Mechanisms of plasmin-catalyzed inactivation of factor VIII

1. A crucial role for proteolytic cleavage at Arg\textsuperscript{336} responsible for plasmin-catalyzed factor VIII inactivation

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Plasmin, not only functions as a key enzyme in the fibrinolytic system but also directly inactivates factor VIII and other clotting factors such as factor V. However, the mechanisms of plasmin-catalyzed factor VIII inactivation are poorly understood. In the current studies, levels of factor VIII activity increased approximately 2-fold within 3 minutes in the presence of plasmin, and subsequently decreased to undetectable levels within 45 minutes. This time-dependent reaction was not affected by von Willebrand factor and phospholipid. The rate constant of plasmin-catalyzed factor VIII(a) inactivation was ~12- and ~3.7-fold greater than those mediated by factor Xa and activated protein C, respectively. SDS-PAGE analysis showed that plasmin cleaved the heavy chain of factor VIII into two terminal products, A\textsubscript{137-336} and A\textsubscript{2} subunits, by limited proteolysis at Lys\textsuperscript{36}, Arg\textsuperscript{336}, Arg\textsuperscript{372}, and Arg\textsuperscript{740}. The 80-kDa light chain was converted into a 67-kDa subunit by cleavage at Arg\textsuperscript{1689} and Arg\textsuperscript{1721}, identical to the pattern induced by factor Xa. Plasmin-catalyzed cleavage at Arg\textsuperscript{336} proceeded faster than that at Arg\textsuperscript{372}, in contrast to proteolysis by factor Xa. Furthermore, breakdown was faster than that in the presence of activated protein C, consistent with rapid inactivation of factor VIII. The cleavages at Arg\textsuperscript{336} and Lys\textsuperscript{36} occurred rapidly in the presence of A\textsubscript{2} and A3-C1-C2 subunits, respectively. These results strongly indicated that cleavage at Arg\textsuperscript{336} was a central mechanism of
plasmin-catalyzed factor VIII inactivation. Furthermore, the cleavages at Arg$^{336}$ and Lys$^{36}$ appeared to be selectively regulated by the A2 and A3-C1-C2 domains, respectively, interacting with plasmin.

Factor VIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, hemophilia A, functions as a cofactor in the tenase complex, which is responsible for anionic phospholipid surface-dependent conversion of factor X to Xa by factor IXa (1). Factor VIII circulates as a complex with VWF$^1$ that protects and stabilizes the cofactor. Factor VIII is synthesized as a single chain molecule consisting of 2,332 amino acid residues with a molecular mass of ~300 kDa (2, 3). The factor VIII molecule can be divided into three domains arranged in the order of A1-A2-B-A3-C1-C2 according to the amino acid content homology. It is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains, plus heterogeneous fragments of a partially proteolyzed B domain, linked to a light chain consisting of the A3, C1, and C2 domains (2-4).

The catalytic efficacy of factor VIII in the tenase complex is enhanced over $10^5$ times by conversion into an active form, factor VIIIa, by limited proteolysis by either thrombin or factor Xa (5). Both enzymes cleave factor VIII at Arg$^{372}$ and Arg$^{740}$ of the heavy chain and produce 50-kDa, A1 and 40-kDa, A2 subunits. The 80-kDa light chain is also cleaved at Arg$^{1689}$ generating a 70-kDa, A3-C1-C2 subunit. Additionally, factor Xa cleaves at Arg$^{1721}$ and produces a 67-kDa, A3-C1-C2 subunit. Proteolysis at Arg$^{372}$ and Arg$^{1689}$ is essential for generating factor VIIIa cofactor activity (6). Cleavage at the former site exposes a functional factor IXa-interactive site within the A2 domain that is cryptic in the unactivated molecule (7). Cleavage at the latter site liberates the cofactor from its carrier protein, VWF (8), contributing to the overall specific activity of the cofactor (9, 10).

Factor VIIIa cofactor activity is down-regulated in the presence of serine proteases such as APC (5), factor Xa (5), and factor IXa (11) by proteolytic inactivation following cleavage at Arg$^{336}$ within the A1 subunit. This inactivation appears to be the result of altered interaction with the A2 subunit and an increased $K_m$ of the truncated A1 for the substrate factor X (12, 13), the latter reaction reflecting loss of a factor X-interactive site within residues 337-372 (14). Factor Xa and APC also cleave at Lys$^{36}$ (13) and at...
Arg$^{562}$ (15), respectively. These events may alter the conformation of A1, limiting the productive interaction with the A2 subunit (13) and impairing the interaction with factor IXa in tenase complex (16).

