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Structure of heavy and light chains of blood coagulation factor VIII (FVIII) involved in the activation of FVIII

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Abbreviations: FVIII, factor FVIII; FVIII-H, FVIII-heavy chain; FXa, activated factor X; FVIII RAg, FVIII related antigen; FVIII-L, FVIII light chain

Abstract

Structure of human blood coagulation factor VIII (FVIII) in relation to its activation process was investigated. FVIII was purified from a commercial FVIII concentrate by immunoaffinity chromatography and its dissociated subunits, heavy and light chains were isolated. The light chain (FVIII-L) was treated with thrombin or factor Xa (FXa) in order to cleave the peptide at Arg^{1689} or Arg^{1721}, respectively. Re-association of FVIII-H with either of FVIII-L derivatives, FVIII-L^{72} (72 kDa) and FVIII-L^{65} (65 kDa) brought about the formation of heterodimers which have similar cofactor activity. The association constant of FVIII-H with FVIII-L^{72} was about two-fold faster than that with FVIII-L^{65}. Cleavage of major FVIII-H with thrombin generated two peptides with molecular weights of 50 kDa (A_1) and 40 kDa (A_2). Formation of heterotrimer by reassociation of A_1, A_2 and FVIII-L^{72}generated FVIII cofactor activity, while the dimers formed from A_1 or A_2 with FVIII-L^{72} had no activity, suggesting that both A_1 and A_2 are required for FVIII activity. Heterotrimers formed from A_1 and A_2 with either of FVIII-L^{72} or FVIII-L^{65} in the presence of $CaCl_2$ (10mM) revealed cofactor activity, and they were dissociated into subunits with the loss of activity when EDTA (10mM) was added, indicating that the formation of heterotrimer, the functional unit of FVIII, from A1, A2 and FVIII-L is calcium dependent and that the cleavage of FVIII-L by FXa does not inactivate FVIII.

Keywords: blood coagulation factors, factor VIII, protein processing

Inroduction

Coagulation factor VIII (FVIII) participates in the activation of factor X (FX) by factor IX as a cofactor in the blood coagulation cascade, and its activation is accomplished by limited proteolysis of both heavy and light chains (Lollar *et al*., 1985; Mertens *et al*., 1985; Kaufman, 1992).

Thrombin and FXa are known to be responsible for the activation of FVIII by cleavage of heavy and light chains. Due to the limited proteolysis by thrombin and FXa at the B domain of FVIII, FVIII heavy chain (FVIII-H) is heterogeneous (Andersson *et al*., 1986). Three major cleaving sites of FVIII by thrombin during activation are Arg^{336}, Arg^{740} and Arg^{1689}. FXa also cleaves at the same sites defined by thrombin and additionally, at Arg^{336} in the heavy chain and at Arg^{1721} in the light chain (Eaton *et al*., 1986). The additional cleavage at Arg^{336}, Arg^{1721} has been thought to inactivate FVIII with the dissociation of subunits. However, recent observation suggested that cleavage at Arg^{1719} or Arg^{1721} in the light chain may be unrelated to FVIII inactivation (Fay, 1993). Therefore inactivation of FVIII by additional cleavage by thrombin or FXa in the light chain remains controversial, and the precise sites of proteolytic cleavage of FVIII in the process of FVIII activation remains unclear.

It is neccessary to define the role of each FVIII subunits in the generation of cofactor activity. In the present study, we attempted to determine the structural requirement of proteolytic subunits for the assembly and regeneration of FVIII activity.

Materials and Methods

Preparation of immunoaffinity column

Polyclonal anti-human FVIII related antigen (FVIIIRAg) antibody (Sigma, St. Louis, U.S.A.) dissolved in a coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) at a concentration of 20 mg/ml was added to CNBr-activated Sepharose-4B gel (Pharmacia, Sweden) swelled in 1 mM HCl and mixed overnight at 4°C with gentle stirring. Uncoupled excess ligands were washed out with 5 gel volumes of coupling buffer and the remaining active groups on the gel were blocked with 0.1 M Tris-HCl buffer, pH 8.0, for two hours. The product was washed with at least three cycles of alternating pH buffers. Each cycle consists of a wash with 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The gel was equilibrated in FVIII buffer (20 mM imidazole/ 0.15 M NaCl/0.1 M L-lysine/0.02% NaN_3, pH 6.8) and packed into an immunoadsorbent column (2 × 12 cm).

