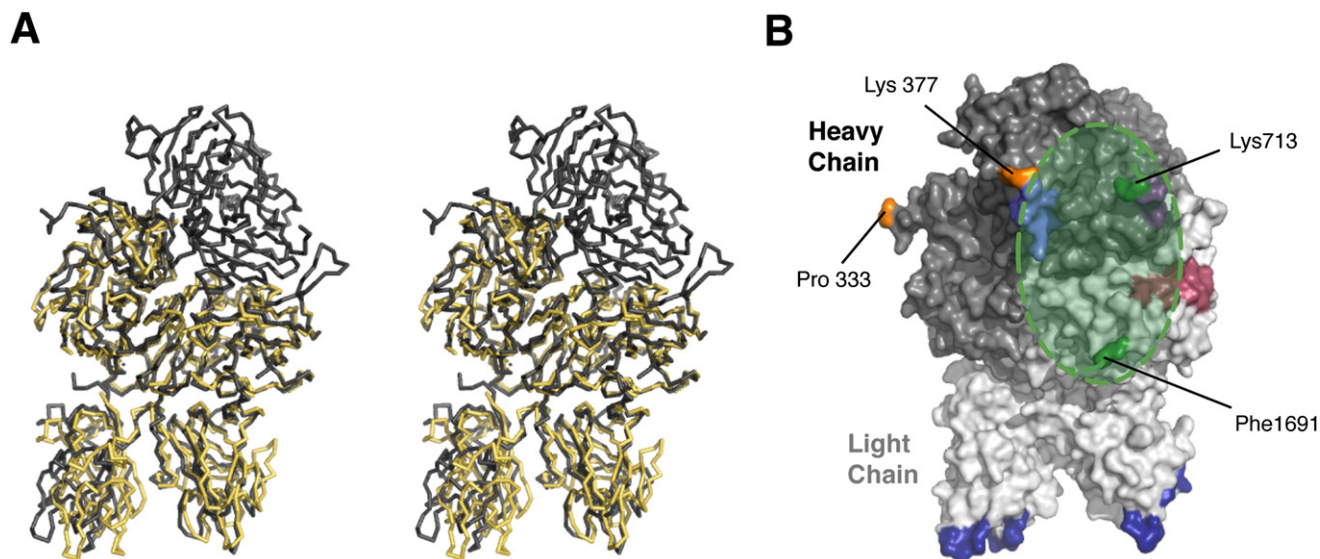


Figure 3. Comparison of Factor VIII and Factor Vai and the Putative Position of the B Domain

(A) Stereo view of the X-ray crystal structure of human B domain-deleted factor VIII compared to that of factor Vai. Superimposition of the carbon backbones of B domain-deleted factor VIII (gray) and factor Vai (yellow; PDB code: 1SDD; Adams et al., 2004). Factor Vai shares the same domain organization as B domain-deleted factor VIII, but does not include the A2 domain.

(B) The model of the heavy chain (dark gray) ends at residue Lys713 and the light chain (light gray) starts at Phe1691. Both termini are colored green, and the putative location of B domain is shaded in green. The region between residues 334 and 376, which includes the activation cleavage site adjacent to Arg372, is disordered and without electron density, and was not modeled. The flanking termini of this region are colored orange. This protein face includes the known interaction sites between factor VIIIa and factor IXa (light blue, purple, and red) based on biochemical studies. The putative membrane binding sites of the C1 and C2 domains are colored dark blue.

Kaufman, 1988). In B domain-deleted factor VIII, conversion of factor VIII to factor VIIIa requires cleavage at Arg1689 to remove the acidic a3 region adjacent to the A3 domain as well as cleavage after Arg372. These regions are located on the front surface of factor VIII (Figures 1A and 3B). Thus, it appears that the B domain and the a3 acidic region peptide are positioned on one face of the triangular heterotrimeric A domains of factor VIII and obstruct a functionally significant surface on the A2 and A3 domains (Figure 3B). This putative B domain interaction surface on factor VIII includes all of the suggested factor IXa binding regions previously described, including residues 558–565, 707–712, and 1811–1818 (Fay et al., 1994; Jenkins et al., 2004; Lenting et al., 1996).

