Articles

Mutations in the Catalytic Domain of Factor IX That Are Related to the Subclass Hemophilia Bm†

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ABSTRACT: Hemophilia Bm, a variant of hemophilia B, results in a marked increase in the ox brain prothrombin time. Mutations known to cause hemophilia Bm occur at residue 180, 181, or 182 near the amino terminus of the heavy chain and at residue 311, 364, 368, 390, 396, or 397 near the activation site of factor IX (Giannelli *et al.*, 1990). In this study we replaced factor IX residues 181, 182, and 390 in separate experiments by site-directed mutagenesis. Valine 181 was replaced by isoleucine or alanine, and valine 182 was replaced by alanine or glycine. Alanine 390 was replaced by valine or aspartic acid. Recombinant factor IXs were expressed in human kidney 293 cells and purified by absorption and elution from a conformational specific monoclonal antibody column. The results show that factor IX Bm is a function not only of the position of the mutated amino acid but also of the particular amino acid substituted. For example, when valine 181 or 182 was replaced by small hydrophobic amino acids (alanine and glycine), factor IXs were found to have significantly decreased clotting activity. Unlike the naturally occurring mutations (Val_{181}→Phe_{181} or Val_{182}→Leu_{182}), however, the small amino acid replacements did not result in prolonged ox brain prothrombin times. Surprisingly, the Ala_{390}→Asp_{390} exchange did not affect clotting activity or binding to the macromolecular inhibitor antithrombin III. The Ala_{390}→Val_{390} exchange resulted in loss of both clotting activity and binding to antithrombin III. These results suggest that residue 390 is not directly involved in binding to antithrombin III. Furthermore, our results suggest that residue 390 does not make significant intermolecular interactions. It is likely those factor IX Bm variants with mutations at 181, 182, or 390 are defective in the conformational change required for activation, which normally occurs upon cleavage between residues 180 and 181.

Factor IX is a blood coagulation protein whose gene is located on the X-chromosome (Thompson, 1986). Defects in factor IX result in the X-linked bleeding disorder hemophilia B. Several point mutations of factor IX cause bleeding disorders subclassified as hemophilia Bm. This classification is based on prolongation of the ox brain prothrombin time (PT), compared with that of normal factor IX. Excepting mutations reported for residue 180 (Table I), all Bm mutations are found in factor IX's catalytic domain.

The activation of serine protease zymogens is well-defined, and one expects the first two residues of the catalytic domain of factor IX (residues 181–182) to flip into the activation pocket after cleavage of the 180–181 peptide bond. Figure 1 depicts the expected three-dimensional structure of factor IX after activation. This process is estimated to take approximately 10 ns (Butz *et al.*, 1982), and the NH^{α} of residue 181 is expected to move as far as 7 Å relative to the zymogen. Aspartic acid (residue 364) is adjacent to the active site serine (residue 365) and forms a new salt bridge with the N-terminus of the catalytic domain (residue 181) when the N-terminus moves into the activation pocket. Glycine (residue 311) is at the bottom of the activation pocket and is a part of the hydrogen bond network, which involves the N-terminus of the catalytic domain (residue 181 and residue 364). Alanine (residue 390) is at the edge of the activation pocket and has van der Waals interactions with the second residue (residue 182) in the heavy chain. Among these sites, residues 181–182 and 390 are probably exposed to solvent. This makes them suitable for investigation by site-directed mutagenesis because changed surface residues are less likely to disrupt the folding process.

Table I: Reported Factor IX_{Bm} Variants

position of mutations	type of amino acid substitutions	reported activity in plasma (%)	reported antigen level (%)
180	R→G	<1	90
180	R→Q[b]	<1	112–120
180	R→W	<1	69–130
181	V→F[c]	<1	130
182	V→L[c]	15	132
182	V→F[c]	<1	120
311	G→E	<1	100
364	D→N	2	ND
368	P→T	<1	156
390	A→V	<1–4	48–140
396	G→R[c]	<1	90–100
397	I→T[c]	<1–5	46–69

[a] Data taken from the database by Giannelli et al. (1991). [b] Mutations are reported to moderately prolong and to significantly prolong the ox brain PT time by different groups. [c] Mutations are reported to moderately prolong the ox brain PT time.