Hemostasis is further regulated by fibrinolysis. Proteolytic degradation of fibrinogen/fibrin by the serine protease, plasmin, occurs at the end stage of the blood coagulation cascade. Previous reports have suggested, however, that plasmin also regulates blood coagulation by proteolysis of several coagulation proteins including factor Va (17, 18), IXa (19), X (20) and factor VIII (21, 22). These findings suggest that plasmin might down-regulate tenase activity by inactivating factor VIII(a), although the exact mechanism of this inactivation is poorly understood.

In the present study, we report on the mechanism of plasmin-catalyzed factor VIII inactivation. Functional assays and SDS-PAGE analysis using isolated subunits of the cofactor, demonstrated for the first time that inactivation was directly associated with unique, limited-proteolysis that led initially to three terminal products, A1$^{37-336}$, A2, and A3-C1-C2$^{2172-2332}$, and subsequently the crucial cleavage at Arg$^{336}$. Cleavage at Arg$^{336}$ and Lys$^{36}$ appeared to be selectively modulated following interaction of the protease with A2 and A3-C1-C2 subunits, respectively.

**MATERIALS AND METHODS**

**Reagents** - Purified recombinant factor VIII preparations were generous gifts from Bayer Corp. (Berkeley, CA). The monoclonal antibodies 58.12 (23) and C5 (24) recognizing the N- and C-terminal end of the A1 domain were gifts from Bayer Corp. and Dr. Carol Fulcher, respectively. The monoclonal antibody JR8 recognizing the A2 domain was obtained from JR Scientific Inc. (Woodland, CA). The monoclonal antibody NMC-VIII/10 recognizing the N-terminal end of the A3 domain was purified as previously described (25). VWF was purified from factor VIII/VWF concentrates using a gel filtration on a Sepharose CL-4B column (Amersham Bio-Science, Uppsala, Sweden) and immune-beads coated with immobilized factor VIII monoclonal antibody as previously reported (25). Enzyme-linked immunosorbent assays of factor VIII demonstrated greater than 95% purity of VWF. Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma, St Louis, MO) were prepared using N-octylglucoside (26). Purified human Lys-plasmin was purchased from Sigma and was shown to be
devoid of factor Xa and APC. Factor Xa, APC and protein S were purchased from Haematologic Technologies Inc. (Essex Junction, VT).

**Isolation of factor VIIIa subunits** - Factor VIII (1.5 µM) was treated overnight at 4 °C in buffer containing 10 mM MES, pH 6.0, 0.25 M NaCl, 50 mM EDTA, and 0.01% Tween 20, and light and heavy chains were isolated following chromatography on SP- and Q-Sepharose columns (Amersham Bioscience) as previously described (7). Purified heavy chain was cleaved by thrombin, and the A2 and A1 subunits were purified using Hi-Trap Heparin column and Mono-Q columns, respectively, as reported previously (12). A3-C1-C2 subunits were purified from thrombin-treated light chain by SP-Sepharose chromatography as previously described (27). Factor VIIIa was isolated from thrombin-treated factor VIII by CM-Sepharose chromatography (Amersham Bioscience) (28). A1/A3-C1-C2 dimers were prepared by reconstitution from isolated A1 and A3-C1-C2 subunits by incubation overnight at 4 °C in 20 mM HEPES, pH 7.2, 0.3 M NaCl, 25 mM CaCl₂, and 0.01% Tween 20 (13). SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL) showed >95% purity. Protein concentrations were determined by the method of Bradford (29).

**Clotting assay** - Factor VIII(a) activity was measured in a one-stage clotting assay using factor VIII-deficient plasma (30). All reactions were performed at 22 °C. All factor VIII products were incubated in buffer (20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM CaCl₂, 100 µg/ml bovine serum albumin, and 0.01% Tween 20) plus phospholipid vesicles (10 µM). Samples were removed from the mixtures at the indicated times and plasmin reaction was immediately terminated by the addition of 0.5 mM pefabloc SC (Roche, Basel, Switzerland) and dilution. The presence of plasmin and pefabloc in the 1000-fold diluted sample was shown not to affect factor VIII activity in the coagulation assay.

**Cleavage of factor VIII(a) and its subunits by plasmin** - Human plasmin was added to factor VIII(a) and its subunits at a 1:25 ratio (mol/mol) in the presence of phospholipid vesicles (10 µM) in buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM CaCl₂, and 0.01% Tween 20 at 22 °C. Samples were taken at the indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min.

**Electrophoresis and Western blotting** - SDS-
PAGE was performed using 8 % gels as described by Laemmli (31). Electrophoresis was carried out using a Bio-Rad minigel apparatus (Hercules, CA) at 150 V for 1 hr. Bands were visualized following staining with GelCode Blue (Pierce). For Western blotting, each protein sample was transferred to a polyvinylidene difluoride membrane using a Bio-Rad mini-transblot apparatus at 50 V for 2 hrs in buffer containing 10 mM CAPS, pH 11 and 10% (v/v) methanol. Protein bands were probed using the indicated anti-factor VIII monoclonal antibodies followed by goat anti-mouse peroxidase-linked secondary antibody (MP Biomedicals, Aurora, Ohio). Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA). Densitometric scans were quantitated using Image J 1.34 (National Institute of Health, USA).

