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Proteolytic Processing of Human Factor VIII. Correlation of Specific Cleavages by Thrombin, Factor Xa, and Activated Protein C with Activation and Inactivation of Factor VIII Coagulant Activity

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ABSTRACT: Human factor VIII was isolated from commercial factor VIII concentrates and found to consist of multiple polypeptides with molecular weights ranging from 80 000 to 210 000. Immunological and amino acid sequence data identified these polypeptides as subunits of factor VIII. N-Terminal amino acid sequence analysis determined that the M_r 210 000 and 80 000 proteins are derived from the N- and C-terminal portions of factor VIII, respectively; M_r 90 000–180 000 polypeptides are derived from the M_r 210 000 polypeptide by C-terminal cleavages. Treatment of purified factor VIII with thrombin resulted in proteolysis of M_r 80 000–210 000 proteins and the generation of polypeptides of M_r 73 000, 50 000, and 43 000. Maximum coagulant activity of thrombin-activated factor VIII was correlated with the generation of these polypeptides. The proteolysis as well as activation of factor VIII by thrombin was found to be markedly dependent on $CaCl_2$ concentration. Proteolysis of factor VIII with activated protein C (APC) resulted in degradation of the M_r 90 000–210 000 proteins with the generation of an M_r 45 000 fragment. This cleavage correlated with inactivation of factor VIII by APC. The M_r 80 000 protein was not degraded by APC. Factor Xa cleaved the M_r 80 000–210 000 factor VIII proteins, resulting in the generation of fragments of M_r 73 000, 67 000, 50 000, 45 000, and 43 000. Factor Xa was found to initially activate and subsequently inactivate factor VIII. Activation by factor Xa correlated with the generation of M_r 73 000, 50 000, and 43 000 polypeptides while inactivation correlated with the cleavage of M_r 73 000 and 50 000 polypeptides to fragments of M_r 67 000 and 45 000, respectively. The cleavage sites in factor VIII of thrombin, factor Xa, and APC were identified by amino acid sequencing of the fragments generated after cleavage of factor VIII by these proteases. Interestingly, factor Xa was found to cleave factor VIII at the same sites as APC and thrombin. This may explain why factor Xa activates as well as inactivates factor VIII.

Purification of factor VIII (antihemophilic factor) from plasma indicates that its coagulant activity is associated with multiple polypeptide chains having molecular weights ranging from 80 000 to 210 000 (Vehar & Davie, 1980; Fass et al., 1982; Fulcher & Zimmerman, 1982; Rotblat et al., 1985). Recently, cDNA clones encoding the entire factor VIII protein sequence have been obtained (Toole et al., 1984; Wood et al., 1984). The amino acid sequence deduced from such clones predicts a mature single-chain protein (2332 amino acids) having a molecular weight of ~300 000 (Wood et al., 1984; Toole et al., 1984). Sequence data obtained from the protein chains of purified factor VIII preparations have been shown to overlap with the sequence predicted from the cDNA clones (Toole et al., 1984; Vehar et al., 1984), and the purification of a single-chain precursor having a M_r >300 000 has been reported (Rotblat et al., 1985). Thus, if factor VIII circulates in plasma as a single-chain form, it is partially degraded during its purification, yielding a form with multiple polypeptide chains.

Amino acid sequence analyses also revealed the orientation of the protein chains associated with factor VIII to the single-chain precursor deduced from the cDNA sequence (Vehar et al., 1984; Toole et al., 1984). Such data show that the M_r 210 000 and 80 000 proteins represent the N-terminal and C-terminal portions of factor VIII, respectively (Vehar et al., 1984; Toole et al., 1984). It is proposed that several proteolytic cleavages on the C-terminal side of the M_r 210 000 protein generate a series of proteins with molecular weights between 90 000 and 180 000 (Vehar et al., 1984; Toole et al., 1984).

Recently, thrombin activation of factor VIII coagulant activity has been shown to be associated with specific proteolysis of factor VIII protein chains (Vehar & Davie, 1980; Fass et al., 1982; Fulcher et al., 1983, 1984; Loller et al., 1984; Rotblat et al., 1985). During thrombin activation of purified

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human factor VIII, proteins with M_r 110 000–210 000 appear to be proteolyzed to generate an M_r 90 000 protein, while the M_r 80 000 protein is cleaved to an M_r 73 000 fragment (Fulcher et al., 1983). Fulcher et al. (1983) suggest that the M_r 90 000 and 73 000 proteins are the active subunits of thrombin-activated factor VIII and that cleavage of the M_r 90 000 protein by thrombin (yielding fragments of M_r ~50 000 and ~43 000) inactivates factor VIII coagulant activity. In contrast, studies with purified porcine factor VIII suggest that cleavage of an M_r 82 000 protein, which is analogous to the M_r 90 000 moiety of human factor VIII, results in further activation of factor VIII coagulant activity (Fass et al., 1982; Loller et al., 1984). Other than species differences, the reason(s) for this discrepancy is (are) unknown.

The inactivation of human factor VIII by activated protein C (APC), a vitamin K dependent plasma protease, has also been correlated with limited proteolysis of the factor VIII protein (Fulcher et al., 1984). Cleavage of factor VIII with APC results in the proteolysis of the M_r 90 000–210 000 proteins with the concomitant appearance of an M_r 45 000 fragment (Fulcher et al., 1984).

The above-mentioned studies clearly show that specific proteolytic processing of factor VIII regulates factor VIII coagulant activity. In this report, we compare the effects of thrombin, factor Xa [also known to activate factor VIII (Vehar & Davie, 1980; Davie et al., 1975)], and APC on factor VIII coagulant activity and correlate the changes in activity with changes in factor VIII subunit structure. Furthermore, most of the cleavage sites of these proteases have been identified by amino acid sequence analyses of the fragments generated by the proteolysis of factor VIII. Knowledge of these sites not only allows the cleavage patterns of these proteases to be compared but also begins to illustrate the basis of the mechanisms that alter factor VIII coagulant activity.