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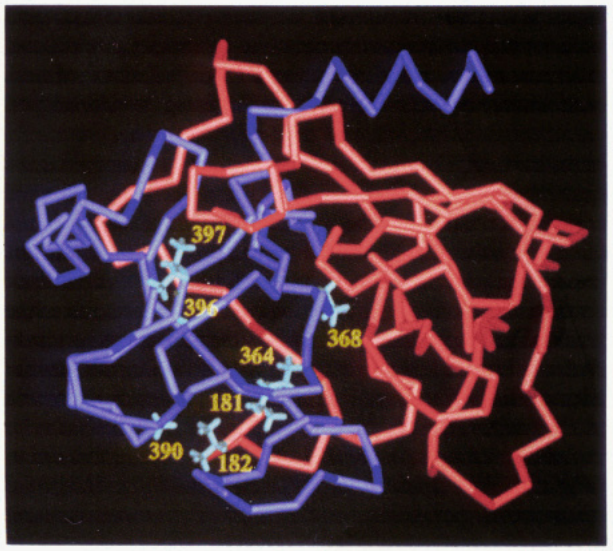

FIGURE 1: Residues implicated in causing factor IX_{Bm} variants in the catalytic domain of factor IXa. The α-carbon traces of residues 181–310 and residues 311–415 are shown in red and blue, respectively. The residues related to factor IX_{Bm} variants are numbered and shown with side chains in green. Illustrated is a view toward the active site pocket in the center of the figure, at the interface between the red and blue traces. The model was developed as described previously by Hamaguchi (1991).

Since the activation sites are relatively conserved among serine proteases, several of our mutations were based on a comparison of their amino acid sequences. This comparison makes evident that residues 181 and 182 must be hydrophobic amino acids. Therefore, isoleucine or alanine was chosen to exchange for the first residue (valine) and alanine or glycine for the second residue (valine). The naturally occurring mutations in factor IX at these sites are valine to phenylalanine at 181 and valine to phenylalanine or leucine at 182. Mutations were designed to maintain a hydrophobic profile similar to that of normal factor IX. Surface complementarity involving intermolecular hydrophobic interactions are nonspecific and can be induced upon binding. It was therefore expected that these mutations would have minor effects on activation of factor IX by factor XIa and would give us insight into the activation process.

At position 390 aspartic acid was chosen to replace the naturally occurring alanine. Alanine is the most common residue found in the comparable position of active serine proteases, although serine and aspartic acid, respectively, are found in chymotrypsin and thrombin. If residue 390 of factor IX is directly involved in binding to other macromolecules such as its substrate factor X or cofactor factor VIIIa, the change from a small hydrophobic amino acid to a bulky, negatively charged amino acid (such as aspartic acid) is unlikely to be tolerated. If, however, the functional importance of this residue is related to a feature common to serine proteases, it should be interchangeable with other amino acids such as aspartic acid and serine.

Mutated factor IXs were produced by *in vitro* mutagenesis. After purification of the mutated proteins from a mammalian cell expression system, the effects of the mutations were examined.

EXPERIMENTAL PROCEDURES

Materials

The oligonucleotides used for *in vitro* mutagenesis were purchased from Oligo etc. (Wilsonvile, OH). The T7 DNA polymerase came from U.S. Biochemicals (Cleveland, OH), and the polynucleotide kinase and T4 DNA ligase were obtained from Bethesda Research Laboratory (Bethesda, MD), New England Biolabs (Boston, MA), and Boehringer Mannheim (Tutzing, Germany).

