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Structural and functional characterization of Factor VIII-ΔII, a new recombinant Factor VIII lacking most of the B-domain

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A recombinant Factor VIII (Factor VIII-ΔII) consists of a unique polypeptide chain of 165 kDa deleted from the major part of the B-domain and from the cleavage site at Arg-1648–Glu-1649 found in plasma-derived Factor VIII. It was expressed in mammalian cells in serum-free medium containing von Willebrand factor and purified by a one-step immunopurification. The recombinant Factor VIII was characterized as a single active peak when subjected to f.p.l.c., in contrast with the plasma-derived molecule. Its coagulant activity was decreased in the presence of EDTA, suggesting that a bivalent ion is required, as for plasma-derived Factor VIII. The activation by thrombin and the inactivation by activated protein C were studied and the resulting molecular forms were analysed by f.p.l.c. and SDS/PAGE. The results clearly demonstrate that, despite the structural differences between plasma-derived and recombinant Factor VIII, activation and inactivation of Factor VIII-ΔII generate proteolysed complexes similar to that described for plasma-derived Factor VIII. Thus this deleted recombinant Factor VIII, which is processed similarly to plasma-derived Factor VIII, should be normally integrated in the regulation system of Factor X activation in the blood-coagulation cascade.

INTRODUCTION

Factor VIII is a glycoprotein which acts as a cofactor of Factor IXa to activate Factor X in the intrinsic pathway of blood coagulation. In plasma, it circulates at a very low concentration and is bound to von Willebrand factor (vWf) (Hoyer, 1981; Kane & Davie, 1988). Some sequence modifications or a deficiency in Factor VIII will cause a bleeding disorder called haemophilia A.

The unproteolysed polypeptide chain of Factor VIII (330 kDa) contains 2332 amino acids organized in three domains, A, B, and C, in the following order: A1–A2–B–A3–C1–C2 (Vehar *et al.*, 1984). When purified from plasma, it is composed of two chains non-covalently associated via a bivalent-cation bridge whose functional importance has been demonstrated (Fass *et al.*, 1982; Tran & Duckert, 1983; Andersson *et al.*, 1986; Burke *et al.*, 1986; Eaton *et al.*, 1987; Fay, 1987; Nordfang & Ezban, 1988). The heavy chain, which corresponds to the *N*-terminus of the molecule, has a molecular mass ranging from 210 to 90 kDa. The 80 kDa light chain corresponds to the *C*-terminus of the protein (Eaton *et al.*, 1986*a*). The B-domain contains most of the potential *N*-linked glycosylated sites of the molecule (19 of the 25) and is not required for the procoagulant activity (Toole *et al.*, 1986; Burke *et al.*, 1986). Furthermore, this domain is susceptible to degradation, generating the different forms of the heavy chain from 210 (including the B-domain) to 90 kDa (without the B-domain).

Factor VIII activity is regulated by some effectors such as thrombin and activated protein C (APC) (Kane & Davie, 1988). Activation by thrombin and inactivation by APC generates specific fragments by proteolytic cleavages. During the activation process, the cleavage of all the high-molecular-mass forms (210–90 kDa) generates fragments of 50 and 45 kDa.

The light chain of 80 kDa is also processed to produce a 70 kDa polypeptide (Fulcher *et al.*, 1983; Toole *et al.*, 1984; Rotblat *et al.*, 1985; Fay *et al.*, 1986). The inactivation of Factor VIII by APC directly correlates with the generation of a 43 kDa fragment from the *N*-terminus of the heavy chain (Fulcher *et al.*, 1984; Eaton *et al.*, 1986*a*).

Cloning of the gene and its expression in mammalian cells have provided a greater insight into its molecular structure and processing (Eaton *et al.*, 1986*b*; Pittman & Kaufman, 1988). Deletion of the heavily glycosylated B-region of Factor VIII improved its secretion by mammalian cells (Kaufman *et al.*, 1988).

The 'deleted' Factor VIII, Factor VIII-ΔII (Meulien *et al.*, 1988), does not contain the sequence Pro-771–Asp-1666 of the natural Factor VIII, which includes the major part of the B-domain as well as the proteolytic cleavage site at Arg-1648–Glu-1649 (Fig. 1). In contrast with some other deleted recombinant Factor VIII molecules (Kaufman *et al.*, 1988), Factor VIII-ΔII is able to bind vWf (Meulien *et al.*, 1988; Leyte *et al.*, 1989). Consequently, this new protein can be stabilized by vWf. Factor VIII-ΔII consists of a single polypeptide chain of 165 kDa. It contains the heavy chain (90 kDa), the *N*-terminal part of the B-domain, and the major part of the light chain corresponding to domains A1–A2–part B–A3–C1–C2. Furthermore, thrombin and APC cleavage sites, described for plasma-derived Factor VIII, remain present.

The present paper describes the immunopurification and characterization of this purified recombinant Factor VIII-ΔII. The effects of thrombin, APC and EDTA are analysed. This new

Abbreviations used: APC, activated Protein C; vWf, von Willebrand factor; PBS, phosphate-buffered saline (0.1 M-phosphate/0.15 M-NaCl, pH 7.2).