Model of the Complex of Factor VIII and Factor IXa

The assembly of the factor IXa-factor VIIIa complex involves binding of factor VIIIa and factor IXa on phospholipid membrane surfaces in the presence of calcium ions. Based upon homology modeling (Autin et al., 2005), the analysis of naturally occurring hemophilia A and B mutations or mutations introduced by site-specific mutagenesis (Mannucci and Tuddenham, 2001; Fay et al., 1994; Nishimura et al., 1993; Hughes et al., 1993), cross-linking studies (Blostein et al., 2003), and inhibition with synthetic peptides (Lenting et al., 1996), the factor VIIIa binding surface for factor IXa is thought to involve the A2 and A3 domains that interact with multiple domains on factor IXa. The A3 domain of the light chain contains a high-affinity binding site ($K_d \sim 2\text{--}15$ nM) for factor IXa (Bajaj et al., 2001). Inhibition studies with synthetic peptides directed against the A3 domain have located this site

to residues 1811–1818 (Lenting et al., 1996). Potential factor IXa binding sites on the A2 domain include residues 558–565 (Fay et al., 1994) and the region around Asp712 (Jenkins et al., 2004). We have mapped these putative binding regions onto our structure of factor VIII (Figures 3B and 4A). All three binding regions are solvent exposed on the front surface of the molecule and surround a portion of the interface between the A2 and A3 domains. One of these binding regions, residues 558–565, bound to the C2 domain of a symmetry-related molecule in the crystal. The crystal interaction involves mainly hydrophobic and basic residues (e.g., Arg2215, Lys2249, Leu2251) from the β -hairpin loops located at the bottom of the C2 domain. Despite the fact that these interacting residues are located on different loops of the C2 domain, their positions closely resemble those in the α -helix region 330–339 in factor IXa (Figures 4B and 4C; Bajaj et al., 2001). This crystal contact may be mimicking the interaction between factor VIIIa and the 330–339 helix of factor IXa, as suggested in previous studies (Bajaj et al., 2001).

Cleavage of the factor VIII heavy chain to the factor VIIIa heavy chain is required to increase catalytic efficiency of factor IXa (Fay, 2004). However, this proteolytic activation of factor VIII appears to lead to only subtle changes in structure (Fay, 2004). Furthermore, both porcine and human factor VIII also bind to porcine factor IXa (Duffy et al., 1992). Based on these observations, we have constructed a model of the factor IXa-factor VIIIa complex with our factor VIII structure and the X-ray crystal structure of the porcine factor IXa backbone with the following constraints: (1) residues 558–565 of factor VIII interact with the 330–339 helix of factor IXa (Bajaj et al., 2001; Kolkman et al.,