Purification of FVIII and FVIII subunits

A commercial FVIII concentrate (HemophilM, Baxter, U.S.A.) containing about 10,000 units was reconstituted with 200 ml of FVIII buffer and was applied to the immunoadsorbent column equilibrated with the FVIII buffer. The column was washed with one liter of FVIII buffer containing 0.5 M NaCl, and FVIII was then eluted from FVIIIRAg bound gel with FVIII buffer containing 0.35 M $CaCl_2$. Fractions containing FVIII activity were pooled and concentrated 100-folds by dialysis through dialysis membrane against the powder form of polyethylene glycol (MW 400,000). The concentrate was then subjected to an extensive dialysis against a buffer containing 0.15 M NaCl, 25 mM EDTA, 10 mM benzamidine and 20 mM Tris (pH 7.4) in order to dissociate FVIII into its subunits. The EDTA-dissociated FVIII-H was isolated by immunoaffinity chromatography using a polyclonal antibody against FVIII-H peptide fragment produced in *E. coli* (Kim and Oh, 1994). FVIII light chain (FVIII-L) was isolated from the FVIII-H removed fraction by immunoaffinity chromatography using a monoclonal antibody against FVIII-L. Thrombin or FXa-cleaved FVIII-L derivatives were prepared from FVIII-L treated with thrombin (2 units/10 μg) or FXa (2 units/10 μg) for one hour at 37°C in the presence of a buffer consisting of 100 mM NaCl, 10 mM $CaCl_2$, and 50 mM Tris, pH 7.4 followed by gel filtration (Sephacryl S 300 column, 1.2×65 cm). Thrombin cleaved FVIII-H derivatives were prepared as A_1 and A_2 complex after immunoaffinity chromatography.

FVIII heterodimer formation assay

FVIII heterodimer formation was assayed by ELISA using FVIII-L specific mouse monoclonal antibody as primary antibody and horse raddish peroxidase-conjugated anti-mouse IgG antibody as secondary antibody. FVIII-H (200 pmol) dissolved in a coating buffer (50 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6) were immmobilized to microtiter plates (100 μl/well) overnight at 4°C. Wells were washed with 10mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4 (TBST) and blocked for 2 h at room temperature with same buffer containing 10% (w/v) non-fat dry milk. Various amount of FVIII-L derivatives dissolved in 100 μl of phosphate buffered saline (pH 7.4) were added to each wells and incubated for 16 h at 4°C. After washing the wells with TBST 3 times, the peroxidase-conjugated secondary antibody was added (2,000-fold dilution in blocking solution) and incubated another two hours. After incubation, wells were washed 4 times with TBST and 100 μl of peroxidase substrate solution, prepared by dissolving (10 mg *o*-phenylenediamine dihydrochloride and 10 μl of H_2O_2 in 25 ml of reaction buffer (20 mM citrate, 20 mM sodium phosphate, pH 5.6) was added to develop the color. After color development, 100 μl of 1.25 M H_2SO_4 was added and absorbance at 490 nm was measured. Standard plasma-derived FVIII (1 units/ml) served as a reference.

FVIII subunits association assay

FVIII subunit association assay was performed with a fixed concentration of FVIII-H subunit and varied in the amount of FVIII-L concentration and incubation time. The incubation buffer (FVIII buffer) contained 40 mM $CaCl_2$ and 400 mM NaCl in the assay. Association of FVIII subunits was monitored by measuring FVIII activity and association constant was determined from the equation; FVIII-H + FVIII-L $\overset{k1}{\leftrightarrow}$ FVIII. $v=k_1$[FVIII-H][FVIII-L] and in integrated expression, $k_1t=1/a_0-b_0 \ln b_0(a_0-p)/a_0(b_0-p)$. k_1 represents the association rate constant and p is the concentration of FVIII dimer formed at time t. a_0 is the initial concentration of FVIII-H and b_0 is the initial concentration of FVIII-L.