**NH\(_2\)**-terminal sequence analysis - NH\(_2\)-terminal sequence analyses of the fragments following plasmin cleavage of factor VIII were performed using an Applied Biosystems Model 491 Sequencer (Foster City, CA). The plasmin-cleaved factor VIII fragments were recovered after electrophoresis and were subjected to ten cycles of automated sequencing.

**RESULTS**

**Factor VIII activation and inactivation by plasmin** - Earlier reports have described factor VIII inactivation by plasmin (21, 22), however, precise inactivation mechanisms remain unclear. We first examined plasmin-catalyzed factor VIII inactivation in the presence of phospholipid and Ca\(^{2+}\) using a one-stage clotting assay. Control experiments confirmed that the presence of either plasmin or pefabloc did not affect these assays at ~1000-fold dilution of the reaction mixture. At concentrations of 100 nM factor VIII and 4 nM plasmin, maximum factor VIIIa activity was observed after 3 minutes and reflected an approximately 1.7-fold increase (Figure 1). This peak activity was followed by a sharp decline to the initial level at 10 min, and finally to an undetectable level within 45 min. Similar results were observed in the absence of phospholipid and/or Ca\(^{2+}\). Factor VIII circulates as a complex with VWF, which protects and stabilizes the cofactor from inactivation, for example, by APC (32, 33). In the current experiments, however, the presence of VWF did not affect the reaction between plasmin and factor VIII.

**Comparison of factor VIII inactivation by plasmin, APC, and factor Xa** - The most potent known serine proteases responsible for factor VIII(a) inactivation are APC and factor...
Xa. We compared, therefore, factor VIII inactivation by plasmin, APC and factor Xa in a one-stage clotting assay. Each protease (4 nM) was incubated with factor VIII (100 nM) in the presence of phospholipid as described above. Control experiments showed that the presence of each protease alone did not affect the assays. APC mixed with protein S (1:10 molar ratio) inactivated factor VIII activity in a time-dependent manner with no initial elevation in activity (Figure 2A). The activity of factor VIII incubated with factor Xa increased approximately 2-fold compared with that obtained after the addition of plasmin. However, the characteristic “spike” of factor VIIIa activity observed in the presence of plasmin was not observed with factor Xa. In contrast, a broad activation plateau was seen for approximately 15 min, and was followed by a slower decrease in activity.

The activity of factor VIIIa at any time-point likely represents contributions from unactivated molecules, activated molecules, and activated molecules that have decayed following factor VIII subunit dissociation. To precisely evaluate the effect of inactivation of factor VIII by these enzymes, experiments were repeated using active factor VIIIa as a substrate. Each protease (4 nM) was incubated with factor VIIIa (25 nM) in the presence of phospholipid. The findings are illustrated in Figure 2B. The data were fitted to a single exponential decay curve and show that the inactivation rate of factor VIIIa activity in the absence of protease, reflecting A2 subunit dissociation from factor VIIIa trimer, was 0.015 ± 0.002 min⁻¹, similar to earlier reports (34). The rate constants of inactivation of factor VIIIa activity by plasmin, APC, and factor Xa under the same conditions were 1.11 ± 0.09 min⁻¹, 0.30 ± 0.04 min⁻¹, and 0.09 ± 0.01 min⁻¹, respectively, suggesting that the effect of factor VIIIa inactivation by plasmin was ~3.7-fold and ~12-fold greater than that with APC and factor Xa, respectively.

Cleavage of factor VIII by plasmin and identification of cleavage sites - The factor VIII activation/inactivation patterns induced by plasmin that was characterized by initial mild elevation followed by a rapid reduction in activity, and the difference in inactivation potential between plasmin, APC and factor Xa, strongly suggest that the mechanism of action of plasmin is different from those of APC and factor Xa. To provide direct evidence for this hypothesis, timed-course changes in electrophoretic mobility of plasmin-treated factor VIII were studied using factor VIII (1.5 µM) and plasmin (20 nM) in the presence of phospholipid. SDS-PAGE showed that plasmin sequentially proteolyzed factor VIII at several cleavage
sites (Figure 3A). The ~180-kDa heavy chain fragments consisting of A1, A2, and full sequenced B domains completely disappeared at 1 min after the addition of plasmin. In contrast, the 90-kDa fragment appeared to increase initially, followed by limited proteolysis into several protein products of apparent 50-, 48-, 42-, 40-, and 38-kDa. The 50-, 48-, and 42-kDa products disappeared gradually with time. The 40- and 38-kDa fragments appeared to persist as terminal products at 30 min, in the absence of factor VIII activity. The 80-kDa light chain fragments were degraded sequentially to 70-kDa products and 67-kDa fragments within 45 min.