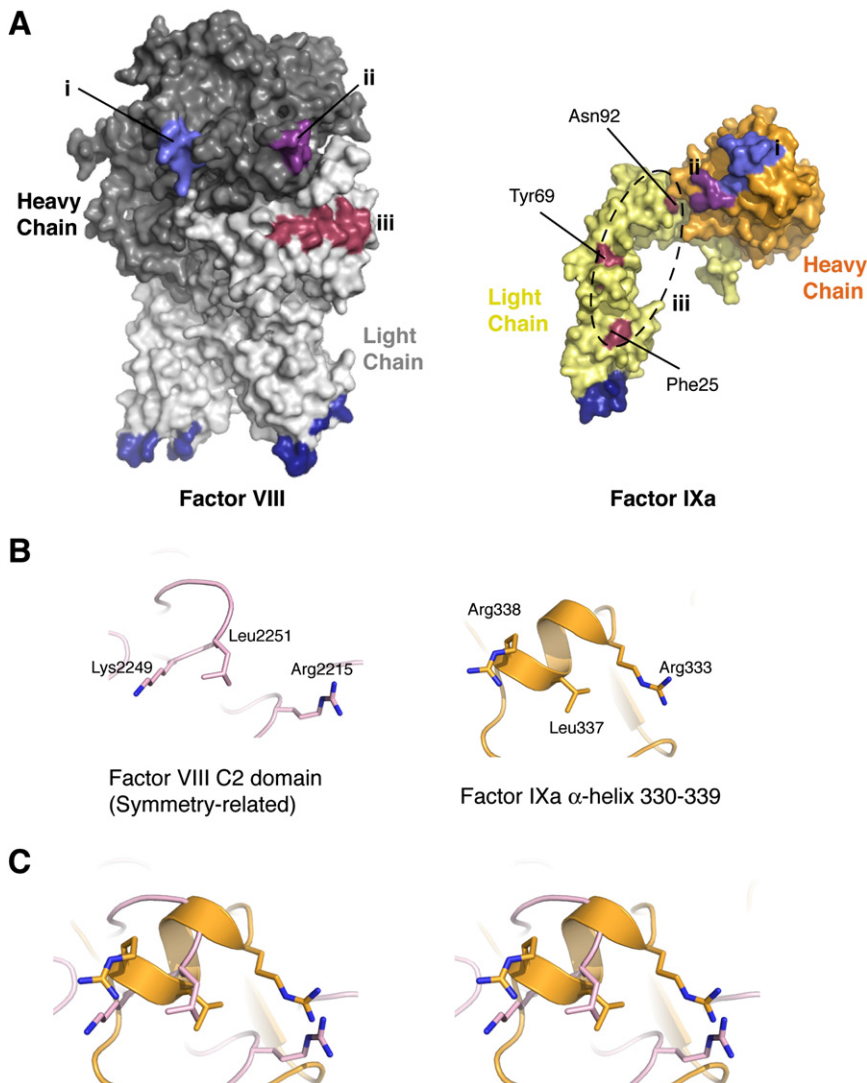


Figure 4. Binding Interface between the Complex of Factor VIII and Factor IXa

(A) Three different interaction sites with factor IXa were identified on factor VIIIa in previous studies, and are colored in blue, purple, and red, respectively. The complementary binding sites on factor IXa are colored accordingly. Region i (light blue) includes residues 558–565 on factor VIIIa and the 330–339 helix on factor IXa. Region ii (purple) includes residues near 712 on factor VIIIa and residues 301–303 on factor IXa. Region iii (red) on factor VIIIa represents the binding site (1811–1818) that has been suggested to be responsible for the high-affinity interaction between factor VIIIa and factor IXa. Residues on the light chain of factor IXa that have been shown to be important for binding include Phe25 (red) within the factor IXa Gla domain, which is known to be juxtaposed to the factor VIIIa light chain, Tyr69 (red; Nishimura et al., 1993) and Asn92 (red; Hughes et al., 1993). The putative binding region on factor IXa that interacts with residues 1811–1818 of factor VIII is highlighted with a dashed line. The putative phospholipid binding sites in the C1 and C2 domains of factor VIIIa and the Gla domain of factor IXa that are responsible for membrane binding are indicated (dark blue).

(B) Positions of residues contributed from different loops of the C2 domain resemble that of the 330–339 α helix of factor IXa.

(C) Stereo view of superimposition of loops of the C2 domain of factor VIII and that of the 330–339 α helix of factor IXa.

1999); (2) 707–712 of factor VIIIa bind to factor IXa residues 301–303 (Kolkman et al., 1999; Liles et al., 1997); (3) residues 1811–1819 of factor VIII interact with the light chain of factor IXa (Lenting et al., 1996); (4) Phe25 in the Gla domain of factor IX is juxtaposed with the light chain of factor VIII (Blostein et al., 2003); (5) the Gla domain of factor IXa is situated within the phospholipid membrane, forming noncovalent interactions between the phosphoserine head group and fatty acid chains of the phospholipid bilayer and the hydrophobic patch and the Gla residues within the Gla domain of factor IXa (Freedman et al., 1996; Huang et al., 2003; Figure 4A).

The program HADDOCK was used for the docking calculations (Dominguez et al., 2003). Figure 5A shows a plot of the intermolecular energy (the sum of intermolecular van der Waals, electrostatic, and AIR energy terms) for 60 complex structures, after water refinement, as a function of their backbone rmsds from the lowest energy structure. Three main clusters, labeled “1,” “2,” and “3,” were obtained after analysis, with a 5 Å rmsd cut-off to distinguish the clusters. After analyzing the representative structures from each cluster based on the con-

straints described above, the representative structure from cluster 1 provided the best model of the factor IXa-factor VIIIa complex. Cluster 1 contains the overall lowest energy structures; the average rmsd values relative to the lowest energy structure is 1.37 ± 0.96 Å. Both factor VIIIa and factor IXa in the model of this complex maintain the same overall fold as in their crystal structures. The model of the factor IXa-factor VIIIa complex illustrates that the light chain of factor IXa is wrapped across the side of the A3 domain of factor VIII, and is on the opposite side from the membrane binding interface of the complex from the C2 domain, which is important for factor VIIIa interaction with the membrane (Figure 5B). This orientation of the factor VIII light chain and factor IX Gla domain suggests that the C2 domain of factor VIII in our model is above the membrane surface, and has to undergo a significant conformational change in order to orient its membrane binding surfaces in the same direction as the Gla domain of factor IXa (Figure 5C). In fact, in addition to our observation that the C2 domain must undergo a conformational change upon activation, the 15 Å resolution cryoelectron microscopy structure of factor VIII bound to phospholipids shows that the A domains are inclined at an angle of 60°–65° to the membrane surface, with the A3 domain positioned close to the membrane surface (Stoylova et al., 1999). Furthermore, factor IXa has been shown to orient with