Materials and Methods

Human factor Xa, human activated protein C (APC), and human α-thrombin were all generous gifts from Dr. Walter Kisiel (The University of New Mexico). Affi-gel 10 was from Bio-Rad; rabbit brain cephalin and phenylmethanesulfonyl fluoride (PMSF) were from Sigma Chemical Co.; Platelin was obtained from General Diagnostics; factor VIII deficient and normal human plasmas were from George King Biomedical; factor VIII chromogenic Coatest assay was from Helena. Bio-Gel A-15m void volume fractions enriched in factor VIII/von Willebrand factor (vWF) complexes were prepared from commercial concentrates and were a generous gift of Cutter Laboratories and Dr. D. Schroeder.

Purification of Human Factor VIII. Commercial factor VIII concentrate from Cutter Laboratories was resolved on a Bio-Gel A-15m column as described by Fay et al. (1982). The V_0 fraction containing factor VIII coagulant activity was made 1 mM PMSF and 35 mM β-mercaptoethanol. This results in the reduction of von Willebrand factor (vWF)/factor VIII complexes, which has been shown to cause their dissociation without significantly affecting factor VIII coagulant activity (Vehar & Davie, 1980; Savidge et al., 1979). Also, the functional and structural properties of the factor VIII preparations isolated here are very similar to factor VIII preparations isolated by others in the absence of reducing agents (Fulcher & Zimmerman, 1982; Rotblat et al., 1985). The reduced V_0 was batch-separated with DEAE-Sepharose that had been equilibrated in a 0.02 M imidazole, pH 6.9, buffer containing 0.15 M NaCl, 0.01 M $CaCl_2$, 0.02 M glycine hydrochloride ethyl ester, 5% glycerol, and 1 mM PMSF (VIII buffer). Twenty milliliters of DEAE-Sepharose was added for every liter of V_0 fraction. After being stirred for 2–3 h at 4 °C, the resin was poured into a column and washed with 5 column volumes of VIII buffer. Factor VIII was step-eluted with VIII buffer containing 0.11 M $CaCl_2$. A factor VIII monoclonal antibody column was prepared by coupling 10 mg of factor VIII monoclonal antibody to 2 mL of Affi-gel 10 (Wood et al., 1984). The resulting column was equilibrated in 0.05 M imidazole, pH 6.9, buffer containing 0.15 M NaCl, 0.01 M $CaCl_2$, 5% glycerol, and 1 mM PMSF. The factor VIII DEAE pool was applied to the antibody column, and the column was washed with 50 column volumes of the above buffer. Factor VIII was eluted with the same buffer containing 1.0 M KI. Fractions containing factor VIII activity were pooled and dialyzed against 0.05 M tris(hydroxymethyl)-aminomethane (Tris), pH 7.5, 0.15 M NaCl, 2.5 mM $CaCl_2$, 5% glycerol, and 1 mM PMSF and stored at −70 °C. Factor VIII activity was measured either by coagulation analysis or by the factor VIII chromogenic Coatest assay as described by Wood et al. (1984). Protein concentration was determined by the method of Bradford (1976).

Cleavage of Factor VIII by Thrombin, Factor Xa, and APC. For N-terminal amino acid sequence analysis, approximately 0.5–1.0 mg of factor VIII was incubated with either thrombin, factor Xa, or APC at a 1/50 ratio (w/w). In the case of factor Xa and APC, $^1/_{10}$th sample volume of rabbit brain cephalin was included in the reaction as a source of phospholipid. After 1–2 h at 37 °C, the reaction was stopped by adding sodium dodecyl sulfate (SDS) to 0.4% and immediately heating the samples to 80 °C. Proteolyzed factor VIII was subsequently resolved on 5–10% polyacrylamide gradient gels in the presence of SDS [SDS–polyacrylamide gel electrophoresis (PAGE)]. Electrophoresis was carried out according to the method of Laemmli (1970). After staining with Coomassie blue, factor VIII peptides were excised and electroeluted according to the method of Hunkapiller et al. (1983). Gel-eluted peptides were subjected to N-terminal amino acid sequence analysis using an Applied Biosystems gas phase sequenator (Hewick et al., 1982) modified for on-line phenylthiohydantoin identification (H. Rodriguez, unpublished results).

For subunit and activity analysis during proteolysis, aliquots of factor VIII (110 μg/mL, 400–700 units/mL) in 0.05 M Tris, pH 7.5, 0.15 M NaCl, 2.5 mM $CaCl_2$, and 5% glycerol were incubated with either thrombin (1.5 μg/mL), factor Xa (2 μg/mL) or APC (4 μg/mL) for 0–120 min (thrombin, Xa) or 0–30 min (APC). Rabbit brain cephalin ($^1/_{10}$th sample volume) was added in reactions containing APC or factor Xa. At the end of each time point, a 10-μL aliquot of the reaction was removed, diluted into 0.05 M Tris, pH 7.3, containing 0.2% bovine serum albumin (BSA), and assayed by coagulation analysis. To the remainder of the aliquot was added SDS to 0.5%, and the sample was immediately heated to 80 °C. Proteolyzed factor VIII was subsequently resolved on 6–12% SDS–polyacrylamide gels. Proteins were visualized by silver staining (Morrissey, 1981).

Results

Purification of Factor VIII. Factor VIII was purified by initially resolving plasma concentrates on a Bio-Gel A-15m column as previously described (Fay et al., 1982). This allowed the partial purification of von Willebrand factor (vWF)/factor VIII complexes which elute in the void volume. These complexes were dissociated by reduction with β-mercaptoethanol and resolved by chromatography on DEAE-Sepharose. Factor VIII obtained from the DEAE-Sepharose chromatograph was subsequently purified to homogeneity by using a factor VIII monoclonal antibody column. A typical purification starting

Table I: Purification of Factor VIII

step	total act. (units)[a]	total protein[b] (mg)	sp act. (units/mg)	% recovery
5 L of V_0 (A-15m)	30000	2800	10	100
DEAE-Sepharose	15000	27.0	550	50
factor VIII monoclonal antibody column	8500	1.86	4500	28

[a] Activity was determined by coagulation analysis using human factor VIII deficient plasma. [b] Protein was measured by the method of Bradford (1976).