To identify the sites of cleavage, all fragments were subjected to automated NH$_2$-terminal sequence analysis and compared with the amino acid sequences derived from human factor VIII cDNA (2, 3). The results indicated that plasmin cleaved the cofactor at four sites in the heavy chain, Lys$^{36}$, Arg$^{336}$, Arg$^{372}$, and Arg$^{740}$, and at two sites in the light chain, Arg$^{1689}$ and Arg$^{1721}$. These sites appeared to be identical to those cleaved by factor Xa (5, 13). The sequences of several factor VIII products generated from cleavage by plasmin were estimated from the NH$_2$-terminal sequence, the molecular weights, specificity for cleavage of plasmin, and Western blotting (as described in next paragraph) using anti-factor VIII monoclonal antibodies analyzed recognizing epitope. The amino acid sequences of the 90-, 50-, 48-, 42-, 40-, and 38-kDa fragments derived from the heavy chain were shown to be residues 1-740, 1-372, 337-740, 1-336, 373-740, and 37-336, respectively and were designated as A1-A2, A1$^{1-372}$, A1$^{337-372}$-A2, A1$^{1-336}$, A2, and A1$^{37-336}$, respectively. The amino acid sequences of the 70- and 67-kDa fragments derived from the light chain were residues 1690-2332 and 1722-2332, respectively, indicating that the light chain was cleaved by plasmin in the order Arg$^{1689}$ and Arg$^{1721}$. The relationship between these fragments is shown schematically in Figure 3B.

**Cleavage of the factor VIII heavy chain by plasmin** - Procoagulant factor VIII activity correlates well with the pattern of limited proteolysis of the heavy chain, as represented by cleavage at Arg$^{372}$ by thrombin and factor Xa for activation, and cleavage at Arg$^{336}$ by APC and factor Xa for inactivation. We focused, therefore, on plasmin cleavage of the factor VIII heavy chain using Western blotting (Figure 4). Products of proteolysis were visualized using two anti-A1 monoclonal antibodies recognizing the N-terminus (58.12, panel A) or C-terminus (C5, panel B) of A1 and one anti-A2 monoclonal antibody (JR8, panel C). The cleavage sites of the heavy chain by plasmin and epitope
regions of factor VIII monoclonal antibodies are schematically illustrated in panel D. In the presence of factor VIII (500 nM), plasmin (10 nM) and phospholipid, the heavy chain (A1-A2-whole B) was initially converted into A1-A2 subunits by cleavage at Arg\textsuperscript{740} (panels A-C). Subsequently, the A1\textsuperscript{337-372}-A2 fragment appeared to be generated more rapidly than the A2 product (inset in panel C), suggesting that cleavage at Arg\textsuperscript{336} occurred more quickly than that at Arg\textsuperscript{372}. Consequently the A1\textsuperscript{1-336} and A1\textsuperscript{337-372}-A2 fragments were generated as intermediate products. These intermediate products were not seen, however, at the 10 and 20 min time-points using the anti A1 antibodies (arrows in panels A and B). Since the 58.12 and C5 antibodies recognize the N- and C-terminal regions in A1, respectively, the failure to detect A1\textsuperscript{1-336} and A1\textsuperscript{337-372}-A2 fragments suggests that complete cleavage at Lys\textsuperscript{36} and Arg\textsuperscript{372} by plasmin generated terminal products A1\textsuperscript{37-336} and A2. An alternative product, A1\textsuperscript{1-372}, generated initially by cleavage at Arg\textsuperscript{372}, was further proteolyzed at Arg\textsuperscript{336} and Lys\textsuperscript{36}, also resulting in the terminal product A1\textsuperscript{37-336}. These results demonstrated that plasmin proteolyzed the heavy chain into A1\textsuperscript{37-336} and A2 subunits by two cleavage pathways, a more predominant cleavage at Arg\textsuperscript{336} and a minor cleavage at Arg\textsuperscript{372}.