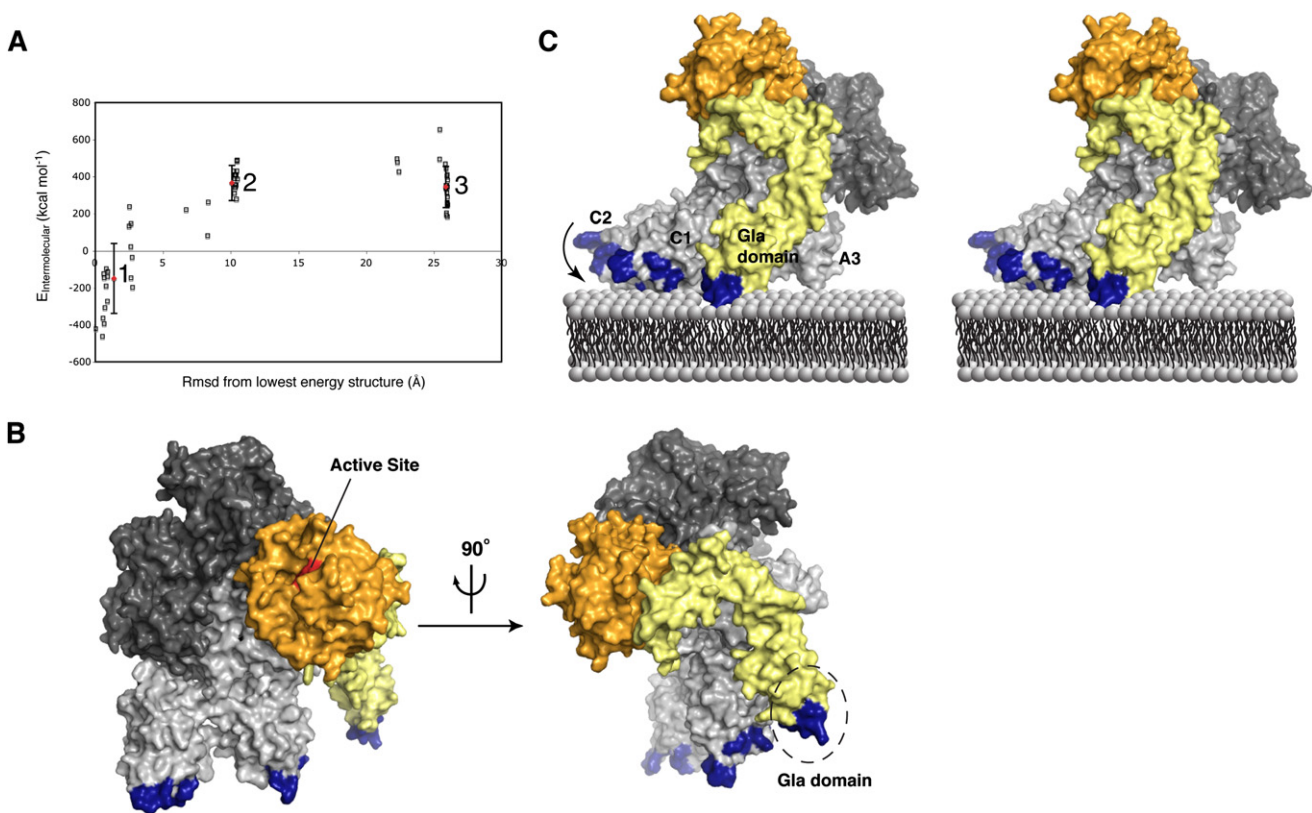


Figure 5. Model of the Factor IXa-Factor VIIIa Complex

(A) Plot of the intermolecular energy as a function of the rmsd from the lowest energy structure for the factor IXa-factor VIIIa complex. Three major clusters were identified, labeled 1, 2, and 3. The values for individual conformations (open squares) and the averages of the clusters (red diamonds) are shown.

(B) Front and side views of the complex of factor VIIIa (heavy chain [dark gray] and light chain [light gray and gray]) and factor IXa (heavy chain [orange] and light chain [yellow]). Four putative membrane binding sites, including the bases of C1 and C2 domains, the A3 domain loop of factor VIII, and the Gla domain of factor IXa, lie on the same plane (blue). The active site of factor IXa is indicated (red).

(C) Stereo view diagram of the possible interaction of the factor IXa-factor VIIIa complex with phospholipid membrane surfaces.

the long axis of the molecule perpendicularly to the membrane, with the Gla domain proximal to the membrane and the active site positioned more than 70 Å above the surface (Mutucumaran et al., 1992). We suggest that, upon binding to factor IXa, the C2 domain of the phospholipid-bound factor VIIIa undergoes a significant conformational change that alters the orientation of the factor VIIIa from an upright position to a bent position, in order for the Gla domain of the bound factor IXa to interact with the phospholipids membrane simultaneously (Figure 5C). The active site of factor IXa in this model of the factor IXa-factor VIIIa complex is positioned on the top of the complex facing into the solution, with an approximate distance of 75–80 Å above the membrane surface.

We have identified four putative phospholipid binding sites in the factor IXa-factor VIIIa complex. Pratt et al. (1999) have previously identified the loops in the C2 domain of factor VIII as likely participants in phospholipid binding. Within the context of the domain organization of C2 in the factor IXa-factor VIII complex, Arg2215 and Lys2249 play a special role. The hairpin loops in the factor VIII C1 domain, including Arg2090, Lys2092, and Arg2159, likely play a similar role, as predicted previously (Liu et al., 2000). In addition, a well-defined loop extends downward from the backside of the factor VIII A3 domain (Figure 5C). This loop, which

includes a β turn, is held together by Cys1899 and Cys1903, and thrusts Arg1900 into a favorable position for electrostatic interaction with acidic phospholipid headgroups. The polypeptide backbone of this loop is structurally similar to the omega loop of the Gla domain of factor IXa. With the prothrombin Gla domain as a prototype, we have previously established that the phosphoserine head group in lysophosphatidylserine interacts with conserved residues within the Gla domain of vitamin K-dependent proteins, including factor IX (Huang et al., 2003). Trp4 within the omega loop of the Gla domain of prothrombin is located 5–7 Å below the membrane surface in the interfacial membrane region (Falls et al., 2001). Based on this observation, the factor IXa in this model was similarly positioned.