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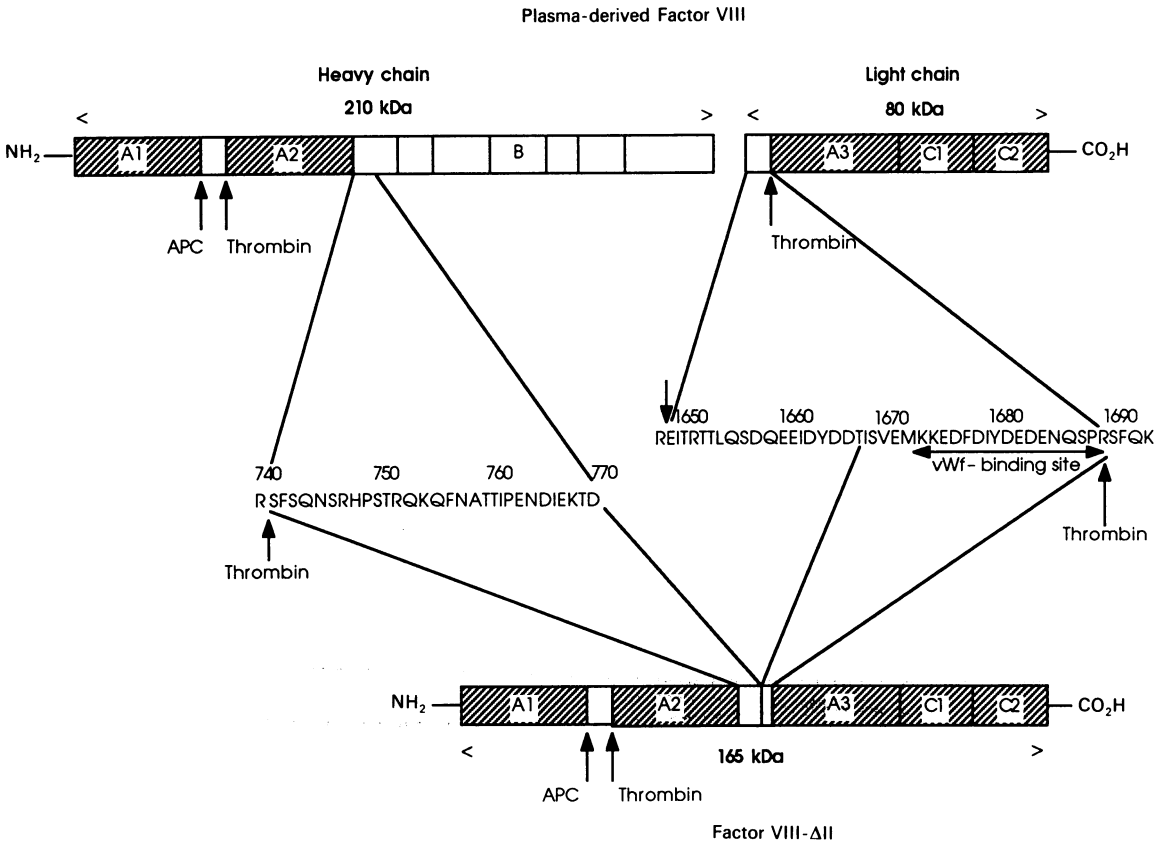


Fig. 1. Comparison of plasma-derived Factor VIII and Factor VIII-ΔII

Boxes correspond to the different domains (A1, A2, A3, B, C1 and C2) of Factor VIII. Thrombin and APC cleavage sites are indicated by the arrows. The single-letter notation is used for the different amino acids.

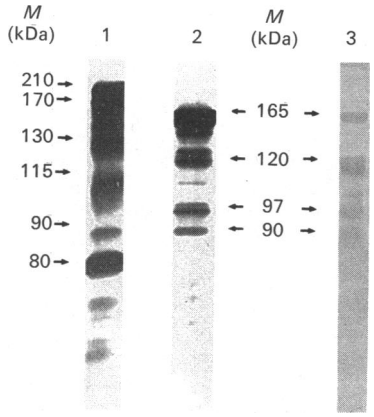


Fig. 2. SDS/PAGE analysis of the immunopurified plasma-derived Factor VIII (lane 1) and Factor VIII-ΔII (lane 2)

A sample of the eluted fraction of Factor VIII was submitted to electrophoresis using a 6–12% (w/v) gradient polyacrylamide gel, and then the gel was silver-stained. Lane 3 represents the corresponding immunoblot of Factor VIII-ΔII revealed by an anti-(heavy chain) monoclonal antibody (CAG1-175A7). Abbreviation in this and succeeding Figures: *M*, molecular mass.

deleted molecule is compared with plasma-derived Factor VIII in terms of structure–function relationships.

MATERIALS AND METHODS

Activity assays

Factor VIII-ΔII procoagulant activity was measured by the 'one-stage clotting' assay using a kaolin-activated method (Langdell *et al.*, 1953), with Factor VIII concentrate (generously given by Dr. A. Gaillandre, Centre National de Transfusion Sanguine) as a standard assessed to 1 i.u./ml. The same assay was used for activated and inactivated Factor VIII.

vWf antigen was determined by an e.l.i.s.a. using a 'sandwich' technique (Asserachrom vWf; Diagnostica Stago, Franconville, France). Protein concentrations were determined as described by Bradford (1976), with BSA (Bio-Rad) as a standard. Specific activity was calculated as coagulant activity ('one-stage clotting' assay)/mg of protein.

Factor VIII antigen

Factor VIII-ΔII antigen was measured by e.l.i.s.a. on microtitre plates (Immulon II; Dynatech). Wells were coated with an anti-(light chain) monoclonal antibody, CAG-1 180-A13 (Croissant *et al.*, 1986; Sauger *et al.*, 1986). The immunoglobulin (1.2 μg of IgG/ml), in 0.1 M-sodium carbonate buffer, pH 9.6 (100 μl/well) was incubated for 2 h at 37 °C and overnight at +4 °C. The plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (v/v) and then saturated with 1% human serum albumin (Biotransfusion, Les Ulis, France) in PBS. Serial dilutions of pooled normal plasma and test samples were incubated for 3 h at 37 °C in 0.05 M-imidazole buffer, pH 7.4, containing 1 M-NaCl, 0.1% BSA (Sigma) and 0.1% Tween 20. The second antibody, CAG-1 175A7, was a peroxidase-conjugated anti-(heavy chain) antibody. It was diluted 1:2000 in 0.05 M-imidazole buffer, pH 7.4, containing 0.15 M-NaCl, 0.1% BSA and 0.1% Tween 20, and added for 2 h at 37 °C.