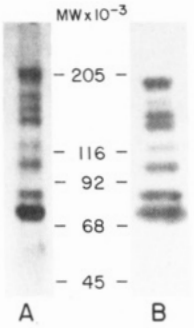

FIGURE 1: SDS–PAGE and western blot analysis of purified human factor VIII. Factor VIII (3–4 μg) was resolved on a 5–10% SDS–polyacrylamide gel and either silver-stained (A) or transferred to nitrocellulose for western blot analysis (B). Two different factor VIII monoclonal antibodies were used for western analyses. One cross-reacts with the M_r 90000–210000 polypeptides, while the other cross-reacts with the M_r 80000 polypeptide (Vehar et al., 1984; Wood et al., 1984).

with material obtained from the Bio-Gel A-15m column is shown in Table I. In this case, factor VIII was purified approximately 5000-fold over plasma concentrates and had a specific activity of 4500 units/mg.

When analyzed by SDS–PAGE, purified factor VIII was resolved into multiple protein chains having molecular weights ranging from 80000 to 210000 (Figure 1). This pattern of proteins is similar to that observed by others who have analyzed purified human factor VIII by SDS–PAGE (Fulcher & Zimmerman, 1982; Rotblat et al., 1985). When resolved under nonreducing conditions, this pattern remained unchanged (data not shown). Western blot analysis demonstrated that all the proteins associated with purified factor VIII cross-reacted with specific factor VIII monoclonal antibodies (Figure 1). Furthermore, as shown below, amino acid sequence analyses of these proteins, and comparison of these sequences with the factor VIII DNA sequence, demonstrate identity.

Presumably, each of the proteins of M_r 90000–210000 forms a complex (perhaps calcium linked) with the M_r 80000 subunit. This is evidenced by the purification of factor VIII consisting of M_r 80000–210000 proteins using a monoclonal antibody that recognizes only the M_r 80000 moiety. We have also found that after factor VIII is bound to a monoclonal antibody column specific for the M_r 80000 moiety, the M_r 90000–210000 proteins can be eluted with ethylendiaminetetraacetic acid (EDTA) (unpublished results). Similar results have been obtained for porcine factor VIII (Fass et al., 1982).

Proteolysis of Factor VIII by Thrombin, APC, and Factor Xa. N-Terminal amino acid sequence analysis of factor VIII proteins reveals that the M_r 90000–210000 proteins have the same N-terminal sequence, while that of the M_r 80000 protein (which sometimes appeared as a doublet) is distinct (Figure 3). Alignment of these sequences with the amino acid sequence deduced from the cDNA for factor VIII shows that the M_r 210000 and 80000 proteins represent the N-terminal and

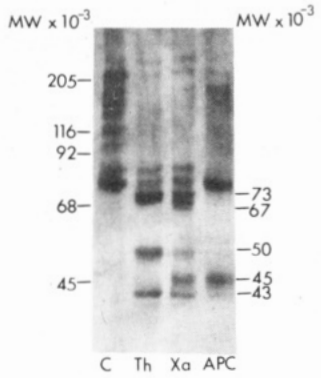

FIGURE 2: Cleavage of factor VIII by thrombin, factor Xa, and APC. Factor VIII (110 μg/mL, ~700 units/mL) was incubated for 1 h at 37 °C with either thrombin (1.5 μg/mL), factor Xa (1 μg/mL), or APC (2 μg/mL). In the case for factor Xa and APC, $^1/_{10}$th volume of rabbit brain cephalin was included in the reaction. The reaction was stopped by the addition of SDS to 0.5% and heating to 80 °C. Proteins were subsequently resolved on a 6–12% SDS–polyacrylamide gel.

C-terminal portions of the factor VIII single-chain precursor, respectively (Figure 3; Toole et al., 1984; Vehar et al., 1984; Wood et al., 1984). Five polypeptide chains were routinely observed with M_r 110000–180000 (Figure 1). Presumably, C-terminal cleavage of the M_r 210000 protein generates these fragments. The sites that are cleaved to generate these five proteins are unknown as is the protease that makes them. It has been shown, however, that cleavage of the M_r 110000–210000 proteins by thrombin at position 740 generates the M_r 90000 protein (Toole et al., 1984).

Proteolysis of factor VIII by thrombin results in the degradation of the M_r 80000–210000 proteins and the appearance of polypeptides of M_r 73000, 50000, and 43000 (Figure 2). N-Terminal sequence analysis of the M_r 50000 and 43000 polypeptides shows that they are derived from the M_r 90000 protein by cleavage by position 372 (Figure 3; Vehar et al., 1984). The M_r 50000 and 43000 polypeptides represent the N-terminal and C-terminal portions of the M_r 90000 protein, respectively. The N-terminal sequence of the M_r 73000 polypeptide shows that it arises from the cleavage of the M_r 80000 protein at arginine-1689 (Figure 3; Vehar et al., 1984). This results in the possible removal of the N-terminal 44 amino acids of the M_r 80000 protein. This 44 amino acid polypeptide is acidic as it contains 15 Asp and Glu residues and only 4 Lys and Arg residues (Vehar et al., 1984). Like the M_r 80000 protein, the M_r 73000 polypeptide also appeared on SDS–PAGE as a doublet.