An intermediate resolution X-ray crystallographic structure of biologically active B domain-deleted human factor VIII has recently been reported, although the coordinates of this structure are not yet available (Shen et al., 2008). The general, overall structure appears similar to our own, specifically the domain organization. One minor difference is that we only identified a single calcium binding site, whereas Shen et al. located two sites. Both of these structures are at similar resolution, and the crystals are characterized by the same space groups. Understanding of the domain organization, the metal binding sites, and the surface

features in this protein, as well as its membrane binding properties, will contribute to the study of the structure-function relationships in the mutations of hemophilia and the assembly of proteases associated with protein cofactors. Furthermore, the model of the factor IXa-factor VIIIa complex and its interaction with membrane surfaces are critically important to detailed understanding of normal hemostasis within the context of the blood coagulation cascade. Knowledge of the structure of factor VIII may also be useful for the design of improved therapies for hemophilia A or venous thromboembolic disorders.

EXPERIMENTAL PROCEDURES

Expression and Purification of B Domain-Deleted Factor VIII

B domain-deleted recombinant factor VIII (Wyeth) was prepared as previously described (Sandberg et al., 2001; Eriksson et al., 2001) with the following modifications. Chinese hamster ovary cells were cultured in medium free of human serum albumin, and purification by monoclonal antibody immunoaffinity chromatography was replaced with peptide ligand affinity chromatography with TN8.2 Sepharose (Kelley et al., 2004). The purified factor VIII used for crystallization was obtained detergent free following the anion exchange step used in the manufacturing process. Purified factor VIII, at a concentration of ~2.5 mg/ml in 50 mM histidine (pH 6.3), 4 mM CaCl₂, 400 mM NaCl, was stored at -80°C.

Formation of B Domain-Deleted Factor VIII Crystals and Data Collection

Crystals were obtained by hanging drop vapor diffusion at 25°C by the Hampton screen (Hampton Research). The drop contained 1 µl of factor VIII at 10 mg/ml mixed with 1 µl of reservoir solution. The optimal condition for crystallization was found to be 100 mM Tris-HCl (pH 8.5), 10% ethanol, and 7% PEG 3350 in the reservoir. All crystals were cryoprotected by sequential addition of 10%, 15%, and, finally, 20% ethylene glycol (v/v) in the presence of the reservoir solution and flash frozen in liquid nitrogen prior to data collection. Factor VIII crystallized in a P4₁2₁2 space group ($a = b = 134.113$ Å, $c = 349.760$ Å, and $\alpha = \beta = \gamma = 90^\circ$) with one molecule per asymmetric unit.

X-ray diffraction data of factor VIII crystals were collected at the National Synchrotron Light Source at Brookhaven National Laboratory and the Northeastern Collaborative Access Team synchrotron beamline 24 of the Advanced Photon Source (APS) at Argonne National Laboratory. All X-ray data were processed using program HKL2000 (Otwinowski and Minor, 1997; Table 1).

Structure Determination and Refinement of Factor VIII

For structural determination by the molecular replacement method, a homology structure model of factor VIII was constructed from the known primary sequence of factor VIII (Schwede et al., 2003) with the template structures of factor Vai (PDB code: 1SDD; Adams et al., 2004) and ceruloplasmin (PDB code: 1KCW; Zaitseva et al., 1996). The structures of the A1 and A2 domains of factor VIII were initially determined with AMoRe (Navaza, 1994) with the A1 and A2 domains of the homology model. This yielded clear rotation function and translation function solutions. The A3 domain was then solved by AMoRe after fixing the solution of the A1 and A2 domains and by using the A3 domain of ceruloplasmin as a search model (PDB code: 1KCW; Zaitseva et al., 1996). After fixing all three A domains together, the positions of the C1 and C2 domains were determined with the program PHASER with a polyalanine model built from the high-resolution structure of the C2 domain of factor VIII (PDB code: 1D7P; Pratt et al., 1999; McCoy, 2007). The structure was refined with several cycles of manual refitting and refinements with REFMAC of the CCP4i suite (Murshudov et al., 1997; Potterton et al., 2003). The R_{cryst} and R_{free} for the factor VIII model were 25.70% and 33.06%, respectively, for data from 50–3.98 Å (Table 1).