The anti-human factor IX monoclonal antibodies used in this study were A-1, A-5, and A-7 (Frazier et al., 1989). Factor X, factor VIIa, and human antithrombin III were purchased from Enzyme Research Laboratories (South Bend, IN). Spectrozyme Xa and human factor IX deficient plasma were purchased from American Diagnostica Inc. (Greenwich, CT). Platelin was obtained from Organon Teknika (Durham, NC). Platelin Plus activator was purchased from General Diagnostics (Morris Plains, NJ). Recombinant human tissue factor (TF) was a gift from Genentech Inc. (San Francisco, CA). Thrombotest was purchased from Nycomed (Oslo, Norway). Factor XIa was a gift from Dr. D. Straight (UNC-CH). All other reagents were of the highest purity available.

Methods

In Vitro Mutagenesis and Construction of the Expression Plasmid. Site-directed mutagenesis was performed as described by Kunkel (1985). The newly created restriction recognition sites in these mutations were used to check the mutations in the expression vectors. Each mutated cDNA was sequenced by the dideoxy-chain termination method (Tabor & Richardson, 1987) and was inserted into the pCMV4 vector (Andersson *et al.*, 1989).

Cell Culture and Transfection of Mutated Factor IX cDNA and Purification of Recombinant Factor IX. Human embryo kidney 293 cells were grown in a mixture of Dulbecco's modified Eagle medium and F-12 medium (1:1), supplemented with 10% fetal calf serum. pCMV4 FIX expression vectors were cotransfected into cells with pSV2neo (selection marker plasmid), using the calcium phosphate coprecipitation method. Selection of cells for production and purification of recombinant factor IXs from culture medium was as described (Hamaguchi *et al.*, 1991).

Clotting Assay. One-stage activated partial thromboplastin time (aPTT) assays were performed according to the manufacturer's instructions (Organon Teknika). The ability of the proteins to correct the clotting time of factor IX deficient plasma was calculated from a standard curve derived from pooled normal plasma. The ox brain prothrombin time assay was performed by mixing 250 μL of Thrombotest (Nycomed, Oslo, Norway), 25 μL of factor IX deficient plasma, and 25 μL of factor IX diluted to 5 μg/mL with TBS. Ox brain PT time was measured according to the manufacturer's instructions.

Activation of Factor IXs by Factor XIa and Binding to p-Aminobenzamidine. The cleavage of factor IX was carried out at a substrate to enzyme ratio of 200:1 at 37 °C. Samples were examined on SDS–PAGE (Laemmli, 1970) and visualized by Coomassie Brilliant Blue R-250 staining.

The cleavage of factor IX by factor XIa was also performed at an enzyme to substrate ratio of 1:14 in the presence of *p*-aminobenzamidine, as described by Monroe *et al.* (1988). The formation of an active site in factor IX was monitored by the fluorescence change of *p*-aminobenzamidine upon activation.

Antithrombin III Binding. Approximately 0.2 μg of factor XIa-cleaved factor IXs was incubated with antithrombin III in the presence of heparin for 30 min before SDS–PAGE analysis under nonreducing conditions. The gels were silver-stained to examine change in mobility.

Table II: Clotting Activity, Ox Brain PT Time, *p*-Aminobenzamidine Binding, and Antithrombin III Binding of Mutated Factors IXs

position of mutations	type of mutation	aPTT activity (%)	ox brain PT time (s)	*p*-ABA[a] binding	ATIII[b] binding
181	V→I	~100	58	+	+
181–182	VV→AA	~30	60	+	+
181–182	VV→IA	~1	60	–	+
181–182	VV→IG	~1	60	–	+
390	A→D	~100	59	+	+
390	A→V	<1	77	–	–
181–182 and 390	VV→AA and A→V	<1	74	–	–
181–182 and 390	VV→IA and A→V	<1	85	–	–
181–182 and 390	VV→IG and A→V	<1	74	–	–

[a] *p*-ABA = *p*-aminobenzamidine. [b] ATIII = antithrombin III.