Factor Xa appears to proteolyze factor VIII more extensively than thrombin (Figure 2). Like thrombin, the M_r 80000–210000 proteins of factor VIII are all cleaved. However, in addition to the polypeptides of M_r 73000, 50000, and 43000, polypeptides of M_r 45000 and 67000 also appear after treatment of factor VIII with factor Xa (Figure 2). N-Terminal sequence analysis shows that the M_r 50000 and 43000 polypeptides originate from cleavage of the M_r 90000 protein at position 372 (Figure 3) as was observed with thrombin. Presumably, the M_r 90000 protein arises from cleavage of the M_r 110000–210000 proteins at position 740 by factor Xa. The M_r 45000 polypeptide has the same N-terminal sequence as the M_r 50000 and 90000–210000 polypeptides (Figure 3). The site at which this cleavage occurs has yet to be determined. However, on the basis of the size difference between the M_r 50000 and 45000 polypeptides, and since factor Xa is specific for arginine residues, cleavage at arginine-336 of the M_r 50000 or M_r 90000–210000 poly-

Polypeptide	No Protease	Thrombin	Factor Xa	APC
110,000-210,000	[A][T]RRYYLGAVELSWD			
90,000	ATRRYYL			
80,000	EITRTTLQSDQ			
73,000		[S]FQKK[T]RHYFIAAVDERL	SFQKKTRHYF	
67,000			AQSGSVPQFKKVVFQ	
50,000		ATRRYYLGAVEL-W-YM	ATRRYYLGAVELS	
45,000			ATRRYYLGAV	ATRRYLGAVEL
43,000		[S]VAKK--K-WV-T	SVAKKHPK	

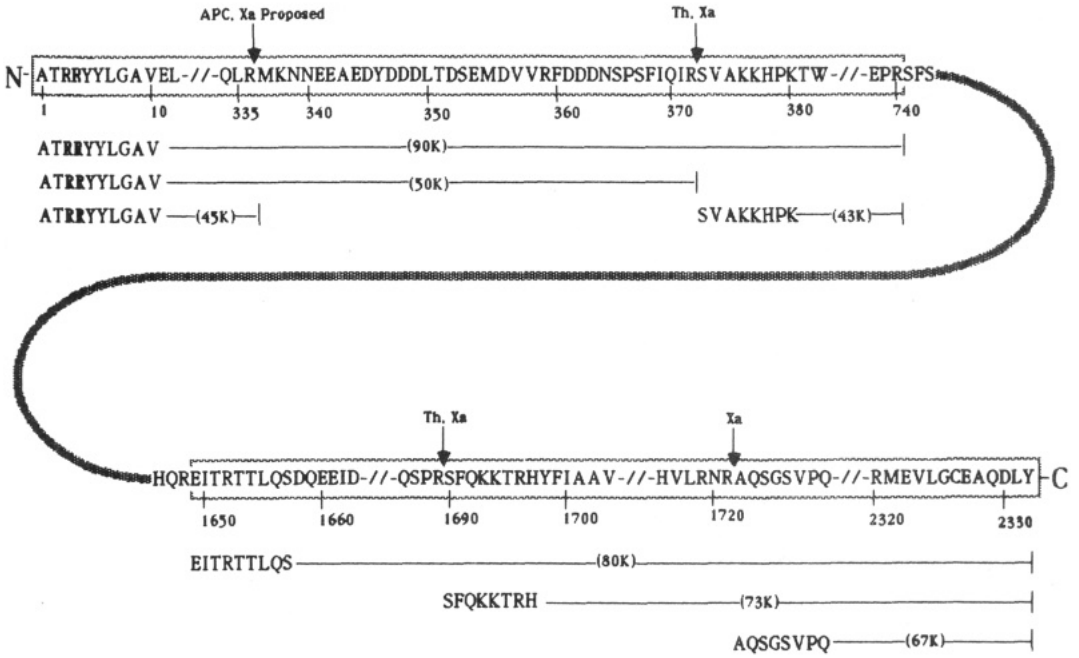

FIGURE 3: N-Terminal sequence of factor VIII polypeptides and their position within the factor VIII molecule. Factor VIII (0.5–1.0 mg) was proteolyzed with either thrombin, factor Xa, or APC, or not at all, and resolved on a 5–10% SDS–polyacrylamide gel. Subsequently, the polypeptides as shown were excised, gel-eluted, and subjected to N-terminal amino acid sequencing as described under Materials and Methods. The N-terminal amino acid sequence shown for the M_r 110 000–210 000 polypeptides was determined by pooling these peptides after gel elution. Only one sequence was obtained from this pool, and quantitation indicates that N-terminal blockage of these polypeptides was minimal (data not shown). Dashes indicate positions where no residue could be identified. Brackets indicate the tentative assignment for that amino acid. The factor VIII sequence shown in the boxes is deduced from the DNA sequence of factor VIII (Wood et al., 1984; Toole et al., 1984; Gitschier et al., 1984). Only that sequence which is necessary to show the orientation of the above-sequenced polypeptides is shown.

peptides may generate the M_r 45 000 fragment (Figure 3). The M_r 73 000 and 67 000 polypeptides are derived by cleavage of the M_r 80 000 protein at positions 1689 and 1721, respectively (Figure 3). The M_r 67 000 polypeptide also appears as a doublet on SDS–PAGE.

Cleavage of factor VIII by APC results in proteolysis of M_r 90 000–210 000 proteins with the appearance of an M_r 45 000 fragment (Figure 2). The M_r 80 000 protein is not cleaved by APC. These results are similar to those of Fulcher et al. (1984). The N-terminal sequence of the M_r 45 000 fragment was found to be the same as the N-terminal sequence of the M_r 90 000–210 000 proteins (Figure 3). Therefore, this fragment is derived from the N-terminal of factor VIII. The site at which APC cleaves factor VIII to generate this fragment has not been determined. This cleavage, however, appears to be the same cleavage made by factor Xa that also generates an M_r 45 000 fragment from the N-terminal of factor VIII. Cleavage at this site by factor Xa is not due to contaminating APC since antibodies against APC did not inhibit factor Xa from making this cleavage (data not shown). By SDS–PAGE, we could not reproducibly detect the C-terminal portions of the M_r 90 000–210 000 proteins after proteolysis of factor VIII by APC. In Figure 6, polypeptides with molecular weights of ~47 000, ~49 000, and 67 000 are apparent after APC cleavage. The appearance of these polypeptides, however, was only transitory. Also, only one sequence was observed when the M_r 45 000 fragment was sequenced.