Modeling of the Factor VIIIa-Factor IXa Complex

Human factor IXa was constructed by homology modeling with the X-ray structure of porcine factor IXa (PDB code: 1PFX) and the program SWISS-

MODEL (Schwede et al., 2003; Brandstetter et al., 1995). The structure of factor VIIIa was docked with factor IXa with the program HADDOCK (Dominguez et al., 2003). In this approach, residues previously reported to be important for factor VIIIa-factor IXa interactions were defined as the ambiguous interaction constraints. Initially, 600 structures for the complex were generated by docking factor VIII and factor IXa as rigid bodies, and 60 of those were followed by semirigid, simulated annealing in torsion angle space, and analyzed with the default settings at all stages. The resultant structures were clustered. The best model was selected based on the convergence of the structures with the largest binding interface and the lowest intermolecular energy while satisfying the constraints defined by previous studies.

ACCESSION NUMBERS

Coordinates have been deposited in the Protein Data Bank with accession code 3CDZ.

ACKNOWLEDGMENTS

We thank Qing Huai for her contributions during the preliminary phase of this study. D.A.R. is an employee of Wyeth. This work was supported by a grant from Wyeth.

Received: January 6, 2008

Revised: February 27, 2008

Accepted: March 1, 2008

Published: April 8, 2008

REFERENCES

- Adams, T.E., Hockin, M.F., Mann, K.G., and Everse, S.J. (2004). The crystal structure of activated protein C-inactivated bovine factor Va: Implications for cofactor function. *Proc. Natl. Acad. Sci. USA* 101, 8918–8923.
- Autin, L., Miteva, M.A., Lee, W.H., Mertens, K., Radtke, K.P., and Villoutreix, B.O. (2005). Molecular models of the procoagulant factor VIIIa-factor IXa complex. *J. Thromb. Haemost.* 3, 2044–2056.
- Bajaj, S.P., Schmidt, A.E., Mathur, A., Padmanabhan, K., Zhong, D., Mastri, M., and Fay, P.J. (2001). Factor IXa:factor VIIIa interaction. Helix 330–338 of factor IXa interacts with residues 558–565 and spatially adjacent regions of the A2 subunit of factor VIIIa. *J. Biol. Chem.* 276, 16302–16309.
- Baker, N.A., Sept, D., Joseph, S., Holst, M.J., and McCammon, J.A. (2001). Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci. USA* 98, 10037–10041.
- Bihoreau, N., Pin, S., de Kersabiec, A.M., Vidot, F., and Fontaine-Aupart, M.P. (1994). Copper-atom identification in the active and inactive forms of plasma-derived FVIII and recombinant FVIII-delta II. *Eur. J. Biochem.* 222, 41–48.
- Blostein, M.D., Furie, B.C., Rajotte, I., and Furie, B. (2003). The Gla domain of factor IXa binds to factor VIIIa in the tenase complex. *J. Biol. Chem.* 278, 31297–31302.
- Brandstetter, H., Bauer, M., Huber, R., Lollar, P., and Bode, W. (1995). X-ray structure of clotting factor IXa: active site and module structure related to Xase activity and hemophilia B. *Proc. Natl. Acad. Sci. USA* 92, 9796–9800.
- Dominguez, C., Boelens, R., and Bonvin, A.M. (2003). HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. *J. Am. Chem. Soc.* 125, 1731–1737.
- Duffy, E.J., Parker, E.T., Mutucumarana, V.P., Johnson, A.E., and Lollar, P. (1992). Binding of factor VIIIa and factor VIII to factor IXa on phospholipid vesicles. *J. Biol. Chem.* 267, 17006–17011.
- Eriksson, R.K., Fenge, C., Lindner-Olsson, E., Ljungqvist, C., Rosenquist, J., Smeds, A.L., Ostlin, A., Charlebois, T., Leonard, M., Kelley, B.D., et al. (2001). The manufacturing process for B-domain deleted recombinant factor VIII. *Semin. Hematol.* 38, 24–31.
- Falls, L.A., Furie, B.C., Jacobs, M., Furie, B., and Rigby, A.C. (2001). The ω-loop region of the human prothrombin γ-carboxyglutamic acid domain penetrates anionic phospholipid membranes. *J. Biol. Chem.* 276, 23895–23902.