Activation of Factor X by Activated Factor IX_{A390V}. Plasma-purified factor IX and factor IX_{A390V} were activated by factor XIa at 37 °C for 2 h in TBS with 5 mM $CaCl_2$ at an enzyme to substrate ratio of 1:100. Activated factor IXs were repurified using an A-7 MoAb column, and yields of the recovered factor IXa from the column were estimated by RIA using ^{125}I-labeled A-7 MoAb. Factor IXa activation of factor X was performed as described (Hamaguchi *et al.*, 1991) at a factor X concentration of 0.8 μM and a factor IXa concentration of 250 nM.

Inhibition of the Factor VIIa–TF Complex Activation of Factor X. Activation of factor X by the factor VIIa–TF complex was measured by the increasing rate of hydrolysis of Spectrozyme Xa. The factor VIIa–TF complex was formed in an equal volume mixture of 0.14 μg/mL factor VIIa, 0.1 μg/mL recombinant TF, and Platelin at 37 °C for 30 min in TBS containing 15 mM $CaCl_2$ and 0.1% of BSA. Each 9 μL of the factor VIIa–TF complex was then added with factor X (final concentration of 180 nM), Spectrozyme Xa (final concentration of 0.5 mM), and factor IX (final concentration of 180 nM). The total volume of reaction was adjusted to 300 μL with TBS. The absorbance at 406 nm was measured, and the relative velocity of the activation of factor X by the factor VIIa–TF complex was calculated by methods described by Geddes *et al.*, (1989).

Molecular Modeling. The model for factor IX was developed from the X-ray crystallographic coordinates of trypsin and bovine thrombin. The structure was energy-minimized using Amber version 3.1 with explicit solvent utilizing periodic boundary condition as described by Hamaguchi *et al.* (1991).

RESULTS

Clotting Activity of Factor IXs. The results of clotting assays are shown in Table II. Factor IX_{V181I} and factor IX_{V390D} retained normal clotting activity. Factor $IX_{VV181AA}$ retained approximately 30% of normal clotting activity. Factor $IX_{VV181IA}$ and factor $IX_{VI181IG}$ had low clotting activity, estimated to be approximately 1% of normal. All of the mutated factor IXs possessing a valine at 390 had no detectable activity even at 100-fold higher concentrations. Specific clotting activities of patients' factor IX were reported as follows: less than 1% for valine to phenylalanine at 181, less than 1% for valine to phenylalanine at 182, and 15% for valine to leucine at 182 (Giannelli *et al.*, 1991). Reported specific clotting activities of factor IX with a mutation at 390 have varied from less than 1% to 4% for the substitution by valine, to 6% for the substitution by glutamic acid (Giannelli *et al.*, 1991).

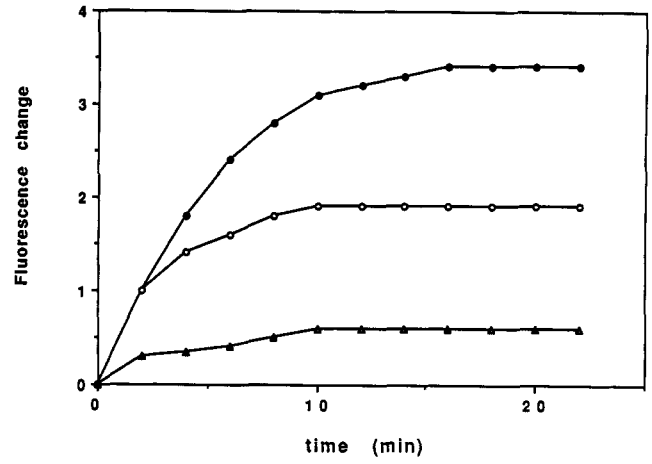

FIGURE 2: Binding of *p*-aminobenzamidine upon activation of factor IX by factor XIa—factor $IX_{VV181AA}$ and factor IX_{A390D}. The reaction solutions contained factor IX (500 nM), factor XIa (7 nM), and *p*-aminobenzamidine (150 mM). Details are described in the text and under Methods. The reactions were started by adding factor XIa. Symbols: (●) plasma-purified factor IX; (○) factor $IX_{VV181AA}$; (△) factor IX_{A390D}.