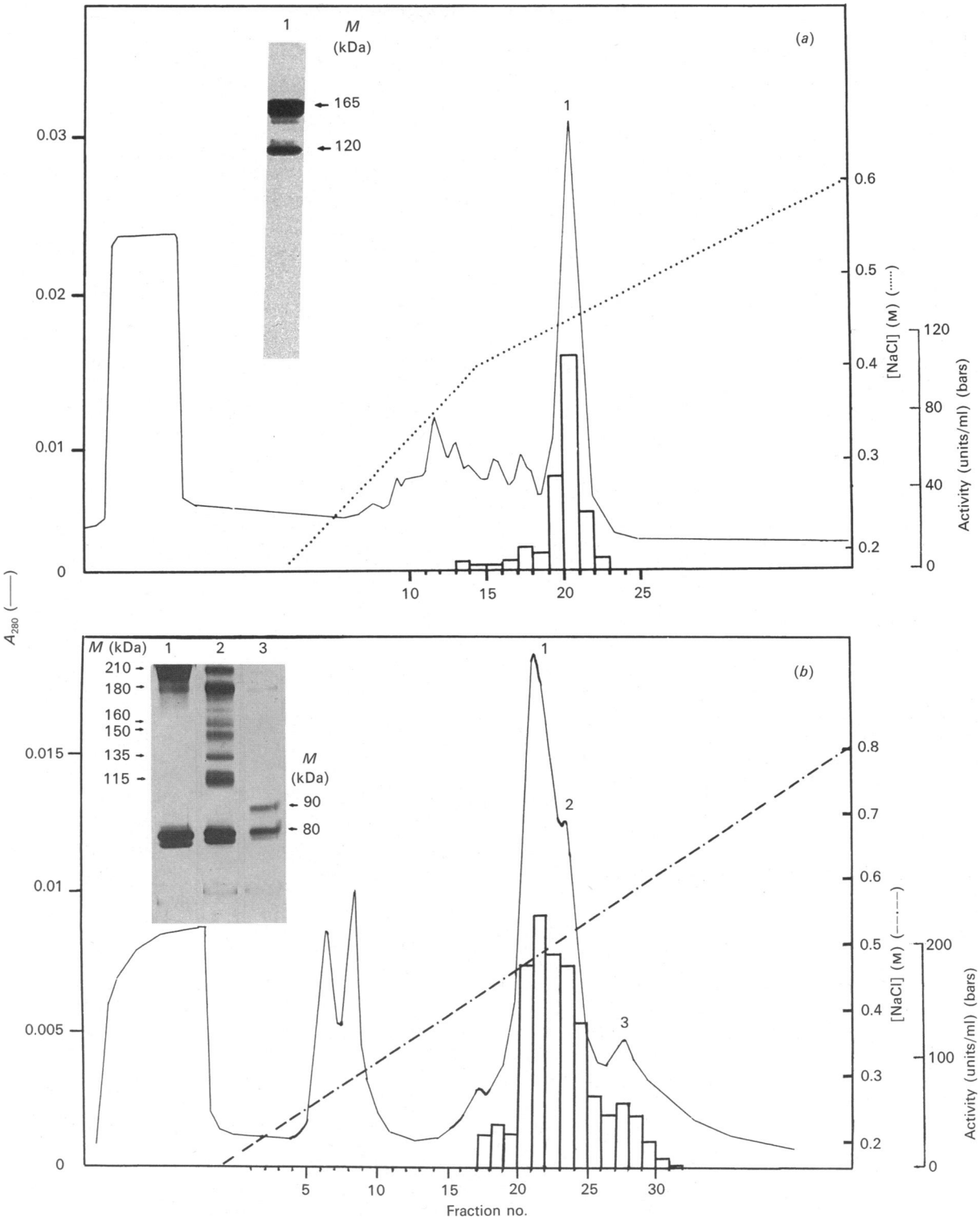


Fig. 3. F.p.l.c. and SDS/PAGE characterization of immunopurified Factor VIII-ΔII and plasma-derived Factor VIII

(*a*) Chromatographic pattern of recombinant Factor VIII on a Mono Q column (HR 5/5); 20 ml (360 units) was applied to the column at a flow rate of 0.5 ml/min. After the final injection, the column was washed with 10 ml of equilibration buffer (20 mM-Tris/HCl/0.15 M-NaCl/10 mM-$CaCl_2$, pH 7.2), and a gradient of 0.15–1 M-NaCl (dotted line) was initiated. Fractions were collected at 1 min intervals and assayed for clotting activity (represented by the histogram bars). Absorbance was measured as a continuous trace. Inset: SDS/PAGE analysis of fractions obtained by f.p.l.c. separation. Aliquots of each fraction were submitted to electrophoresis using a 6–12 % (w/v) gradient gel; then the gel was silver-stained. Lane 1 represent the corresponding peak from the Mono Q chromatography step. (*b*) Chromatographic pattern and SDS/PAGE analysis of plasma-derived Factor VIII. Lanes 1, 2 and 3 correspond to peaks 1, 2 and 3 of the f.p.l.c. pattern respectively.

Peroxidase activity was revealed with *o*-phenylenediamine in 0.05 M-citrate buffer, pH 5.6, containing 0.03 % (v/v) H_2O_2. The enzymic reaction was stopped with 2 M-H_2SO_4 and the A_{492} was read (Multiscan II Labsystem, Les Ulis, France). The calibration was linear between 5 and 250 munits/ml and had a lower limit of detection of 5 munits/ml.

Culture medium

Factor VIII-ΔII was expressed in a Chinese-hamster ovary (CHO) cell line in a serum-free medium containing a recombinant vWf (1 unit/ml). The ratio of vWf to Factor VIII antigen was 1:4.

Purification of recombinant Factor VIII-ΔII

Recombinant Factor VIII was purified from cell-culture supernatants by using a monoclonal immunosorbent column. The monoclonal antibody AMC-463 (CAG1 463A8), elicited against Factor VIII light chain (Croissant *et al.*, 1986; Sauger *et al.*, 1986), was purified from mouse ascites using DEAE-Affi-gel Blue column (Bio-Rad). Immunosorbent was obtained by coupling monoclonal antibody AMC-463 to CNBr-activated Sepharose 4B (Pharmacia) at a ratio of 0.5 mg of IgG/ml of gel; 700 units of recombinant Factor VIII were loaded/ml of immunosorbent.

After adsorption the column was washed with a 200 mM-imidazole buffer, pH 7.2, containing 150 mM-NaCl, 100 mM-lysine, and 350 mM-$CaCl_2$ to release the vWf (Cooper *et al.*, 1973).

Factor VIII-ΔII was eluted with a buffer containing 10 mM-histidine, 20 mM-imidazole, 50% (v/v) ethylene glycol, 1 mM-$CaCl_2$ and 2 M-NaCl, pH 7.0, which was then removed by gel filtration on a Sephadex G-25 (Pharmacia) column in a 20 mM-Tris buffer, pH 7.2, containing 150 mM-NaCl and 10 mM-$CaCl_2$ (referred to as 'buffer A').

Plasma-derived Factor VIII was purified from commercial concentrates under the same conditions.

F.p.l.c. characterization

Immunopurified Factor VIII-ΔII and plasma-derived Factor VIII were subsequently subjected to f.p.l.c. on an anion-exchanger (Mono Q) column (HR 5/5; Pharmacia), equilibrated in buffer A and eluted with a gradient (0.15–1 M-NaCl) at a flow rate of 0.5 ml/min at room temperature. Some of the eluted fractions were analysed by SDS/PAGE and tested for coagulant activity. Immunopurified activated and inactivated Factor VIII-ΔII were chromatographed under the same conditions.