Measurement of FVIII activity and protein content

Cofactor activity of FVIII was assayed by Coamatic factor FVIII kit (Chromogenix AB, Stockholm, Sweden). The principle of the method is the measurement of the FVIII-dependent generation of FXa from FX in the presence of factor IXa (Carlebjork *et al*., 1987). Total protein content was determined by Bradford method (Bradford, 1976) and the amount of FVIII and its subunit derivatives were quantified by ELISA using anti-FVIII-H and anti-FVIII-L antibodies according to the method of Johnston and Thorpe (1987). A purified FVIII preparation with known concentration was used as the reference.

Western blot analysis of FVIII

Western blot analysis of FVIII and its subunit derivatives was performed according to the method described by Burnette (1981). SDS-PAGE was performed according to the method of Laemmli (1970). FVIII subunits in the gel were electrotransferred to nitrocellulose membrane and immunodetection was performed using a polyclonal anti-FVIII-H rabbit antiserum or monoclonal anti-FVIII-L mouse antibody as primary antibodies and peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies as secondary antibodies (Sigma). Immunodetection of FVIII and its subunits was performed by ECL Western blotting protocol (Amersham, Buckinghamshire, U.K.).

Results

Purification of FVIII

A 2,413-fold purification of FVIII from a commercial FVIII concentrate was achieved by immunoaffinity column chromatography (Table 1). Specific activity of the purified FVIII was 2,485 units/mg and thrombin treatment increased FVIII activity about 2 folds. The peak fraction eluted from the immunoaffinity column contained about half of total FVIII activity and the presence of two subunits (FVIII-H and FVIII-L) were identified by Western blot immunodetection analysis in the present study (Figure 1).

Table 1. Purification of FVIII from FVIII concentrate

Step	Total protein (mg)	Total activity (unit)*	Specific activity (unit/mg)	Fold purification
Commercial FVIII concentrate	4,500	3,408	0.76	-
Affinity column (pooled)	1.10	2,010	1,827.30	2,403
Affinity column (peak fraction)	0.45	1,121	2,485.00	3,270

*: FVIII activity was assayed by coamatic factor VIII assay kit and unit of activity represents the amount of cofactor activity of FVIII in 1 ml of standard reference plasma (Sigma Chem. Co., St. Louis, USA).

Treatment of EDTA (25 mM) dissociated FVIII into its subunits, FVIII-H and FVIII-L and thrombin treatment to a major FVIII-H (96 kDa) resulted in 2 peptides of 50 kDa and 40 kDa. Treatment of FVIII-L with thrombin and FXa resulted in the appearance of FVIII-L dervatives of 72 kDa and 65 kDa, respectively (Figure 2).

Restoration of FVIII activity

Recombination of FVIII-H with FVIII-L^{72} restored FVIII activity and the activity was slightly decreased when FVIII-L^{65} was reassembled with FVIII-H (Table 2). Heavy chain treated with thrombin was cleaved into two peptides of 50 kDa (A_1) and 40 kDa (A_2) as shown in Figure 3, and reassociation of A_1, A_2 and FVIII-L^{72} in the presence of $CaCl_2$ (10 mM) and NaCl (100 mM) bought about cofactor activity suggesting the formation of a heterotrimer. Treatment of EDTA (10 mM) to the heterotrimers formed from A_1, A_2 and FVIII-L^{72} or FVIII-L^{65} abolished FVIII activity indicating that the formation of heterotrimer is calcium-dependent.