Activation of Factor VIII by Thrombin. A time course treatment of factor VIII with catalytic amounts of thrombin resulted in a 36-fold increase in factor VIII coagulant activity (Figure 4). After maximum activity was reached, thrombin-activated factor VIII appeared to remain stable for at least 1 h at 37 °C. Figure 4 does show a slight decrease in activity at the 2-h time point; however, in other experiments, this decrease was not seen. Factor VIII that was not activated with thrombin remained stable throughout the 2-h time course (data not shown). Analysis of factor VIII subunit structure during thrombin activation shows that factor VIII coagulant activity dramatically increases with the generation of fragments of M_r 73 000, 50 000, and 43 000 (Figure 5). Thrombin, therefore, appears to activate factor VIII by initially cleaving M_r 110 000–210 000 proteins to generate the M_r 90 000 protein, which is subsequently cleaved to polypeptides of M_r 50 000 and 43 000. Occurring concomitantly is cleavage of the M_r 80 000 protein to an M_r 73 000 polypeptide. These results indicate that fully activated factor VIII may consist of subunits

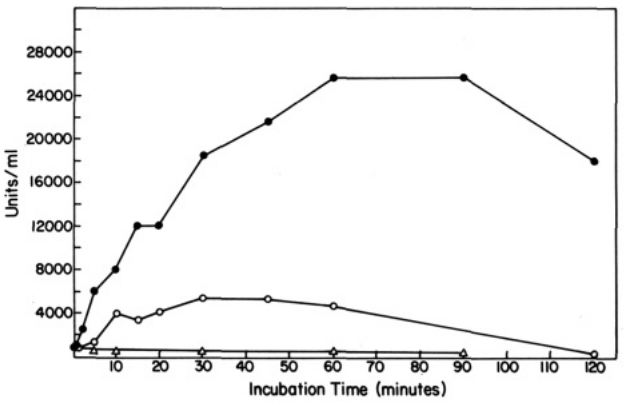

FIGURE 4: Activation of factor VIII by thrombin and the effect of $CaCl_2$ on thrombin activation. Factor VIII (110 μg/mL, ~700 units/mL) was incubated for the times shown at 37 °C with thrombin (1.5 μg/mL) in the presence of 2.5 (●), 10 (○), or 50 mM $CaCl_2$ (△). At each time point, factor VIII coagulant activity was determined, and the reaction was stopped by the addition of SDS as described under Materials and Methods.

of M_r 73 000, 50 000, and 43 000, with the latter two originating from the N-terminal portion and the M_r 73 000 from the C-terminal portion of the factor VIII precursor protein.

As shown in Figure 4, increasing the $CaCl_2$ concentration significantly alters the extent to which factor VIII is activated by thrombin. At 10 mM $CaCl_2$, only a 13-fold activation is observed, while at 50 mM $CaCl_2$ factor VIII is not activated by thrombin (Figure 5). These results are similar to the recent findings of Hultin (1985), who has shown that activation of partially pure factor VIII by thrombin in inhibited by $CaCl_2$. Comparison of the subunit structure of factor VIII activated by thrombin at 2.5, 10, and 50 mM $CaCl_2$ shows that at the higher $CaCl_2$ concentrations (10 and 50 mM) the proteolytic processing of factor VIII by thrombin is limited (Figure 5). At 10 mM $CaCl_2$, the M_r 90 000 and 80000 polypeptides are only partially cleaved to the M_r 73 000, 50 000, and 43 000 subunits, while at 50 mM $CaCl_2$ factor VIII is not cleaved at all by thrombin (Figure 5). Interestingly, at 10 mM $CaCl_2$, after maximum activity is achieved there appears to be very little change in factor VIII subunit structure yet coagulant activity greatly diminishes (Figures 4 and 5). The reasons for this decrease in activity are presently unknown. Here we show that maximum activation and proteolytic processing of factor VIII by thrombin appear to occur at physiological $CaCl_2$ concentration (2.5 mM), while partial activation or proteolysis occurs at higher $CaCl_2$ concentrations (Figures 4 and 5). In the absence of $CaCl_2$, factor VIII was proteolyzed and activated by thrombin similarly to factor VIII treated by thrombin in the presence of 2.5 mM $CaCl_2$ (data not shown).

Inactivation of Factor VIII by APC. Treatment of factor VIII with APC resulted in a dramatic decrease in coagulant activity (Figure 6). Correlated with the decrease in activity is the cleavage of M_r 90 000–210 000 proteins with the concomitant generation of an M_r 45 000 polypeptide (Figure 6, inset). The M_r 80 000 protein is not significantly proteolyzed. As previously discussed, the N-terminal amino acid sequence of the M_r 45 000 polypeptide is identical with the N-terminal sequence of the M_r 90 000–210 000 proteins (Figure 3), and the exact site at which this cleavage occurs is presently unknown. However, as proposed above for factor Xa, APC may cleave at position 336. This site precedes a very acidic region (15 Asp–Glu; 4 Lys/Arg; total of 42 amino acids) of the M_r 90 000–210 000 proteins. Significantly, cleavage of the M_r 90 000 protein at position 372, immediately following this acidic region, generates the M_r 50 000 and 43 000 subunits of thrombin–activated factor VIII. Taken together, this suggests that the acidic region between positions 336 and 372, shown in Figure 3, is of functional importance.

Activation of Factor VIII by Factor Xa. Similar to thrombin, factor Xa cleaves factor VIII at position 372 of the M_r 90 000 protein and at position 1689 of the M_r 80 000 protein (Figures 2 and 3). These cleavages would result in activation of factor VIII, as is the case for thrombin. However, factor Xa also appears to cleave factor VIII at the same site that APC proteolyzes factor VIII (Figure 2 and 3). This cleavage at position 336 would inactivate factor VIII. These results suggest that factor Xa would at best only moderately activate factor VIII and ultimately cause inactivation. Indeed, over a 2-h time course, factor Xa initially activated factor VIII only 3-fold and eventually inactivated factor VIII (Figure 7a).