Electrophoresis

SDS/PAGE was performed as described by Laemmli (1970), a 3% (w/v) stacking gel with a 6–12% (w/v) gradient separating gel being used. To samples (150 μl) were added 10% (w/v) SDS (30 μl) and Bromophenol Blue (10 μl); the mixture was heated for 5 min at 95 °C under reducing conditions and applied to the gels. A mixture of high- and low-molecular-mass proteins (Bio-Rad) were used as markers. Gels were run at 70 V for 16 h and silver-stained (Morrisey, 1981). Gels were then read with a laser scanning densitometer (Preference; Sebia Issy-les-Moulineaux, France).

Western blotting

Proteins were transferred on to a nitrocellulose membrane (Bio-Rad) using a Novablot system (LKB) at a constant current of 0.8 mA/cm² for 5 h in 0.025 M-Tris/HCl/0.192 M-glycine/20% (v/v) methanol/0.1% (w/v) SDS buffer, pH 8.3.

The membrane was incubated with an anti-(heavy chain) monoclonal antibody, CAG1-175A7 (Sauger *et al.*, 1986) and a goat anti-mouse IgG–horseradish peroxidase conjugate (Bio-Rad). H_2O_2 and 4-chloro-1-naphthol (Sigma) were added as substrate and chromogen respectively to reveal reacting proteins.

Thrombin activation

Purified human α-thrombin (Behring; 1100 units/mg specific activity; 37 and 29 kDa) was added to purified recombinant Factor VIII-ΔII (1 unit of thrombin/10 units of Factor VIII-ΔII) in buffer A. The solution was incubated at room temperature. Coagulant activity was assessed, and SDS/PAGE analysis was performed, on samples removed every 5 min.

Inactivation by APC

Human APC was generously given by Dr. J. Amiral (Diagnostica Stago). Plasma-derived Factor VIII and Factor VIII-ΔII (30 units/ml) in a 0.02 M-Tris buffer, pH 7.5, containing 0.15 M-NaCl, 0.01 M-$CaCl_2$ and 5% (v/v) glycerol, were incubated at 37 °C with purified APC (4 μg/ml) and with rabbit brain cephalin (Sigma) (1 vol. to 20 sample vol.). Proteolytic fragments were characterized by SDS/PAGE.

EDTA treatment

Purified samples of activated or non-activated Factor VIII-ΔII were incubated at room temperature for 150 min in buffer A supplemented with 50 mM-EDTA. The coagulant activity was measured every 10 min.

RESULTS

Purification of Factor VIII-ΔII and f.p.l.c. analysis

The immunopurification of Factor VIII-ΔII increased its specific activity by a factor of 1000. The plasma-derived Factor

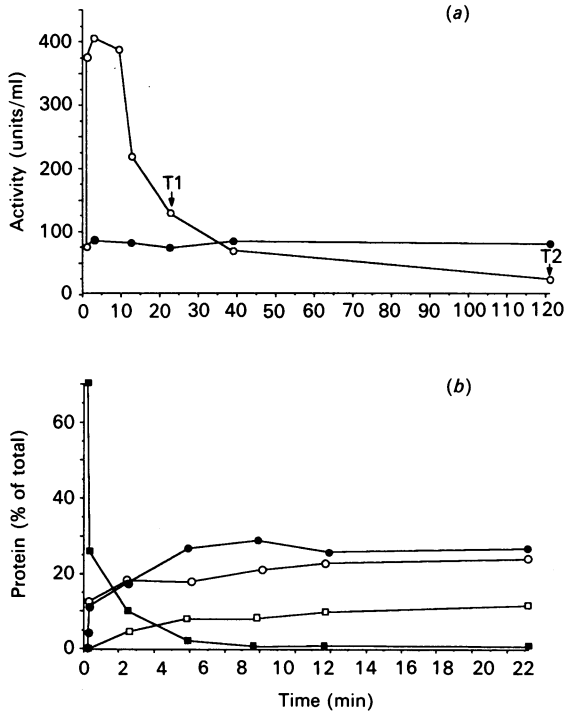


Fig. 4. Activation by thrombin of the immunopurified Factor VIII-ΔII

(*a*) The stability of recombinant Factor VIII was measured by the 'one-stage clotting' assay at different time intervals for 120 min at 4 °C (●). Factor VIII (2 ml; 80 units/ml) was incubated with thrombin (1 unit/10 units of Factor VIII), and the activity was measured at each time (○). After 5 min, coagulant activity was 420 units/ml, which represented a 5-fold activation, and was only 19 units/ml after 2 h. (*b*) Kinetics of activation. At different time intervals the different polypeptide chains were characterized by SDS/PAGE and the intensity of each band was scanned. Also at different time intervals the concentrations of the 165 kDa Factor VIII-ΔII (■) and the cleaved 70 kDa (●), 50 kDa (○) and 45 kDa (□) forms were measured.