The ox brain PT time assays were performed as described under Methods. Without factor IX, the PT time was 53 (±1) s, while the addition of plasma-purified factor IX at the normal plasma level prolonged the ox brain PT time to 61 (±1) s. The results obtained in the presence of recombinant factor IXs are shown in Table II. Only recombinant factor IXs with valine at 390 significantly prolonged the ox brain PT time. Factor IX_{A390D} exhibited no more inhibition than normal factor IX. Factor IX mutated at position 181 (phenylalanine) or position 182 (phenylalanine or leucine) are Bm variants. However, mutations to different amino acids at 181 (isoleucine and alanine) or 182 (alanine and glycine) (Table II) did not result in the Bm phenotype.

Activation of Factor IXs by Factor XIa and Binding to p-Aminobenzamidine. All mutated factor IXs displayed similar time courses of activation by SDS–PAGE analysis (data not shown). Factor XIa activation of factor IX_{V181I}, factor $IX_{VV181AA}$, and factor IX_{A390D} could also be shown with *p*-aminobenzamidine (Figure 2). *p*-aminobenzamidine is an active site inhibitor that changes fluorescence intensity upon binding to the active site (Monroe *et al.*, 1988). Although the observed fluorescence changes of factor IX_{V181I}, factor $IX_{VV181AA}$, and factor IX_{A390D} were significantly lower than that of plasma-purified factor IX, the fluorescence change occurred during a similar time period, suggesting the formation of the active site. The other recombinant factor IXs, factor $IX_{VV181IG}$, factor $IX_{VV181IA}$, factor $IX_{VV181IA,390V}$, factor $IX_{VV181IG,390V}$, and factor IX_{A390V}, exhibited no significant fluorescence change compared with the background obtained without factor XIa.

Antithrombin III Binding. The bindings of antithrombin III and recombinant factor IXs were examined by SDS–PAGE analysis as described under Methods. The results are summarized in Table II. Factor IXs, mutated at the first two residues of the catalytic domain, could bind antithrombin III after activation. Factor IX, mutated to valine at position 390, failed to bind to antithrombin III; however, factor IX_{A390D} reacted with antithrombin III in the same manner as normal factor IXa.

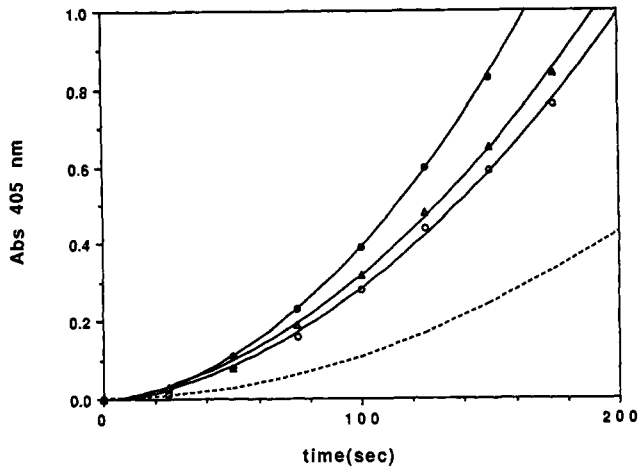

FIGURE 3: Factor X activation by the factor VIIa–TF complex in the presence of plasma factor IX and factor IX_{A390V}. The factor VIIa–TF–phospholipid complex (25 pM) and Spectrozyme Xa (0.5 mM), with and without factor IX (180 nM), were mixed in TBS containing 0.1% PEG 8000 and 5 mM CaCl2. The reactions were started by adding factor X (180 nM). Symbols: (●) without factor IX; (○) with purified plasma factor IX; (△) with factor IX_{A390V}. The dotted line is a simulated reaction curve in the presence of factor $IX_{Deventer}$, based on previous data (Bertina *et al.*, 1990).