**Cleavage of the A1 subunit of factor VIII(a) by plasmin, APC, and factor Xa** - The cleavage sites within the A1 subunit are Arg\textsuperscript{336} for APC and Arg\textsuperscript{372}, Arg\textsuperscript{336}, and Lys\textsuperscript{36} for factor Xa and plasmin. The changes associated with the activity of factor VIII incubated with proteases in the current studies were different, however, suggesting alternative mechanisms. To investigate this further, therefore, the A1 cleavage pattern mediated by plasmin, APC, and factor Xa, were analyzed by Western blotting using anti-A1 antibody 58.12 (Figure 5A). The addition of APC mixed with cofactor protein S cleaved factor VIII at Arg\textsuperscript{336} and resulted in the appearance of A1\textsuperscript{1-336} fragments in a time-dependent manner (panel b). Factor Xa cleaved initially at Arg\textsuperscript{740} followed by cleavages at Arg\textsuperscript{372} and Arg\textsuperscript{336} (panel c), consistent with the earlier reports (5). However, plasmin cleaved the A1 subunit at Arg\textsuperscript{336} much more rapidly than at Arg\textsuperscript{372}, followed by cleavage at Lys\textsuperscript{36} at an early time-point (panel a). These data were consistent with distinct rate-dependent cleavage patterns of the A1 subunit by plasmin, APC and factor Xa, although the cleavage sites appeared to be similar or identical.

Additional experiments, using active factor VIIIa, in place of inactive factor VIII, were devised to quantitate the kinetics of A1
cleavage. Western blotting using 58.12 antibody (Figure 5B) was utilized to assess both the proteolysis of the A1 subunit in factor VIIIa (left panels) and the rate of loss of intact A1\textsuperscript{1-372} subunit (right panel). The Western blots were analyzed by scanning densitometry and the data points extrapolated using a single exponential decay equation. The rate of cleavage of A1 in factor VIIIa (100 nM) by plasmin (4 nM) was 1.53 ± 0.16 min\textsuperscript{-1}, and was approximately 3.2 to more than 20-fold greater than that mediated by APC (0.48 ± 0.06 min\textsuperscript{-1}) and factor Xa (0.06 ± 0.01 min\textsuperscript{-1}). The findings were in keeping with earlier reports (15, 35) that inactivation of factor VIIIa can be attributed to A1 proteolysis at Arg\textsuperscript{336} (and Lys\textsuperscript{36}) and that similar cleavage governs plasmin-catalyzed inactivation of factor VIII.

**Role of individual factor VIIIa subunits in A1 subunit cleavage by plasmin**  
In order to further study the roles and mechanisms of each subunit in factor VIII(a) for plasmin-mediated proteolysis, various factor VIII(a) fragments (100 nM) were used as substrates for plasmin (4 nM), followed by the Western blotting using the anti-A1 58.12 antibody for detection (Figure 6). Since this antibody, recognizing the N-terminus of the A1 subunit, detects A1\textsuperscript{1-336} fragments but not A1\textsuperscript{37-336} fragments, the appearance or disappearance of A1\textsuperscript{1-336} can be attributed to cleavage at Arg\textsuperscript{336} or Lys\textsuperscript{36} respectively. The A1\textsuperscript{1-336} fragments derived from factor VIII (panel A), factor VIIIa (panel B), and intact heavy chain (panel C) were identified at an early time-point after the addition of plasmin. With A1/A3-C1-C2 dimers (panel D) little A1\textsuperscript{1-336} was detected and with isolated A1 subunits (panel E), the A1\textsuperscript{1-336} fragment was generated very slowly after the addition of plasmin, respectively. Factor VIII(a) and the intact heavy chain contain the A2 domain, but this is not present in A1/A3-C1-C2 dimers or isolated A1 subunits. Our results supported the view that cleavage at Arg\textsuperscript{336} may be modulated by the A2 domain (panel a). Interestingly, the A1\textsuperscript{1-336} fragments derived from factor VIII (panel A) and factor VIIIa (panel B) disappeared within ~5 min after the addition of plasmin. In contrast, with the intact heavy chain (panel C) and isolated A1 subunit (panel E), the A1\textsuperscript{1-336} fragments persisted strongly even at the 30 min-time point. With the A1/A3-C1-C2 dimer (panel D), the A1\textsuperscript{1-372} fragment disappeared after the addition of plasmin, without the appearance of A1\textsuperscript{1-336}. It seemed likely, therefore, that cleavage at Lys\textsuperscript{36} in the dimer form was predominant, probably with little cleavage at Arg\textsuperscript{336}. The factor VIII(a) molecule and the dimer contain A3-C1-C2 subunits but these are not constituents of the heavy chain or isolated A1. Our findings suggest, therefore, that cleavage at Lys\textsuperscript{36} was likely regulated by
the presence of the A3-C1-C2 subunit (panel b). Taken together, these data demonstrated that specific cleavages at Arg^{336} and Lys^{36} by plasmin appeared to be selectively modulated following interaction of plasmin with the A2 and A3-C1-C2 subunit, respectively.