- Fay, P.J. (2004). Activation of factor VIII and mechanisms of cofactor action. *Blood Rev.* 18, 1–15.
- Fay, P.J., Beattie, T., Huggins, C.F., and Regan, L.M. (1994). Factor VIIIa A2 subunit residues 558–565 represent a factor IXa interactive site. *J. Biol. Chem.* 269, 20522–20527.
- Fowler, W.E., Fay, P.J., Arvan, D.S., and Marder, V.J. (1990). Electron microscopy of human factor V and factor VIII: correlation of morphology with domain structure and localization of factor V activation fragments. *Proc. Natl. Acad. Sci. USA* 87, 7648–7652.
- Freedman, S.J., Blostein, M.D., Baleja, J.D., Jacobs, M., Furie, B.C., and Furie, B. (1996). Identification of the phospholipid binding site in the vitamin K-dependent blood coagulation protein Factor IX. *J. Biol. Chem.* 271, 16227–16236.
- Furie, B., and Furie, B.C. (1988). The molecular basis of blood coagulation. *Cell* 53, 505–518.
- Furie, B., and Furie, B.C. (1990). Molecular basis of hemophilia. *Semin. Hematol.* 27, 270–285.
- Gitschier, J., Wood, W.I., Goralka, T.M., Wion, K.L., Chen, E.Y., Eaton, D.H., Vehar, G.A., Capon, D.J., and Lawn, R.M. (1984). Characterization of the human factor VIII gene. *Nature* 312, 326–330.
- Graw, J., Brackmann, H.H., Oldenburg, J., Schneppenheim, R., Spannagl, M., and Schwaab, R. (2005). Haemophilia A: from mutation analysis to new therapies. *Nat. Rev. Genet.* 6, 488–501.
- Huang, M., Rigby, A.C., Morelli, X., Grant, M.A., Huang, G., Furie, B., Seaton, B., and Furie, B.C. (2003). Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. *Nat. Struct. Biol.* 10, 751–756.
- Hughes, P.E., Morgan, G., Rooney, E.K., Brownlee, G.G., and Handford, P. (1993). Tyrosine 69 of the first epidermal growth factor-like domain of human factor IX is essential for clotting activity. *J. Biol. Chem.* 268, 17727–17733.
- Jenkins, P.V., Dill, J.L., Zhou, Q., and Fay, P.J. (2004). Contribution of factor VIIIa A2 and A3-C1-C2 subunits to the affinity for factor IXa in factor Xase. *Biochemistry* 43, 5094–5101.
- Kane, W.H., and Davie, E.W. (1986). Cloning of a cDNA coding for human factor V, a blood coagulation factor homologous to factor VIII and ceruloplasmin. *Proc. Natl. Acad. Sci. USA* 83, 6800–6804.
- Kelley, B.D., Tannatt, M., Magnusson, R., Hagelberg, S., and Booth, J. (2004). Development and validation of an affinity chromatography step using a peptide ligand for cGMP production of factor VIII. *Biotechnol. Bioeng.* 87, 400–412.
- Key, N.S., and Negrier, C. (2007). Coagulation factor concentrates: past, present, and future. *Lancet* 370, 439–448.
- Kolkman, J.A., Christophe, O.D., Lenting, P.J., and Mertens, K. (1999). Surface loop 199–204 in blood coagulation factor IX is a cofactor-dependent site involved in macromolecular substrate interaction. *J. Biol. Chem.* 274, 29087–29093.
- Lenting, P.J., van de Loo, J.W., Donath, M.J., van Mourik, J.A., and Mertens, K. (1996). The sequence Glu1811-Lys1818 of human blood coagulation factor VIII comprises a binding site for activated factor IX. *J. Biol. Chem.* 271, 1935–1940.
- Liles, D., Landen, C.N., Monroe, D.M., Lindley, C.M., Read, M.S., Roberts, H.R., and Brinkhous, K.M. (1997). Extravascular administration of factor IX: potential for replacement therapy of canine and human hemophilia B. *Thromb. Haemost.* 77, 944–948.
- Liu, M.L., Shen, B.W., Nakaya, S., Pratt, K.P., Fujikawa, K., Davie, E.W., Stoddard, B.L., and Thompson, A.R. (2000). Hemophilic factor VIII C1- and C2-domain missense mutations and their modeling to the 1.5-angstrom human C2-domain crystal structure. *Blood* 96, 979–987.
- Mannucci, P.M., and Tuddenham, E.G. (2001). The hemophilias—from royal genes to gene therapy. *N. Engl. J. Med.* 344, 1773–1779.
- McCoy, A.J. (2007). Solving structures of protein complexes by molecular replacement with Phaser. *Acta Crystallogr. D Biol. Crystallogr.* 63, 32–41.
- Messerschmidt, A., and Huber, R. (1990). The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. *Eur. J. Biochem.* 187, 341–352.
- Mosesson, M.W., Fass, D.N., Lollar, P., DiOrto, J.P., Parker, C.G., Knutson, G.J., Hainfeld, J.F., and Wall, J.S. (1990). Structural model of porcine factor VIII and factor VIIIa molecules based on scanning transmission electron microscope (STEM) images and STEM mass analysis. *J. Clin. Invest.* 85, 1983–1990.