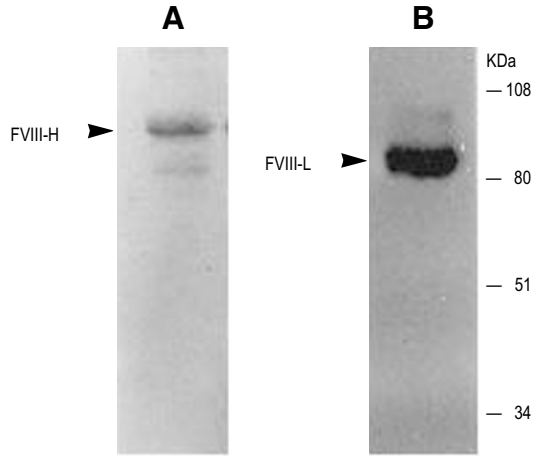

Figure 1. Western blot immunodetection of FVIII protein purified from FVIII concentrate. Protein (10 μg) was subjected to SDS-PAGE and electrotransfered to nitrocellulose membrane. **(A)** Polyclonal anti-FVIII-H antibody was used as a primary antibody. **(B)** Monoclonal anti-FVIII-L antibody was used as a primary antibody. Horse raddish peroxidase-conjugated anti-IgG antibodies corresponding to each primary antibodies were used as secondary antibodies.

Association of FVIII subunits

Reassociation of heavy chain with either of light chain derivatives brought about the formation of heterodimers which have high cofactor activity. The association of FVIII-H and FVIII-L needed $CaCl_2$ (40 mM), and the addition of 2-mercaptoethanol (1.0 mM) increased the rate of association. The association constant of heavy and light chain was 39 ± 7 $M^{-1}sec^{-1}$, and the association constant (28 ± 6 $M^{-1}sec^{-1}$) of heavy chain with thrombin-cleaved light chain (FVIII-L^{72}) was 2 folds faster (Table 3, Figure 4) compared to that (12 ± 3 $M^{-1}sec^{-1}$) of heavy chain with FXa-cleaved light chain (FVIII-L^{65}).

Table 2. Generation of FVIII activity from calcium-dependent reassociation of FVIII subunits and derivatives. FVIII subunits or derivatives were combined and incubated for 16 h at 20°C in a buffer containing 400 mM NaCl, 1% human serum albumin, 20 mM Hepes (pH 7.2) with or without $CaCl_2$ (40 mM). All values are mean of duplicate determinations.

FVIII subunits and derivatives	Ca^{2+} (40mM)	FVIII cofactor activity (unit/ml)
FVIII-H + FVIII-L	+	3.4
FVIII-H + FVIII-L	-	0.4
FVIII-H + FVIII-L^{72}	+	4.1
FVIII-H + FVIII-L^{65}	+	3.7
FVIII-H^{50} + FVIII-H^{40} + FVIII-L^{72}	+	3.3
FVIII-H^{50} + FVIII-H^{40} + FVIII-L^{72}	-	<0.1

Table 3. Association rate constant (k_{assoc}) of FVIII-L derivatives for FVIII-H. Values were obtained from the first order kinetics of association velocity plotted in Figure 4. and from equation, FVIII-H + FVIII-L↔FVIII, $v=k_{assoc}[FVIII\text{-}H][FVIII\text{-}L]$ or $k_{assoc}t=1/a_o\text{-}b_o \ln b_o(a_o\text{-}p)/a_o(b_o\text{-}p)$, where p is the concentration of dimer formed at time t and a_o is the initial concentration of FVIII-H and b_o is the initial concentration of FVIII-L derivative.

FVIII-L derivatives	$k_{assoc}(M^{-1}.sec^{-1})$
FVIII-L (whole)	39 ± 7
FVIII-L72	28 ± 6
FVIII-L65	12 ± 3