DISCUSSION

In normal hemostasis there is a controlled balance between coagulation and anticoagulation or fibrinolysis. An imbalanced state between these systems leads to pathologic thrombosis or hemorrhage consequent to the excessive activation and inactivation of coagulation and fibrinolytic factors (36, 37). In clinical disseminated intravascular coagulation (DIC), the course of the disease is complicated by uncontrolled proteolysis of important clotting factors (38). During the development of DIC, numerous proteases are likely to be active simultaneously, and in particular, plasmin is likely to be a major instigator of the observed proteolysis (39, 40). In this context, the proteolysis of fibrinogen and/or fibrin by plasmin has been extensively studied and documented in the literature (41). It is less widely appreciated, however, that other mechanisms involving thrombolytic proteases may promote a hemorrhagic response, and that plasmin may have an anticoagulant effect by moderating the activity of coagulation cofactors factor V or factor VIII and limiting excessive activation of the coagulation system. An earlier study reported that an increase in the plasma concentration of plasmin correlated with decreased factor VIIIa activity (42). The present study was undertaken, therefore, to examine the relationship between factor VIII and plasmin.

We observed that plasmin inactivated factor VIII activity rapidly after an initial weak increase (~2-fold) in activity in a clotting based-assay. This phospholipid-independent response was similar for both native factor VIII and active factor VIIIa (data not shown). However, the phospholipid-independent action of plasmin on factor VIII was different from that reported for other clotting factors. For example, the inactivation of factor Va by plasmin appeared to be phospholipid/Ca^{2+}-dependent (17), and the mechanism has been described to be associated with critical residues 307-348 of the factor V molecule (18). Similarly, plasmin-induced cleavage and inactivation of factor X appeared to be lipid-dependent (20). Factor VIII and factor V are structurally homologous (43), and our data imply distinct similarities in the action of plasmin on factor VIII and factor V.

Factor VIII circulates in a noncovalent
complex with VWF. Critical sites for factor VIII interaction with VWF have been localized to the N-terminal acidic region of the A3 domain (8), to the C-terminus of the C2 domain (44) and within the A3-C1 domain (32, 45), supporting the view that the A3-C1-C2 environment forms an extended surface for interaction with VWF. In our experiments neither VWF nor phospholipid affected plasmin-catalyzed factor VIII inactivation. It appeared, therefore, that plasmin interaction with factor VIII did not involve VWF and phospholipid-interactive sites, and was not dependent on activated and/or unactivated forms of the cofactor. These findings indicated that plasmin may be a unique anticoagulant protease for factor VIII. Activation and inactivation of factor VIII by other proteases, APC (32, 33) and factor Xa (46, 47), are markedly affected by the presence of VWF. Interestingly, an earlier report by Rick et al. (22) demonstrated that factor VIII activation and inactivation by plasmin was affected by the presence of platelets but not by phospholipid vesicles. The reason for this difference was not clear, but it may be that the conformation of factor VIII bound to platelets may be more susceptible to plasmin compared with that bound to isolated phospholipid moieties. Our studies were performed using phospholipid vesicles and further experiments are in progress to investigate possible differences in reaction between phospholipid and intact platelets.

A combination of SDS-PAGE and NH$_2$-terminal sequence analysis confirmed that limited proteolysis of factor VIII occurred at four positions in the heavy chain, Lys$^{36}$, Arg$^{336}$, Arg$^{372}$, and Arg$^{740}$ and at two sites in the light chain, Arg$^{1689}$ and Arg$^{1721}$, resulting in the generation of three terminal products, A1$^{37-336}$, A2, and A3-C1-C2$^{1722-2332}$ within one hour of adding plasmin. The amino acid specificity of these cleavage sites is in agreement with the known preference of the protease for hydrolyzing either arginine or lysine residues (48). The six plasmin cleavage sites included those lysed by thrombin and factor Xa which result in activation of factor VIII cofactor, and those lysed by APC and factor Xa which result in inactivation. Surprisingly, the plasmin cleavage sites were identical to those observed after interaction of factor VIII with factor Xa (5, 13), but one of the APC cleavage sites, Arg$^{562}$, was not affected by plasmin. We also observed the A2 doublet with unidentified band (Figure 4C). The N-terminal sequences of the double bands were identical (data not shown). Therefore, this is unclear at the present time. Fay et al. (7) reported that although the doublet bands derived by the heavy chain cleavage by thrombin and factor Xa could be observed,
the origin of the bands was unclear.