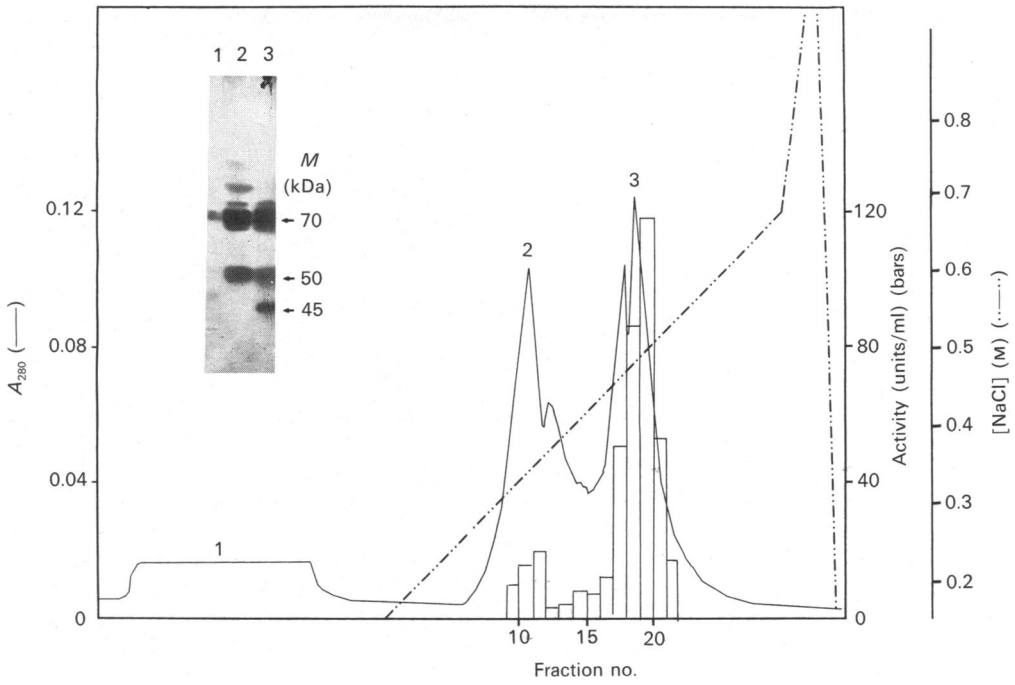


Fig. 5. F.p.l.c. and SDS/PAGE analysis of the activated immunopurified Factor VIII-ΔII

F.p.l.c. analysis was performed by using the same conditions as those in Fig. 3(*a*); 36 ml of activated Factor VIII (1800 units) were applied to the mono Q column. Under these conditions, thrombin was not retained on the ion-exchanger. Inset: SDS/PAGE analysis of activated Factor VIII-ΔII: lane 1, non-adsorbed fraction; lanes 2 and 3, peaks 2 and 3 of the f.p.l.c. pattern respectively.

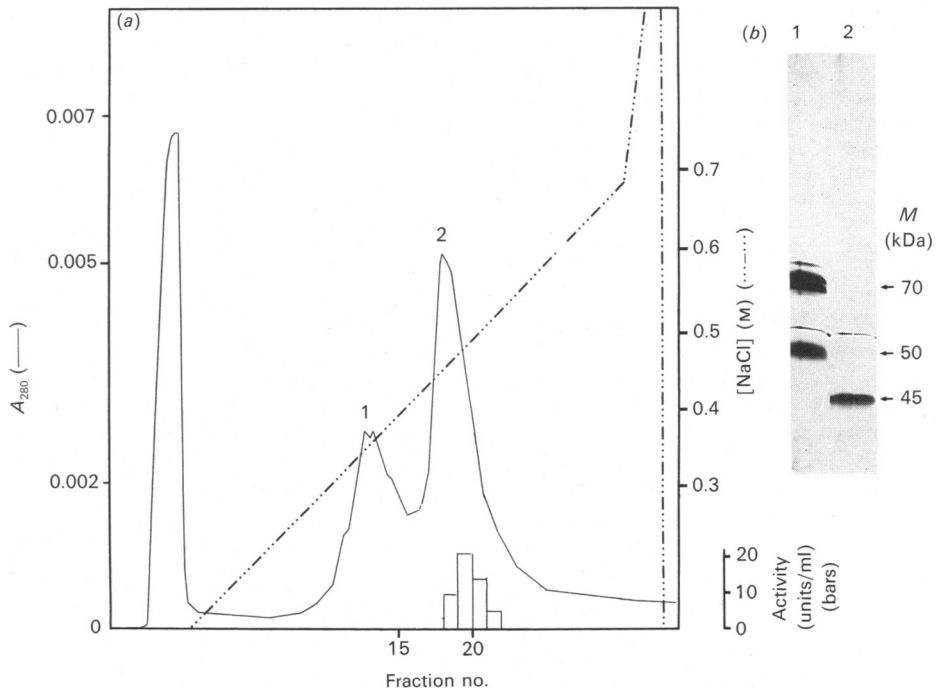


Fig. 6. F.p.l.c. and SDS/PAGE analysis of inactive immunopurified Factor VIII-ΔII

(*a*) F.p.l.c. analysis was performed using the same conditions as those in Fig. 3(*a*); 4 ml of inactivated Factor VIII (T2, Fig. 4*a*) was applied to the Mono Q column. (*b*) SDS/PAGE analysis. Lanes 1 and 2 correspond to peaks 1 and 2 of the f.p.l.c. pattern.

VIII and Factor VIII-ΔII purified under the same conditions were compared by SDS/PAGE (Fig. 2). Different heavy chains of molecular masses ranging from 210 to 90 kDa, and the 80 kDa light chain of the plasma-derived product, were seen (Fig. 2, lane 1). The recombinant Factor VIII exhibited two major bands at 165 and 120 kDa and two minor bands at 97 and 90 kDa (Fig. 2, lane 2). The Western-blot analysis (Fig. 2, lane 3) confirmed that these different bands correspond to Factor VIII-ΔII.

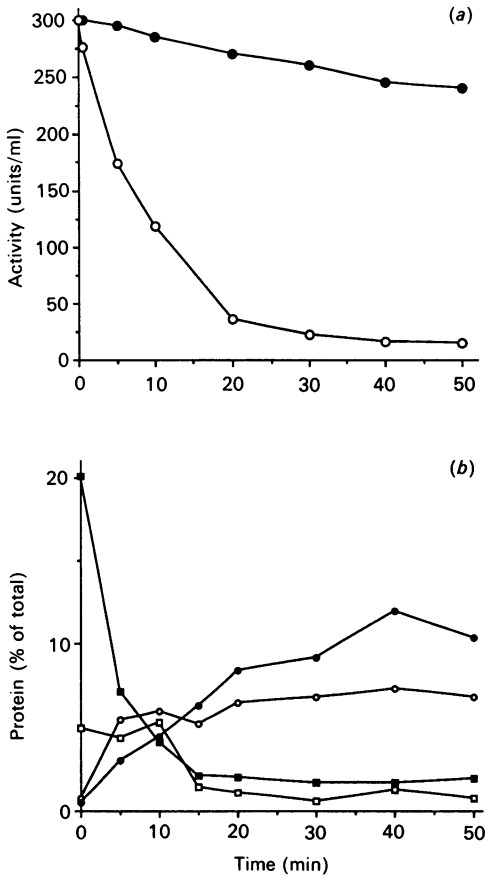


Fig. 7. Inactivation of the immunopurified Factor VIII-ΔII by APC

(*a*) Activity was measured by the 'one-stage clotting' assay, at different time intervals for 50 min during incubation with (○) and without (●) APC (4 μg/ml). (*b*) Kinetics of inactivation. The different species were characterized by SDS/PAGE. The gel was analysed as described in Fig. 4(*b*). Concentrations of 165 kDa (■), 120 kDa (□), 78 kDa (●) and 43 kDa (○) polypeptides are expressed with respect to the total protein concentration for each lane.