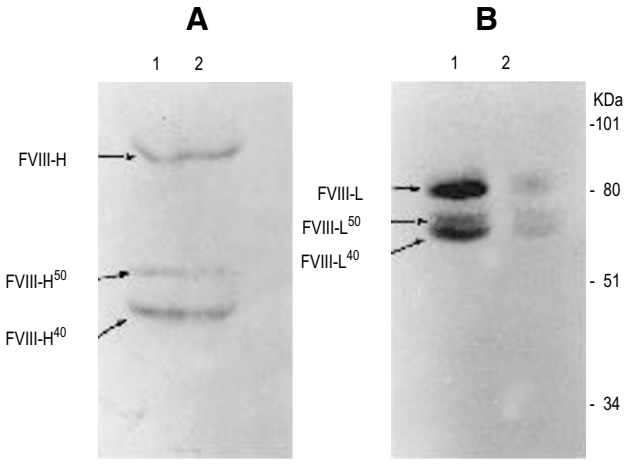

Figure 2. Western blot immunodetection of FVIII-H and FVIII-L proteolyzed with thrombin or FXa. A, FVIII-H (10 μg) treated with α-thronbin (2 units) for 2 h at 37˚C. B, FVIII-L (10 μg) treated with FXa (2 units) for 2 h at 37˚C. The proteolyzed peptides in duplicate (lane 1 and 2 each) were subjected to SDS polyacrylamide gel electrophoresis and electrotransfered to nitocellulose membrane. FVIII-H or FVIII-L was detected using FVIII-H and FVIII-L specific antibodies as in Figure 1.

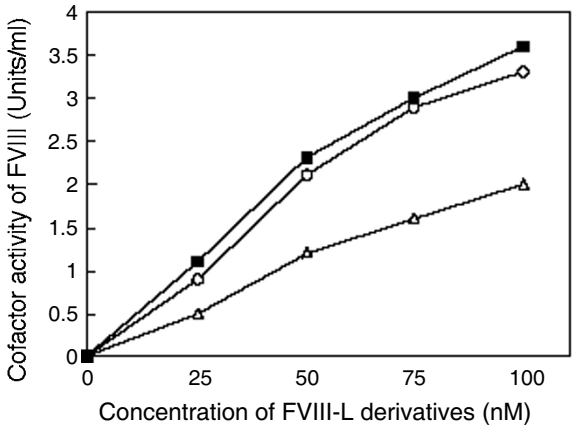

Figure 3. Generation of FVIII activity from complexes of FVIII-H and FVIII-L derivatives. Varying concentration of FVIII-L (■), FVIII-L^{72} (○), and FVIII-L^{65} (△) were reassembled with FVIII-H (200 nM). FVIII subunit and its derivatives were incubated for 1 h in 400 mM NaCl, 40 mM $CaCl_2$,1% human serum albumin, 20 mM Hepes (pH 7.2) and FVIII cofactor activity was assayed according to Coamatic FVIII assay method (Carlebjok *et al*., 1987).

Discussion

An extensive purification of FVIII from FVIII concentrate was achieved by immunoaffinity chromatography in the present study, although it was partially pure. The final product had a specific activity of 2,485 units/mg protein, but further purification has not been attempted in the present study due to the shortage of the concentrate. The overall recovery of FVIII activity during purification was about 60% and the activity in the peak fraction was about 30% with 3,270 folds purification, indicating that immunoaffinity column chromatography was an efficient step to prepare FVIII in a nearly pure form.

The use of an anti-FVIIIRAg (anti-FVIII-related antigen) monoclonal antibody in the preparation of immunoaffinity column had an advantage over anti-FVIIIc antibody since FVIII molecules are tightly bound to von Willebrand factor (vWF) in a mole ratio of 50:1 in human plasma as well as in commercial FVIII concentrate. FVIII molecules are stabilized in plasma in association with von Willebrand factor and they are unlikely to be in free forms (Leyte *et al*., 1989).

FVIII is synthesized as a single peptide having a sequencial domain of A1-A2-B-A3-C1-C2 (Tool *et al*., 1984) of which B domain is known to be unrelated to cofactor activity(Vehar *et al*., 1984). Initial proteolytic activation of FVIII involves the formation of heterodimer of FVIII-H (A1-A2-B) and FVIII-L (A3-C1-C2) after limited proteolysis by thrombin or FXa. A1 is associated noncovalently with A2 and FVIII-L (A3-C1-C2) and is divalent metal ion dependent (Andersson *et al*., 1986).