Proteolysis at Arg^{372} and Arg^{1689} is essential for generating factor VIIIa cofactor activity (6). Recently, Nogami et al. reported that failure of proteolysis at Arg^{740} resulted in markedly low cofactor activity, indicating that cleavage at the A2-B junction may be an essential step in the process of procofactor activation (49). In our studies, the initial activation of factor VIII by plasmin appeared to be associated with three cleavage sites. We focused attention, therefore, on inactivation of factor VIII, and it was notable that cleavage by plasmin at Arg^{336} within A1 subunit was rapid compared with that at Arg^{372} between A1-A2 junction. This predominant cleavage at Arg^{336} rather than at Arg^{372} was in contrast to the cleavage process reported for factor Xa. Furthermore, the cleavage at Arg^{336} was very rapid compared with similar cleavage induced by APC. It is well-known that serine proteases including APC and factor Xa inactivate factor VIII(a) following cleavage at Arg^{336} (15, 35). Inactivation occurs due to an altered interaction between the A2 subunit and the truncated A1, and results in the loss of a factor X-interactive site within residues 337-372 (14) and an increase in the $K_m$ for substrate factor X (12, 13). Our data using a clotting-based assay and SDS-PAGE supported the concept that the degree of factor VIII inactivation is likely to be dependent on the proportion of unactivated molecules, activated molecules and decay following subunit dissociation, the more rapid the cleavage at Arg^{336} in the A1 subunit, the more rapid the inactivation of factor VIII(a).

A relatively recent report described that additional cleavage at Lys^{36} altered the conformation of the A1 subunit and limited productive interaction with the A2 subunit (13). Consequently, there appeared to be approximately half the level of activity of factor VIIIa compared with the A1^{1-336} subunit (13). In the present study, proteolysis by plasmin at Lys^{36} occurred more rapidly than that observed by factor Xa. These results suggested that the proteolysis at Lys^{36} might contribute to some extent to the rapid inactivation of factor VIII(a) by plasmin. No influence on inactivation of factor VIII by cleavage at Arg^{1721} in the light chain has been reported (50). In this context our data again demonstrated that cleavage at Arg^{336} was a major mechanism responsible for plasmin-catalyzed inactivation of factor VIII.

A comparison of the proteolysis of the factor VIII heavy chain by plasmin, APC, and factor Xa is schematically illustrated in Figure 7. These mechanisms have been well described.
APC cleaves factor VIII at Arg$^{336}$ within the A1 domain, followed by Arg$^{562}$ within A2 (51). Factor Xa cleaves initially at Arg$^{372}$ and then at Lys$^{36}$ and Arg$^{336}$ (35). Interestingly, the terminal products derived from plasmin-cleavage are identical to those produced by factor Xa. Although plasmin-catalyzed cleavage of the heavy chain may involve the two sites, Arg$^{336}$ and Arg$^{372}$, within the A1 domain, cleavage at Arg$^{336}$ would appear to be the predominant pathway.

The factor VIII-APC interactive sites have also been localized to the A3 domain (32, 52). In addition, Nogami et al. demonstrated that factor Xa-catalyzed reactions at Arg$^{336}$ and Lys$^{36}$ are likely selectively regulated by interactions with the A3-C1-C2 and A1 subunits, respectively (35, 47). The data from the present study using gel analysis, indicated that cleavage at Arg$^{336}$ was selectively enhanced by plasmin associated with A2 subunit, and cleavage at Lys$^{36}$ was regulated by the A3-C1-C2 subunit, although conformational changes of the A1 subunit associated with factor VIII(a) could not be completely excluded. On this basis, we suggest that this mechanism of plasmin activity is distinct from that of factor Xa, although the cleavage sites are identical for both proteases. Plasmin is comprised of a heavy chain containing five kringle domains and a light chain containing the catalytic domain. It is reactive with numerous proteins represented typically by lysine-binding site interaction with fibrin (41). It is attractive to speculate, therefore, that clustered basic residues of lysine (and arginine) found within both A2 and A3-C1-C2 sequences but not the A1 domain (2, 3) provide the natural target for plasmin in the factor VIII molecule.

The physiological significance of plasmin-catalyzed cleavage of factor VIII, resulting in activation and inactivation of cofactor function, remains to be fully determined. However, even very low concentrations (4 nM) of protease, generated from high plasma concentrations of proenzyme, plasminogen (~2.4 µM), would be sufficient to promote a catalytic rate approximately 3.7- and 12-fold greater than APC and factor Xa, respectively. Our data imply that small amounts of plasmin generated in the fibrinolytic response might contribute to the up- and down-regulation of blood coagulation. Furthermore, the plasmin-catalyzed inactivation mechanism of other clotting factors such as factor Va (18) and factor IX (19) has been also reported. Especially, factor V has similar conformation to factor VIII and similar activation/inactivation mechanisms. Therefore, we also suggest the presence of regulatory role of plasmin through direct proteolytic reaction in the coagulation
reaction as well as fibrinolytic activity. 