The immunopurified Factor VIII-ΔII preparation was further purified by f.p.l.c. The clotting activity was eluted at 0.45 M-NaCl, and the recovery was 70% (Fig. 3*a*).

By contrast, the immunopurified plasma-derived preparation (Fig. 3*b*) produced three peaks of activity that were eluted between 0.5 and 0.55 M-NaCl. This chromatographic step resulted in a 6-fold concentration of Factor VIII-ΔII activity, and the specific activity reached 10000 units/mg (as found for plasma-derived Factor VIII). vWf, described as the natural carrier and stabilizer of Factor VIII (Weiss *et al.*, 1977), was measured in all the eluted fractions. The results indicate that the ratio of Factor VIII to vWf was increased 8000-fold after the purification process. Different eluted fractions were stored at 4 °C in order to study the relative stability of the protein. After 24 h, 80% of the initial activity of this highly purified recombinant Factor VIII remained.

Fractions corresponding to peak 1 were characterized by SDS/PAGE. Fig. 3(*a*) shows that they consisted of a major polypeptide of 165 kDa. A fragment of 120 kDa was also observed, but its concentration was not correlated with Factor VIII activity. By contrast, a good correlation was observed between the intensity of the 165 kDa polypeptide stained on the gel and the activity measured for each fraction of the peak.

Characterization of plasma-derived Factor VIII (Fig. 3*b*) shows that the active fractions contained two different polypeptides, ranging from 210 to 80 kDa (peak 1) and from 90 to 80 kDa (peak 3).

Thrombin activation of purified recombinant Factor VIII

Kinetics of activation. Purified Factor VIII-ΔII was activated by human thrombin. The kinetics of activation (Fig. 4*a*) showed a rapid increase in Factor VIII activity, reaching a maximum 5 min after the addition of thrombin. The activity measured after 22 min was almost the same as the initial activity (T1) and fell to 24% after 2 h (T2).

These changes in coagulant activity correlated with changes in protein structure, as revealed by the densitometric scans of the electrophoresis gel (Fig. 4*b*). During the activation step, the 165 kDa polypeptide fragment gradually disappeared. Simultaneously, polypeptides of 50, 45 and 70 kDa appeared. After 5 min, and during the whole of the inactivation phase after the activation process (Fig. 4*a*), these three fragments were not further cleaved by thrombin (Fig. 4*b*).

Analysis of activated recombinant Factor VIII. Activated recombinant Factor VIII (T1, Fig. 4*a*) was characterized by f.p.l.c. (Fig. 5). The chromatographic pattern showed two main peaks which were eluted at 0.35 and 0.5 M-NaCl. The clotting activity was essentially found in fractions 17–22 (Fig. 5, peak 3). These activated fractions, resolved by f.p.l.c., consisted of 70, 50 and 45 kDa polypeptides. Earlier fractions (fractions 10–14; peak 2) corresponded to inactive complexes of 70–50 kDa.

Furthermore, the 70 kDa proteolytically cleaved chain was not retained by the anion-exchanger under these experimental conditions (Fig. 5, lane 1). Previous results described for activated plasma-derived Factor VIII (Bihoreau *et al.*, 1989) have shown a similar f.p.l.c. pattern, with two peaks also eluted at 0.35 and 0.5 M-NaCl. They corresponded respectively to the same inactive form of 70–50 kDa and the activated 70 kDa–50 kDa–45 kDa complex.

Factor VIII-ΔII obtained at the end of the inactivation phase (Fig. 4*a*, T2) was characterized by f.p.l.c., as shown in Fig. 6. A residual activity was found in fractions 19–22, corresponding to peak 2 eluted at 0.5 M-NaCl. SDS/PAGE analysis indicated that, similar to the previous characterization (Fig. 5), the 70–50 kDa inactive complexes were present in the first peak eluted at 0.35 M-NaCl (Fig. 6, lane 1). In the fractions eluted at 0.5 M-NaCl, only a 45 kDa fragment was observed (Fig. 6, lane 2). The residual activity in those fractions could be due to a minor proportion of active complexes not revealed by staining.

The inactivation process was also studied from the previously purified 70 kDa–50 kDa–45 kDa complex (Fig. 5, fraction 20). After a 4-fold decrease in activity, fraction 20 was subjected to f.p.l.c. The chromatographic pattern, similar to the previous one, consisted of three peaks: the non-adsorbed fraction and two peaks eluted at 0.35 and 0.5 M-NaCl. This result suggests that the polypeptides in these two peaks came from the initial activated 70 kDa–50 kDa–45 kDa complex.

Inactivation of purified recombinant Factor VIII by APC

Kinetics of inactivation. Purified Factor VIII-ΔII was inactivated by APC. Kinetic studies revealed that 90% of the initial Factor VIII activity was lost within 30 min (Fig. 7*a*). SDS/PAGE analysis showed that this loss of activity was correlated with modification in the polypeptide distribution. Fig. 7(*b*) presents the densitometric scans of the electrophoresis gel. Factor VIII inactivation led to the disappearance of the 165 and 120 kDa proteins to undetectable amounts within 20 min. This occurred with the appearance of 78 and 43 kDa polypeptides