Activation of Factor X by Activated Factor IX_{A390V}. The activation of factor X without any cofactors was examined for factor IX_{A390V}. The observed acceleration rate of *p*-nitroaniline released from Spectrozyme Xa for plasma factor IXa was 6.4 ODU/min, consistent with previous experiments (Hamaguchi *et al.*, 1991): no measurable increase was found in the acceleration rate by activated factor IX_{A390V}. This suggests that the defect of the molecule is related to catalytic activity and probably not to binding to its cofactors.

Inhibition of the Factor VIIa–TF Complex Activation of Factor X. The activation of factor X by the human factor VIIa–TF complex was performed as described under Methods. There was no significant hydrolysis of Spectrozyme Xa by the factor VIIa–TF complex (<1%). The rate of activation of factor X by factor VIIa–TF, in the presence of plasma-purified factor IX and factor IX_{A390V} (180 nM), was calculated to be 64% and 67%, respectively, compared with the activation rate without factor IX (Figure 3). The reaction rate (dotted line in Figure 3) in the presence of factor $IX_{Deventer}$ was simulated from previous data (Bertina *et al.*, 1990). This shows that factor IX_{V390} is not inhibitory in the presence of human TF.

DISCUSSION

Factor IXs with naturally occurring mutations at 181 and 182 (phenylalanine at 181 and phenylalanine or leucine at 182) are considered Bm variants (Bertina *et al.*, 1990; Taylor *et al.*, 1990; Sakai *et al.*, 1989). In contrast to these naturally occurring mutations, none of the recombinant factor IXs with smaller residues (alanine, glycine) at these sites prolonged the ox brain PT time. This shows that not only the position of the mutation but also the type of substituted residue determines if the mutation has a Bm phenotype.

Besides exhibiting a Bm phenotype, the mutation at 390 was expected to disrupt the macromolecular binding site since it is probably on the surface (Spitzer *et al.*, 1988); however, factor IX_{A390D} retained full activity in the clotting assay. If alanine 390 were involved in the macromolecular binding site as a part of a hydrophobic cluster, as suggested (Spitzer *et al.*, 1988), the introduction of a highly charged residue at this position should decrease the binding energy significantly (Fersht *et al.*, 1985). Thus, it is reasonable to conclude that residue 390 is not directly involved in the macromolecular binding site. This is also consistent with the findings that patients' factor IX with glutamic acid at 390 had a higher specific activity (clotting activity/antigen level) than patients' factor IX with valine at 390 (Giannelli *et al.*, 1991).

One can assume that there is no significant disruption in the overall structure by the substitution of valine at 390 because the mutated molecule is cleaved normally by its activators; moreover, it reacts with four different monoclonal antibodies for different regions of the heavy chain of factor IX (data not shown). The mechanism of the defects in mutations at residue 390 may be explained by the properties of its side chain. The most significant difference between alanine, aspartic acid, and valine is the branching of the side chain at the β-carbon. There is no side chain branching at the β-carbon in alanine and aspartic acid; however, two methyl groups branch off at the β-carbon in valine. Furthermore, all of the amino acids found at this position among serine proteases have no branching side chains at the β-carbon positions although the electrostatic properties of amino acids vary: aspartic acid in thrombin, asparagine in elastase, serine in chymotrypsin, and glycine in kallikrein. In the computer model of factor IX, as well as the X-ray crystallographic structures of trypsin, there are van der Waals interactions between the β-carbon of residue 390 (alanine) and the side chain of residue 182 (valine). The substitution of leucine at valine 182 in factor IX also caused a loss of clotting activity (1%). Kallikrein, which has isoleucine at a position equivalent to 182 in factor IX, is the only serine protease with a smaller residue (glycine) substituted at the position equivalent to alanine 390 in factor IX. We surmised that these two residues are complementary and a molecule might be active since one residue is large and the other small. Thus, we attempted to recover the activity of factor IX with valine at 390 by introducing a second mutation at residue 182. Unfortunately, no smaller residue (alanine or glycine) at 182 could compensate for the mutation at 390 (factor $IX_{V182A,A390V}$) (Table II). It is possible that the first residue (181) in the catalytic domain may also interact with residue 390 during the activation process. To form the active conformation, the first residue must pass through the opening (including residue 390) of the activation pocket. However, mutated factor IX containing alanines at 181 and 182 and valine at 390 (factor $IX_{VV181AA,I390V}$) lacked activity.