During the time course treatment of factor VIII with factor Xa, the M_r 110 000–210 000 proteins were initially cleaved, with the major product being the M_r 90 000 protein (Figure 7b). This protein was cleaved to generate M_r 50 000, 45 000, and 43 000 polypeptides (Figure 7b). Subsequently, the M_r 50 000 polypeptide appeared to be cleaved to the M_r 45 000 fragment. This proteolysis correlates with the inactivation of factor VIII by factor Xa (Figure 7). Occurring concomitantly with cleavage of the M_r 90 000 protein is the cleavage of the M_r 80 000 protein to the M_r 73 000 polypeptide, which is subsequently cleaved to generate an M_r 67 000 polypeptide. This latter cleavage also correlates with factor VIII inactivation (Figure 7). Whether this cleavage, itself, is sufficient to inactivate factor VIII has not been determined.

Discussion

Recently a detailed understanding of the primary structure of factor VIII was made possible due to the isolation of factor

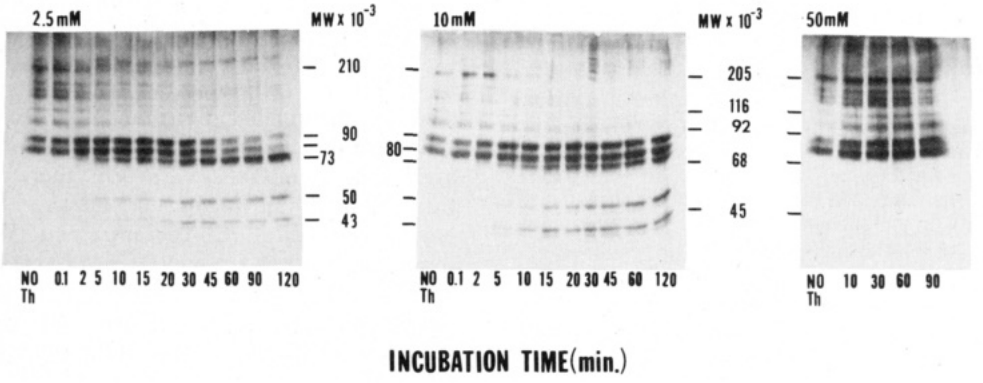

FIGURE 5: Subunit structure of thrombin-activated factor VIII. Factor VIII samples from Figure 4 that had been activated by thrombin at either 2.5, 10, or 50 mM $CaCl_2$ and subsequently made 0.5% SDS were heated to 80 °C for 5 min. The proteins were resolved on SDS–PAGE as described under Materials and Methods.

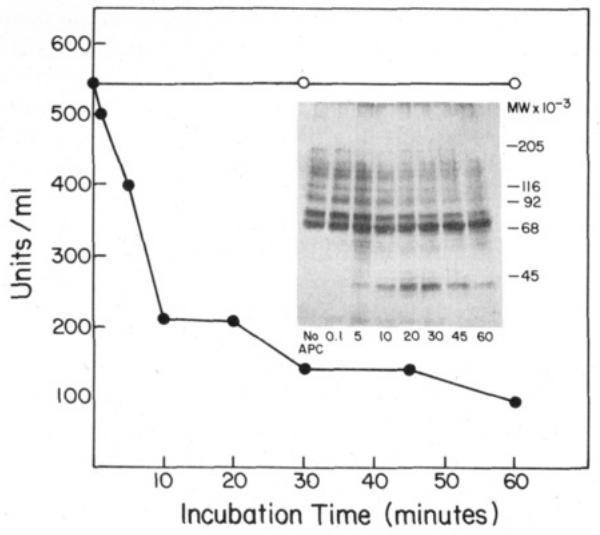

FIGURE 6: Inactivation of factor VIII by APC. To 40 μL of factor VIII (110 μg/mL) was added 5 μL of rabbit brain cephalin and subsequently incubated at 37 °C with APC (4 μg/mL) (●) or with no addition (○) for the times shown. At the end of each time point, factor VIII coagulant activity and subunit structure (inset) were determined as described in Figure 4 and under Materials and Methods.

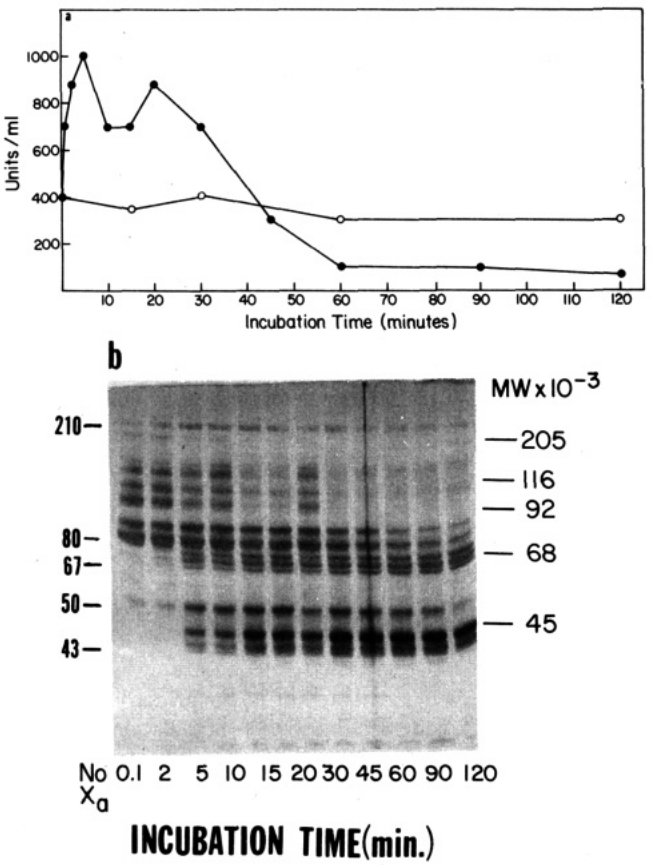

FIGURE 7: Activation of factor VIII by factor Xa. To 40 μL of factor VIII (110 μg/mL) was added 5 μL of rabbit brain cephalin and subsequently incubated with factor Xa (1 μg/mL) (●) or with no addition (○) at the times shown. At the end of each time point, factor VIII coagulant activity (a) and subunit structure (b) were determined as described in Figures 4 and 5 and under Materials and Methods.