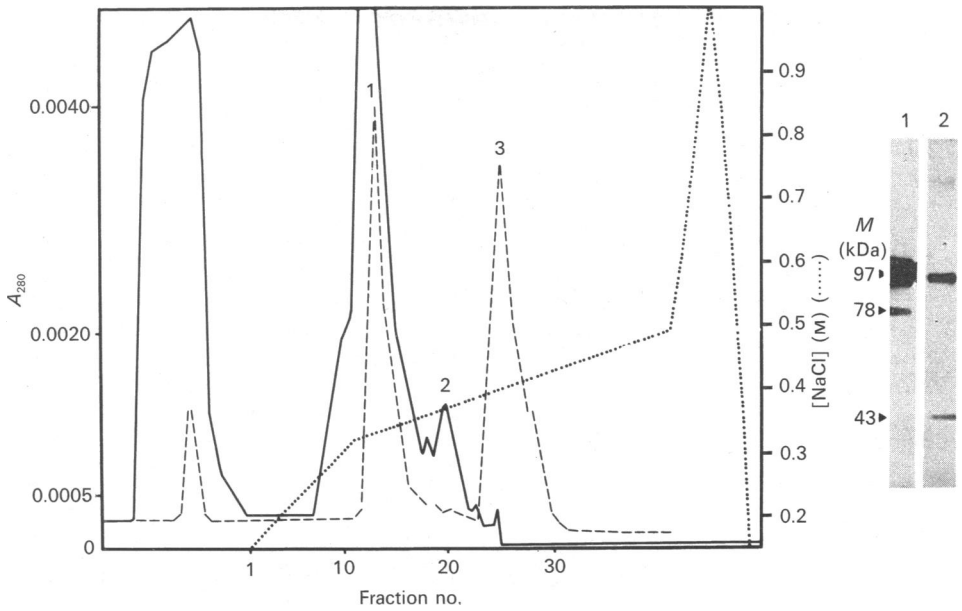


Fig. 8. Analysis of the immunopurified Factor VIII-ΔII inactivated by APC

(*a*) F.p.l.c. analysis of non-treated (broken line) and inactivated (continuous line) Factor VIII-ΔII was performed using the same conditions as those described in Fig. 3(*a*). (*b*) SDS/PAGE analysis: lanes 1 and 2, peaks 1 and 2 of the f.p.l.c. pattern.

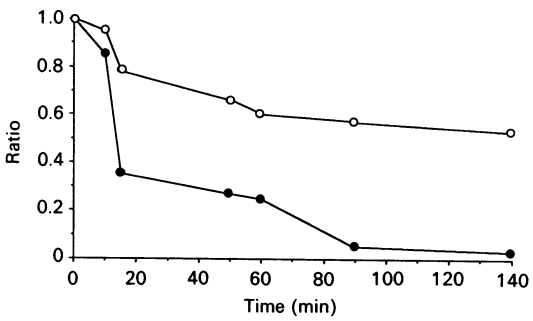


Fig. 9. Effect of EDTA on Factor VIII-ΔII activity

Activated (●) or normal (○) immunopurified Factor VIII was incubated with EDTA. The activity was measured for 140 min. At each time the ratio is defined as Factor VIII activity with EDTA/Factor VIII activity without EDTA.

(Fig. 7*b*). In contrast, the 97 kDa fragment was not proteolytically cleaved by APC.

Analysis of inactivated recombinant Factor VIII. The f.p.l.c. profiles obtained with the APC-treated preparation and non-treated Factor VIII-ΔII are shown in Fig. 8. After the action of APC, the initial active peak, which corresponded to the 165 kDa Factor VIII-ΔII, disappeared (peak 3), and a new inactive peak, eluted at 0.37 M-NaCl, was generated (peak 2). This new peak corresponded to the cleaved fragment of 43 kDa, as revealed by SDS/PAGE analysis (Fig. 8, lane 2).

The presence of the 97 kDa polypeptide in the same fraction was due merely to a residual contamination from the previous peak (peak 1). The 78 kDa polypeptide, generated after APC treatment, was also found in the peak 1 (Fig. 8, lane 1).

Effect of EDTA

The sensitivity of purified Factor VIII-ΔII to a bivalent-ion-chelating agent was tested. Fig. 9 shows that the treatment of recombinant Factor VIII (165 kDa) with EDTA resulted in a decrease of the coagulant activity to 40% in 140 min. The purified activated complex (Fig. 5, fraction 20) was tested under the same conditions. During incubation with EDTA a rapid decrease of the coagulant activity was also observed.

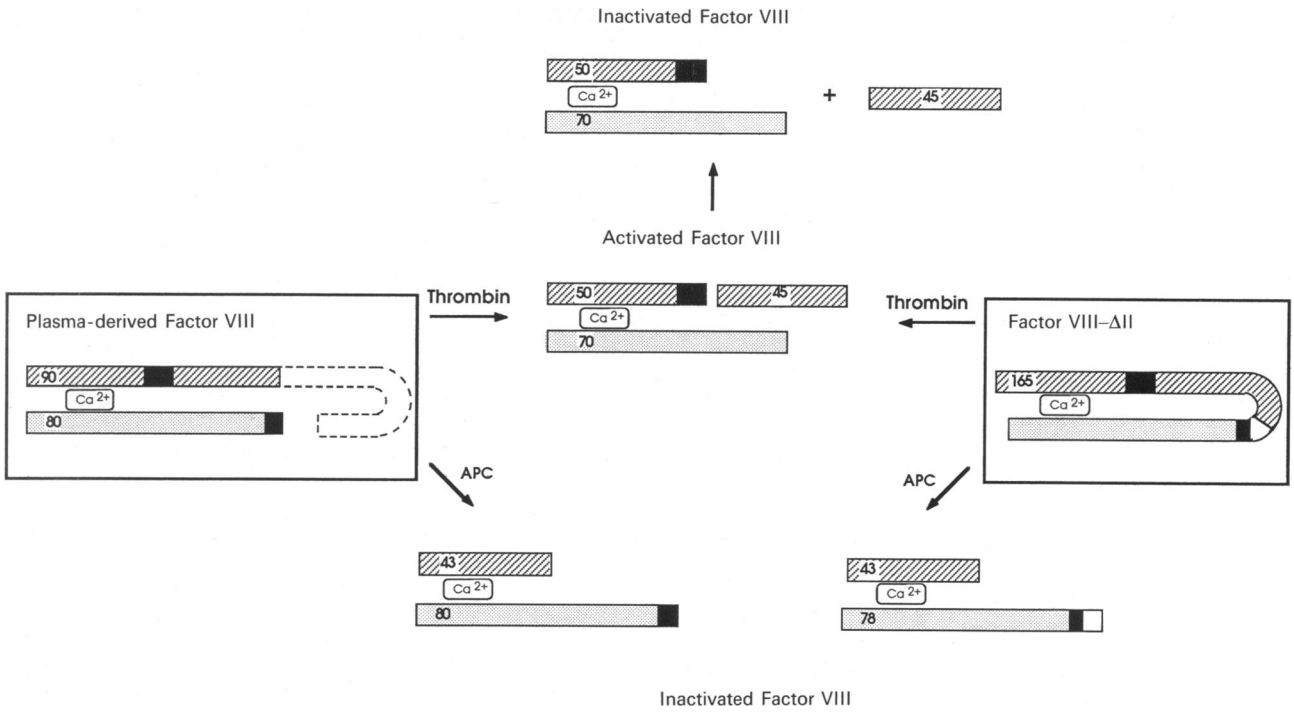
DISCUSSION

The recombinant Factor VIII-ΔII molecule is structurally different from plasma-derived Factor VIII. This single-chain protein lacks the major part of the B-domain and the protein-cleavage site Arg-1648–Glu-1649. Considering that Factor VIII acts in the coagulation cascade in its proteolytically cleaved form, it was important to compare the functional and structural features of the activated Factor VIII-ΔII with those of plasma-derived Factor VIII.