A recent study indicates that surface loops of residues 221–224 (in the chymotrypsin numbering system, these correspond to residues 390–393 in factor IX) of trypsin and chymotrypsin involve a structural feature that determines the substrate specificity of these enzymes (Hedstrom *et al.*, 1992). It is clear from the known crystallographic structures of enzyme–substrate or enzyme–inhibitor complexes that this surface loop is not involved in substrate binding. Therefore, they proposed that this loop affects the structural flexibility required by the reaction dynamics or is a crucial structural feature near or at the active site. This hypothesis is consistent with our proposal that the mutation at 390 in factor IX affects the activity by disturbing the active site rather than the substrate binding site.

The major structural change accompanying activation of the serine proteases is at the N-terminus in the catalytic domain. The preferable amino-terminal primary structure in the catalytic domain of serine proteases for the change was studied by Bode using oligopeptides and trypsinogen (1979) (Table III). This study shows that occupation of the activation

Table III: Properties of the Dipeptides Studied as the First Two Residues in the Catalytic Domain

sequence	factor IXa activity (aPTT assay) (%)	K_a to trypsinogen[a] (M)	surface area (Å²)	hydrophobicity
IV	~100	20300	537	1.27
VV	~100	710	516	1.08
AA	~30	0.5	428	0.5
IA	~1	130	494	1.27
IG	~1	~5	471	1.89
VA	NS[b]	~2.6	472	0.79
AV	NS	~0.1	472	0.79

[a] Reported by Bode (1979). [b] NS = not studied.

pocket by a small peptide of appropriate sequence can induce a pseudo-active site conformation in trypsinogen; this occurs without cleavage at the activation site. Moreover, it shows that the contributions to the free energies of the binding of the first and the second peptide residues are almost additive, showing the independence of these subsites.

The clotting activities of factor IXs with mutations at the first two amino acids of the heavy chains share similarities with Bode's previous study; however, there are differences. First, although dipeptides with isoleucine at the first position bind tighter to the activation pocket than dipeptides with amino-terminal valine, isoleucine at the amino terminus of the catalytic domain of factor IX did not increase clotting activity. This suggests that the clotting activity of factor IX is governed by factors other than efficiency (turnover number at the active site) or that the activation pocket is slightly different from trypsin. Second, in Bode's study, activation of trypsinogen with the isoleucine–alanine dipeptide was more effective than was the alanine–alanine dipeptide. In contrast, factor IX with alanine–alanine at 181–182 retained 30% activity, while factor IX with isoleucine–alanine at 181–182 retained only 1% clotting activity. This difference in clotting activities is consistent with the *p*-aminobenzamidine binding of the factor IX mutants. These contrasting results suggest differences in the activation pockets between trypsin and factor IX. It is also possible that differences are due to experimental conditions. In Bode's experiments, the N-terminal residues were supplied as free oligopeptides, while in our experiments the mutated N-terminal residues were covalently attached to the catalytic domain of factor IX. Since the alanine–alanine dipeptide has the lowest hydrophobicity, it may require much higher concentrations relative to other hydrophobic peptides.