VIII cDNA and genomic clones (Wood et al., 1984; Toole et al., 1984; Gitschier et al., 1984). The deduced amino acid sequence predicts a mature single-chain protein consisting of 2332 amino acids which, after accounting for 25 potential N-linked glycosylation sites, indicates that the single-chain form of factor VIII has an M_r >300 000. This is supported by the purification of single-chain factor VIII having M_r >300 000 from plasma (Rotblat et al., 1985). The single-chain precursor form of factor VIII appears to be readily proteolyzed in vivo and/or in vitro, yielding a species consisting of multiple subunits with M_r 80 000–210 000. As stated previously, the protease which cleaves at position 1648 to generate the M_r 80 000 protein is unknown as are the protease(s) and sites which generate the M_r 110 000–180 000 proteins seen when factor VIII is resolved by SDS–PAGE (Figures 1 and 8).

Detailed analysis of the factor VIII sequence revealed a triplicated domain structure. These domains each consist of approximately 330 amino acids and are approximately 30% homologous (Vehar et al., 1984). Interestingly, these domains also share approximately 30% homology with the triplicated domains of the plasma copper binding protein ceruloplasmin (Vehar et al., 1984). The importance of this homology as it pertains to factor VIII function is as yet not understood. The location of these domains within the factor VIII precursor is shown in Figure 8.

Thrombin activation of purified factor VIII correlates with proteolysis at positions 740, 372, and 1689 (Figure 8). These cleavages ultimately generate the M_r 73 000, 50 000, and 43 000 subunits. Cleavage at position 740 removes the C-terminal region of the M_r 110 000–210 000 proteins, generating the M_r 90 000 protein (Toole et al., 1984). Subsequently, the M_r 90 000 protein is cleaved at position 372 to generate the M_r 50 000 and 43 000 subunits. This cleavage site is between two ceruloplasmin-like domains and follows an acidic spacer region (336–372) (Figures 3 and 8). Cleavage of the M_r 80 000 protein at position 1689 to generate the M_r 73 000 subunit also follows an acidic region (positions 1649–1689) of factor VIII, which has some sequence homology with the region between positions 336 and 372 (Vehar et al., 1984).

Similar to results presented here, porcine factor VIII has been shown to be activated 70-fold by thrombin in the presence of 5 mM $CaCl_2$ (Fass et al., 1982; Loller et al., 1984). This activation was correlated with the cleavage of an M_r 82 000 protein to M_r 44 000 and 35 000 polypeptides and the cleavage of an M_r 76 000 protein to a fragment of M_r 69 000. The M_r 82 000, 44 000, and 35 000 proteins of porcine factor VIII are analogous to the M_r 90 000, 50 000, and 43 000 proteins of human factor VIII, while the M_r 76 000 and 69 000 polypeptides of porcine factor VIII are analogous to the M_r 80 000 and 73 000 proteins of human factor VIII (Fass et al., 1985). Interestingly, thrombin-activated porcine factor VIII was found to be unstable even though its subunit structure remained unchanged (Loller et al., 1984). However, it could be stabilized by factor IXa and phospholipid, suggesting that changes other than proteolysis may cause inactivation of factor VIII coagulant activity (Loller et al., 1984). This is consistent with the results of Hultin & Jesty (1981) and Rick & Hoyer (1977), who observed that inactivation of thrombin-activated factor VIII was not mediated by active thrombin. Factor VIII activated by thrombin at 10 mM $CaCl_2$ becomes inactive with time even though there is little change in subunit structure after activation (Figure 5); it is therefore possible that thrombin-activated factor VIII may not be stable at non-physiological high $CaCl_2$ concentrations. We show here that thrombin-activated factor VIII was stable for at least 1 h at 37 °C. This stability is probably a reflection of the high concentration (110 μg/mL) of factor VIII as well as the $CaCl_2$ concentration (2.5 mM) used in thrombin activation experiments.

In contrast to our results and those of Fass et al. (1982), Fulcher et al. (1983) observed that thrombin activation of

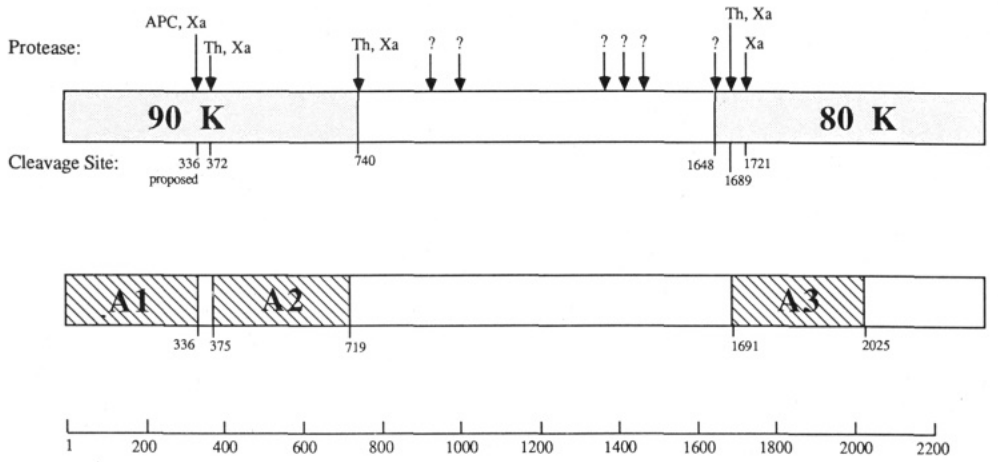

FIGURE 8: Schematic representation of proteolytic cleavage sites of thrombin (Th), factor Xa (Xa), and APC (upper diagram). The ceruloplasmin-like domains (A1, A2, and A3) and their position within the factor VIII precursor are shown in the lower diagram. The position within the factor VIII sequence is indicated by the scale.

factor VIII correlates with the generation of M_r 90 000 and 73 000 proteins. They suggest that cleavage of the M_r 90 000 protein to M_r 50 000 and 43 000 polypeptides results in inactivation of factor VIII. These investigators, however, activated factor VIII in the presence of 40 mM $CaCl_2$. As shown here and as reported by Hultin (1985), this $CaCl_2$ concentration should limit the activation of factor VIII by thrombin (Figures 4 and 5). Indeed, these authors only observed a 5-fold activation.