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FOOTNOTES

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1The abbreviations used are: VWF: von Willebrand factor, APC: activated protein C, SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis, MES: 4-morpholineethanesulfonic acid, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, CAPS: 3-(cyclo-hexylamino)-1-propanesulfonic acid, DIC: disseminated intravascular coagulation
REFERENCES

FIGURE LEGENDS

Figure 1. Time course of activation of factor VIII following reaction with plasmin.
Factor VIII (100 nM) was incubated with plasmin (4 nM) in the presence (open circles) or absence (closed circles) of Ca\(^{2+}\) (5 mM) for the indicated times, after which the reaction was terminated by pefabloc. Each sample was tested immediately for factor VIIIa activity in a one-stage clotting assay. In addition, factor VIII was preincubated with VWF (100 nM, open squares) or phospholipid vesicles (10 \(\mu\)M, closed squares) prior to addition of plasmin. The zero point was taken prior to addition of plasmin. The initial activity of factor VIII was \(\sim\)50 units/ml and designated as 100%. Experiments were performed at least three separate times and average values are shown.

Figure 2. Comparisons of the time course of factor VIII(a) inactivation by plasmin, APC, and factor Xa
Factor VIII (A, 100 nM) or factor VIIIa (B, 25 nM) was incubated with 4 nM of plasmin (open circles), APC (closed circles) together with protein S (40 nM), factor Xa (open squares), and buffer only (closed squares) in the presence of phospholipid vesicles (10 \(\mu\)M). Factor VIIIa activity was measured at indicated times using a one-stage clotting assay. The initial activities of factor VIII or factor VIIIa (100% level) was \(\sim\)50 units/ml or \(\sim\)80 units/ml, respectively. The values of factor VIIIa activity were plotted as a function of incubation time and the data in (B) fitted to an equation of single exponential decay. Experiments were performed at least three separate times and average values are shown.

Figure 3. Time course of plasmin-catalyzed proteolysis of factor VIII.
(A) SDS-PAGE of factor VIII (1.5 \(\mu\)M) was incubated with plasmin (20 nM) in the presence of phospholipid vesicles (10 \(\mu\)M) as described under “Materials and Methods”. At the indicated times, the reaction was terminated, and samples were run on an 8% gel and stained with GelCode Blue. The molecular weight and amino acid sequence of each fragment were obtained from given molecular weight marker (MW. lane 1) and NH\(_2\)-terminal sequence analysis. (B) Schematic representation of the cleavage sites in factor VIII by plasmin and the generated cleaved factor VIII fragments. The characters from (a) to (i) corresponded in figures (A) and (B).
Figure 4. Time course of plasmin-catalyzed cleavage of the heavy chain of factor VIII.
Factor VIII (500 nM) was incubated with plasmin (10 nM) in the presence of phospholipid vesicles (10 µM) for the indicated times as described under “Materials and Methods”. Samples were run on 8% gel followed by Western blotting using anti-A1 (58.12, A), anti-A1 (C5, B), or anti-A2 (JR8, C) monoclonal antibodies. Panel D shows a schematic presentation of the domain organization of the heavy chain, location of plasmin-catalyzed cleavage sites, and epitope regions of monoclonal antibodies. The inset in panel C represents the appearance of A1337-372-A2 and A2 bands at an early time-phase (within 60 sec). The arrow in panel A or B shows the disappearance of A11-336 or A1337-372-A2 band by proteolytic cleavage at Lys36 or Arg372, respectively.

Figure 5. Comparison with A1 cleavage in factor VIII(a) by plasmin, APC, and factor Xa.
Factor VIII (A, 100 nM) or factor VIIIa (B, 100 nM) was incubated with 4 nM of plasmin (panel a), APC (panel b) together with protein S (40 nM), and factor Xa (panel c) in the presence of phospholipid vesicles (10 µM) for the indicated times as described under “Materials and Methods”. Samples were run on 8% gel followed by Western blotting using an anti-A1 antibody (58.12). Right panel in B shows the data obtained by quantitative densitometry of the intact A11-372. The symbols used are; open circles; plasmin, closed circles; APC, and open squares; factor Xa. The band density of A11-372 at time zero point was designated as 100%. Data were extrapolated using a single exponential decay curve.

Figure 6. Effects of factor VIII(a) subunit on Lys36 and Arg336 cleavages in A1 subunit by plasmin.
Equivalent concentrations (100 nM) of factor VIII (A), factor VIIIa (B), intact heavy chain (C), A1/A3-C1-C2 dimer (D), or A1 subunit (E) were incubated with plasmin (4 nM) in the presence of phospholipid vesicles (10 µM) for the indicated times as described under “Materials and Methods”. Samples were run on 8% gel followed by Western blotting using an anti-A1 antibody (58.12). Right panels schematically illustrate the relationship between the cleavage at Arg336 (panel a) or Lys36 (panel b) and the A2 or A3-C1-C2 subunit (bold), respectively.

Figure 7. A schematic comparison of the proposed pathways for the cleavage of factor VIII heavy chain by plasmin, APC, and factor Xa.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 5
Figure 6
Figure 7
Mechanisms of plasmin-catalyzed inactivation of factor VIII. A crucial role for proteolytic cleavage at Arg 336 responsible for plasmin-catalyzed factor VIII inactivation

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