- Murshudov, G.N., Vagin, A.A., and Dodson, E.J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr.* 53, 240–255.
- Mutucumarana, V.P., Duffy, E.J., Lollar, P., and Johnson, A.E. (1992). The active site of factor IXa is located far above the membrane surface and its conformation is altered upon association with factor VIIIa. A fluorescence study. *J. Biol. Chem.* 267, 17012–17021.
- Navaza, J. (1994). AMoRe: an automated package for molecular replacement. *Acta Crystallogr. A* 50, 157–163.
- Nesheim, M.E., and Mann, K.G. (1979). Thrombin-catalyzed activation of single chain bovine factor V. *J. Biol. Chem.* 254, 1326–1334.
- Nishimura, H., Takeya, H., Miyata, T., Suehiro, K., Okamura, T., Niho, Y., and Iwanaga, S. (1993). Factor IX Fukuoka. Substitution of ASN92 by His in the second epidermal growth factor-like domain results in defective interaction with factors VIIIa/X. *J. Biol. Chem.* 268, 24041–24046.
- Otwinowski, Z., and Minor, W. (1997). Processing of x-ray diffraction data collected in oscillation mode. In *Methods Enzymol., Volume 276*, C.W. Carter, Jr. and R.M. Sweet, eds. (New York: Academic Press), pp. 307–326.
- Pan, Y., DeFay, T., Gitschier, J., and Cohen, F.E. (1995). Proposed structure of the A domains of factor VIII by homology modelling. *Nat. Struct. Biol.* 2, 740–744.
- Pellequer, J.L., Gale, A.J., Griffin, J.H., and Getzoff, E.D. (1998). Homology models of the C domains of blood coagulation factors V and VIII: a proposed membrane binding mode for FV and FVIII C2 domains. *Blood Cells Mol. Dis.* 24, 448–461.
- Pemberton, S., Lindley, P., Zaitsev, V., Card, G., Tuddenham, E.G., and Kembell-Cook, G. (1997). A molecular model for the triplicated A domains of human factor VIII based on the crystal structure of human ceruloplasmin. *Blood* 89, 2413–2421.
- Pittman, D.D., and Kaufman, R.J. (1988). Proteolytic requirements for thrombin activation of anti-hemophilic factor (factor VIII). *Proc. Natl. Acad. Sci. USA* 85, 2429–2433.
- Potterton, E., Briggs, P., Turkenburg, M., and Dodson, E. (2003). A graphical user interface to the CCP4 program suite. *Acta Crystallogr. D Biol. Crystallogr.* 59, 1131–1137.
- Pratt, K.P., Shen, B.W., Takeshima, K., Davie, E.W., Fujikawa, K., and Stoddard, B.L. (1999). Structure of the C2 domain of human factor VIII at 1.5 Å resolution. *Nature* 402, 439–442.
- Saenko, E.L., Scandella, D., Yakhyayev, A.V., and Greco, N.J. (1998). Activation of factor VIII by thrombin increases its affinity for binding to synthetic phospholipid membranes and activated platelets. *J. Biol. Chem.* 273, 27918–27926.
- Sandberg, H., Almstedt, A., Brandt, J., Castro, V.M., Gray, E., Holmquist, L., Lewin, M., Oswaldsson, U., Mikaelsson, M., Jankowski, M.A., et al. (2001). Structural and functional characterization of B-domain deleted recombinant factor VIII. *Semin. Hematol.* 38, 4–12.
- Schwede, T., Kopp, J., Guex, N., and Peitsch, M.C. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* 31, 3381–3385.
- Shen, B.W., Spiegel, P.C., Chang, C.H., Huh, J.W., Lee, J.S., Kim, J., Kim, Y.H., and Stoddard, B.L. (2008). The tertiary structure and domain organization of coagulation factor VIII. *Blood* 111, 1240–1247.
- Stoylova, S.S., Lenting, P.J., Kembell-Cook, G., and Holzenburg, A. (1999). Electron crystallography of human blood coagulation factor VIII bound to phospholipid monolayers. *J. Biol. Chem.* 274, 36573–36578.
- Toole, J.J., Knopf, J.L., Wozney, J.M., Sultzman, L.A., Buecker, J.L., Pittman, D.D., Kaufman, R.J., Brown, E., Shoemaker, C., Orr, E.C., et al. (1984). Molecular cloning of a cDNA encoding human antihemophilic factor. *Nature* 312, 342–347.

- Toole, J.J., Pittman, D.D., Orr, E.C., Murtha, P., Wasley, L.C., and Kaufman, R.J. (1986). A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. *Proc. Natl. Acad. Sci. USA* *83*, 5939–5942.
- Wakabayashi, H., Koszelak, M.E., Mastri, M., and Fay, P.J. (2001). Metal ion-independent association of factor VIII subunits and the roles of calcium and copper ions for cofactor activity and inter-subunit affinity. *Biochemistry* *40*, 10293–10300.
- Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996). The X-ray structure of human serum caeruloplasmin at 3.1 Å: nature of the copper centre. *J. Biol. Inorg. Chem.* *1*, 15–23.