Inactivation of factor VIII by APC correlates with cleavage of M_r 90 000–210 000 proteins and generation of an M_r 45 000 fragment (Figure 6). This result is similar to those reported by Fulcher et al. (1984). The site at which APC cleaves has yet to be identified; however, as discussed previously, this cleavage could occur at arginine-336 (Figures 3 and 8). Since thrombin cleavage at position 372 of the M_r 90 000 protein causes activation (Figure 4), the acidic region between positions 336 and 372 may be functionally important in regulating the activity of factor VIII. Recently, Guinto & Esmon (1984) have shown that factor V proteolyzed by APC does not interact with prothrombin or with factor Xa. It is tempting to speculate that this acidic region of factor VIII may be involved in protein/protein or protein/phospholipid interactions.

Cleavage of factor VIII by factor Xa results in a modest activation followed by inactivation of factor VIII coagulant activity (Figure 6). This observation is consistent with the fact that factor Xa cleaves at the same sites as thrombin (372, 1689) as well as APC (336) (Figure 8). The inactivation of human factor VIII by factor Xa has also been observed by Triantaphyllopoulus (1979). However, purified bovine factor VIII has also been shown to be activated and stable by factor Xa at a nonphysiological low pH (Vehar & Davie, 1980). Activation of human factor VIII by factor Xa appears to correlated with the generation of M_r 73 000, 50 000, and 43 000 fragments, while inactivation appears to correlate with the cleavage of the M_r 50 000 subunit to the M_r 45 000 polypeptide. Presumably, this cleavage occurs at the same site that APC cleaves factor VIII (Figure 3). Interestingly, it has been shown that inactivation of thrombin-activated porcine factor VIII by APC correlates with the cleavage of the M_r 44 000 subunit, which is analogous to the M_r 50 000 subunit of human factor VIII (Fass et al., 1984). These results suggest that the M_r 50 000 subunit is indeed a functional subunit of activated factor VIII. Factor Xa also cleaves the M_r 73 000 subunit at position 1722 to generate an M_r 67 000 polypeptide. Whether this cleavage alters factor VIII activity has not been determined. However, this cleavage occurs within a ceruloplasmin-like domain and may release the first 20 amino acids of the A3 domain (Figure 8).

The functional as well as structural similarities of factors V and VIII have recently been recognized (Vehar et al., 1984; Fulcher et al., 1983, 1984; Church et al., 1984; Fass et al., 1985). Both of these proteins function as cofactors in the intrinsic coagulation pathway. Factor VIII in a complex with factor IXa, calcium ions, and a phospholipid surface functions in the activation of factor X. Factor V in a complex with factor Xa, calcium ions, and a phospholipid surface functions in the conversion of prothrombin to thrombin. Both proteins appear to circulate in plasma as large (M_r 300 000) single-chain precursors which are proteolytically processed to yield active cofactors (Mann et al., 1981; Rotblat et al. 1985). In both cases, the N-terminal (M_r ~90 000) and C-terminal (M_r ~80 000) portions represent the functional regions of these cofactors (Vehar et al., 1984; Toole et al., 1984; Esmon, 1979; Hibbard & Mann, 1980; Nesheim et al., 1984). Separating these regions in both factor V and factor VIII is a highly glycosylated region (M_r ~100 000). The function of this region is as yet undetermined (Vehar et al., 1984; Church et al., 1984). Amino acid sequencing also shows that factor V has homology with both factor VIII and ceruloplasmin (Church et al., 1984; Fass et al., 1985). Factor V has also been shown to contain at least a duplication of the ceruloplasmin-like domain (Fass et al., 1985). Factors VIII and V are also both activated by thrombin and factor Xa and inactivated by APC (Foster et al., 1983; Suzuki et al., 1983). Thrombin activation of either factor V or factor VIII results in the generation of polypeptides of M_r ~90 000 (from the N-termini) and ~70 000 (from the C-termini). In the case for factor VIII, the M_r 90 000 polypeptide appears to be proteolyzed further to M_r 50 000 and 43 000 polypeptides. Factor Xa proteolysis of factor V is unlike that by thrombin, and factor Xa is less efficient than thrombin in activating factor V (Foster et al., 1983). The same appears true for the activation of factor VIII by factor Xa (Figure 7). APC appears to inactivate both factor VIII and factor V by proteolyzing the N-terminal portion (molecular weight of approximately 90 000 subunit) of these factors (Figure 6; Suzuki et al., 1983). These data support the notion that factors VIII and V are regulated by thrombin, APC, and factor Xa in a very similar manner.

In this study, we have identified the cleavage sites in factor VIII of thrombin, factor Xa, and APC. This has allowed us to determine how factor VIII is qualitatively processed by these proteases. Correlation of the changes in factor VIII subunit

structure due to proteolysis by thrombin, factor Xa, or APC with changes in factor VIII coagulant activity allowed the tentative identification of the functional subunits of activated factor VIII. The results presented here suggest that the generation of the M_r 50 000, 43 000, and 73 000 subunits correlates with complete activation of factor VIII.

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Registry No. Ca, 7440-70-2; blood coagulation factor VIII, 9001-27-8; thrombin, 9002-04-4; blood coagulation factor Xa, 9002-05-5; blood coagulation factor XIVa, 42617-41-4.

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