It has been reported that the molecular weight of FVIII is ranged between 85 and 285 kDa (Hoyer and Trabold, 1981) and that FVIII-H appears as heterogeneous in

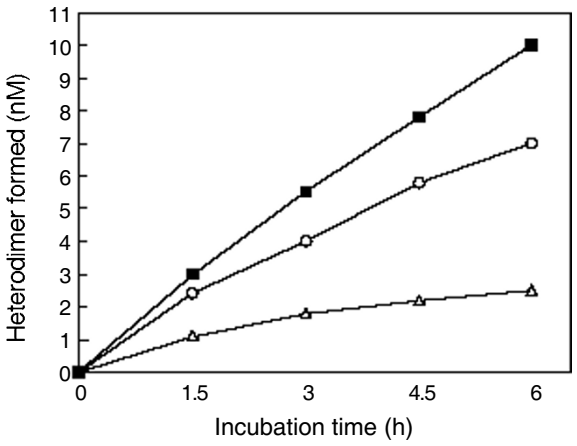

Figure 4. Reassociation of FVIII-H with FVIII-L or its derivatives. FVIII-H (200 nM) was incubated with FVIII-L (■), FVIII-L^{72} (○), or FVIII-L^{65} (△) at 20˚C and heterodimers formed at various incubation time were estimated by assaying the binding of FVIII-L subunits to FVIII-H. The detailed method is described in the text.

molecular weights (122 kDa, 96 kDa and 40 kDa). The purified FVIII in the present study showed FVIII-H as 96 kDa and FVIII-L as 81 kDa when Western blot immunodetection of the EDTA-dissociated subunits of FVIII was performed. This result is consistent with the values reported by Fulcher and Zimmerman (1982).

Thrombin treatment to a major form of FVIII-H (96 kDa) in the present study released two peptides with molecular masses of 50 kDa and 40 kDa indicating the selective cleavage of FVIII-H at Arg^{336} by thrombin. Thrombin treatment to FVIII-L (81 kDa) released a peptide with molecular weight of 72 kDa, a little smaller in size, indicating the cleavage at Arg^{1689} of FVIII-L. Treatment of FXa to FVIII-L released a peptide with molecular weight of 65 kDa and small peptides with molecular weight of 10 kDa, indicating that FXa cleaves at Arg^{1721} of FVIII-L in addition to Arg^{1689}.

The activation process of FVIII is known to involve the limited proteolytic cleavage of FVIII protein by specific proteases such as thrombin and FXa (Harris *et al*., 1981) and further proteolysis by thrombin activated protein C (APC) inactivates FVIII as well as FV (Eaton *et al*., 1986; Fass, 1991). FXa activates FVIII by limited proteolysis at Arg^{336} of FVIII-H which is cleaved also by thrombin, APC, and FIXa. Proteolytic cleavage at both Arg^{336} and Arg^{373} of FVIII-H by thrombin or FXa may release a peptide comprising Ser^{337}-Arg^{372} that will eventually inactivate FVIII (Eaton *et al*., 1986; O'Brien *et al*., 1992; Fay *et al*., 1993).

The increased cofactor activity by addition of sulfhydryl agent (2-mercaptoethanol) to FVIII subunits complex indicates the involvement of disulfide bond formation during the reassembly of FVIII which may increase the stability of subunit complex. The reassociation of thrombin treated FVIII-H and FVIII-L in the presence of calcium suggests that initial activation process of FVIII requires the limited proteolysis at Arg^{336} of FVIII H and Arg^{1689} of FVIII-L and calcium ion dependent heterodimer formation of these subunits.

Restoration of FVIII activity by FVIII-H subunits (50 kDa and 40 kDa) with either of FVIII-L^{72} or FVIII-L^{65} in the present study indicates that activation of FVIII involves the further proteolytic cleavage of FVIII peptides with thrombin or FXa and calcium-mediated heterotrimer formation.

Proteolytic cleavage of FVIII-L at Arg^{1689} by thrombin or at Arg^{1721} by FXa did not influence on the FVIII cofactor activity but the lower association constant of FVIII-L^{72} with FVIII-H compared to that of FVIII-L^{65} with FVIII-H indicates that FVIII-L^{72} has a higher binding affinity to FVIII-H than that of FXa cleaved FVIII-L^{65} has. This result is consistent with that reported by Donath *et al*. (1995).

Although the FVIII cofactor activities between FVIII-L^{65} bound form and FVIII-L^{72} bound form were not much different, amino acid sequence (32 amino acids) between FVIII-L^{65} and FVIII-L^{72} has an important role in the association of subunit molecules to form heterodimer or heterotrimer.

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