Modeling the three-dimensional structure of the heavy chain of factor IX suggests that most of the factor IX Bm variants with point mutations in the catalytic domain involve the activation site. The most complete study on factor IX Bm to date (Bertina *et al.*, 1990) suggested that partially cleaved factor IX Bm becomes a competitive inhibitor due to the lack of cleavage at 180/181. In the same study they proposed that, since the mutation at the first cleavage site (145/146) does not prolong ox brain PT time, the abnormality of the cleavage at 180/181 and the lack of a proper conformational change is related to the Bm phenotype. Our results with substitutions at the first two residues in the catalytic domain suggest that the ability to prolong the ox brain PT time (Bm phenotype) is more closely related to loss of the ability to bind ATIII rather than loss of the ability to bind a small active site inhibitor and loss of activity. We propose that the conformational change responsible for the Bm phenotype is strictly related to the relocation of the first residues, which occurs before the formation of the active site. Mutations at residues 181 and 182 and mutations that change the conformation of the activation pockets, such as 311, 364, and 390 (which interfere with relocation of the first two residues of the heavy chain following cleavage at 180/181), appear to cause a significant prolongation of the ox brain PT time. We also found that recombinant factor IX_{I397T} (same substitution as in factor $IX_{Vancouver}$ Bm) did not prolong the ox brain PT time more than plasma-purified normal factor IX (Hamaguchi, unpublished data).

The factor IX hemophilia Bm phenotype is characterized by prolonged ox brain PT assays that are known to vary with assay conditions. The type of mutation and the antigen level in factor IX and other coagulation factors in the sample plasma are crucial. The Bm variant factor $IX_{Deventer}$ has a tryptophan for arginine substitution at 180 and is not activated by its normal activators, the factor VIIa–TF complex, or factor XIa. Furthermore, in a reconstituted reaction system, this abnormal factor IX inhibits the activation of factor X by the factor VIIa–TF complex 3 times (with human TF) to 10 times (with bovine TF) more effectively than normal factor IX (Bertina *et al.*, 1990). Factor $IX_{Lake\ Elsinore}$, with a point mutation alanine 390 to valine, inhibits the activation of factor X by the factor VIIa–TF (bovine) complex 5 times more than normal factor IX in a purified system; however, it is cleaved by the factor VIIa–TF complex as efficiently as normal factor IX (Osterud *et al.*, 1981; Usharani *et al.*, 1985). In this study, factor IX_{A390V} (same substitution as in factor $IX_{Lake\ Elsinore}$) prolonged the ox brain PT time and did not inhibit the factor VIIa–recombinant human TF complex activation of factor X more than normal factor IX (Osterud *et al.*, 1981). While the human factor VIIa–human TF complex and the human factor VIIa–bovine TF complex have the same K_m for human factor X, human factor IX has an affinity 20 times higher for the human factor VII–bovine TF complex than for the human factor VIIa–human TF complex (Bertina *et al.*, 1990). This appears to explain the greater inhibition by factor IX of the bovine TF–factor VIIa complex. Since factor $IX_{Lake\ Elsinore}$ has been reported to prolong the ox brain PT time less than factor $IX_{Deventer}$, factor IX_{A390V} (factor $IX_{Lake\ Elsinore}$), it may inhibit the human TF only slightly more than normal factor IX.

The activation of factor IX is accompanied by a conformational change, which leads to its ability to bind the active site inhibitor, *p*-aminobenzamidine, the macromolecule inhibitor, antithrombin III, the substrate factor X, and its cofactor, factor VIIIa. Our results showed that the activation process of factor IX shares common features with trypsin and other serine proteases and that factor IX_{Bm} variants are probably defective in completing the activation process. We conclude that factor IX_{Bm} results from changes that prevent the normal conformational change at the amino terminus of the heavy chain.

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