Factor VIII-ΔII has been previously identified in cell-culture supernatant as a unique polypeptide chain of 165 kDa (Meulien *et al.*, 1988). It was purified by a one-step immunopurification. Western-blot analysis showed that the highly purified product contained a major polypeptide of 165 kDa. Other polypeptides, of 120, 97 and 90 kDa, were also identified as Factor VIII, indicating proteolysis of the product. Since the 97 and 90 kDa fragments have been revealed by the CAG-175A7 antibody, they should correspond to the A1–A2–part B and A1–A2 heavy chains respectively. The 120 kDa polypeptide was also revealed by the anti-(heavy chain) antibody, and since it is cleaved by APC, it should contain the full light chain. The fragment of 120 kDa could correspond to A2–part B–A3–C1–C2.

The effect of EDTA on recombinant Factor VIII suggested that this active single chain contains a bivalent cation, as well as plasma-derived Factor VIII (Nordfang & Ezban, 1988), and it may be a structural prerequisite for coagulant activity.

Characterization of the recombinant protein by f.p.l.c. and SDS/PAGE showed that all the active Factor VIII-ΔII was eluted in a single peak corresponding to the 165 kDa polypeptide (Fig. 3*a*). In contrast, plasma-derived Factor VIII has been resolved in multiple active peaks related to different active complexes (Fig. 3*b*). These results reflect the homogeneity of the recombinant molecule caused by the absence of the B-domain.



Scheme 1. Effect of thrombin and APC on plasma-derived Factor VIII-ΔII

For each form, the heavy chain ((▨), light chain (▩), B domain (⬚) and acidic regions (■) are indicated. The values represent the molecular masses (kDa) of each fragment.

The structural differences between Factor VIII-ΔII and plasma-derived Factor VIII were also revealed by their ability to bind to an ion-exchange matrix. It was reported previously that the 90–80 kDa complex from plasma-derived Factor VIII (without the B-domain), which is structurally close to Factor VIII-ΔII, was eluted from the Mono Q column at 0.55 M-NaCl. The 165 kDa recombinant protein was comparatively less retained on the anion-exchanger under the same conditions. This result can be correlated with differences in the global charge of the two proteins. The increase of Factor VIII-ΔII basicity is consistent with the loss of three acidic residues and the gain of three basic ones, compared with the 90–80 kDa polypeptide. The stability of purified Factor VIII-ΔII observed, without addition of any stabilizing reagent and in the presence of only trace amounts on vWf, is similar to that of plasma-derived Factor VIII (results not shown). This result suggests that, for the stabilization of Factor VIII, the B-domain is dispensable.

Thrombin activation of purified Factor VIII-ΔII was shown to be associated with specific proteolysis. Similar to plasma-derived Factor VIII (Eaton *et al.*, 1986*a*, 1987; Bihoreau *et al.*, 1989), recombinant Factor VIII is cleaved by thrombin, generating the 50 and 45 kDa fragments from the heavy chain and the 70 kDa cleaved light chain.

The activatability of Factor VIII-ΔII, which does not contain the cleavage site at Arg-1648–Glu-1649, suggests that the generation of a 80 kDa fragment, observed in plasma-derived Factor VIII, is not required for the formation of the 70 kDa fragment. This result fully agrees the observation that a variant obtained by site-directed mutagenesis at Arg-1648 (A1648I) was activated without the formation of the 80 kDa light chain (Pittman & Kaufman, 1988). The structural analysis of Factor VIII-ΔII cleaved by thrombin also confirms that the B-domain is not required for activation. Nevertheless, several authors (see, e.g., Eaton *et al.*, 1986*b*) have shown that this region inhibits thrombin action.

The recombinant and plasma-derived 70 kDa–45 kDa–50 kDa activated complexes showed identical behaviour on f.p.l.c. (Fig. 5; Bihoreau *et al.*, 1989), suggesting close similarity. This similarity is essential, since Factor VIII acts, as the cofactor of Factor IXa, in this activated form of 70 kDa–45 kDa–50 kDa. The decrease in activity of Factor VIII-ΔII after the addition of EDTA indicated that a bivalent cation maintains its functional role in the activated 70 kDa–45 kDa–50 kDa complex.

The inactivation phase, observed after a prolonged thrombin action, corresponded to the dissociation of the 70 kDa–45 kDa–50 kDa complex to form an inactive 70 kDa–50 kDa complex and a 45 kDa fragment. The same process has been described for plasma-derived Factor VIII (Eaton *et al.*, 1986*a*).

Recombinant and plasma-derived 70 kDa–50 kDa complexes were both eluted at the same ionic strength on an anion-exchange matrix (Figs. 5 and 6; Bihoreau *et al.*, 1989). Furthermore, the 70 kDa light chain, after dissociation from the complex, was not retained on the anion-exchanger, a finding that is in full agreement with the basic pI of the cleaved light chain reported by Eaton *et al.* (1987). These results, concerning the activation of Factor VIII, suggest a close similarity between plasma-derived and 'deleted' recombinant fragments generated by thrombin.

Likewise, the inactivation of Factor VIII-ΔII by APC resulted in the proteolysis of the 165 kDa protein and the concomitant generation of 43 and 78 kDa polypeptides. Similar fragments have been identified for plasma-derived Factor VIII after APC inactivation (Fulcher *et al.*, 1984; Eaton *et al.*, 1986*a*), showing that Factor VIII-ΔII is processed by the APC in the same way as plasma-derived Factor VIII. The 97 kDa polypeptide was not cleaved by APC, confirming that this fragment corresponds to a free heavy chain of Factor VIII (Fay & Walker, 1989).

The generation by thrombin and APC of very closely related fragments from two structurally different Factor VIII species may explain the identical efficacy observed *in vivo* of these anti-haemophilia factors (Van de Pol *et al.*, 1989). A model describing the processing of the complete and 'deleted' Factor VIII deduced from the present work is presented in Scheme 1.

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