Factor IX Amagasaki: A New Mutation in the Catalytic Domain Resulting in the Loss of both Coagulant and Esterase Activities†

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ABSTRACT: Factor IX Amagasaki (AMG) is a naturally occurring mutant of factor IX having essentially no coagulant activity, even though normal levels of antigen are detected in plasma. Factor IX AMG was purified from the patient's plasma by immunoaffinity chromatography with an anti-factor IX monoclonal antibody column. Factor IX AMG was cleaved normally by factor VIIa–tissue factor complex, yielding a two-chain factor IXa. Amino acid composition and sequence analysis of one of the tryptic peptides isolated from factor IX AMG revealed that Gly-311 had been replaced by Glu. We identified a one-base substitution of guanine to adenine in exon VIII by amplifying exon VIII using the polymerase chain reaction method and sequencing the product. This base mutation also supported the replacement of Gly-311 by Glu. In the purified system, factor IXa AMG did not activate factor X in the presence of factor VIII, phospholipids, and Ca^{2+}, and no esterase activity toward Z-Arg-*p*-nitrobenzyl ester was observed. The model building of the serine protease domain of factor IXa suggests that the Gly-311 → Glu exchange would disrupt the specific conformational state in the active site environment, resulting in the substrate binding site not forming properly. This is the first report to show the experimental evidence for importance of a highly conserved Gly-142 (chymotrypsinogen numbering) located in the catalytic site of mammalian serine proteases so far known.

Human factor IX is a vitamin K dependent plasma protein that consists of 415 amino acids (McGraw et al., 1985; Yoshitake et al., 1985). It is activated by either factor XIa or factor VIIa–tissue factor complex to give rise to a two-chain active serine protease (factor IXa). Factor IX can also be activated by the factor X activating enzyme (RVV-X)[1] from Russell's viper venom. Factor IXa proteolytically converts factor X to factor Xa in the presence of Ca^{2+} and factor VIIIa on the surface of phospholipid vesicles (Furie & Furie, 1988).

A deficiency or functional defect of factor IX causes an X-linked recessive bleeding tendency known as hemophilia B (Thompson, 1986). Some of the affected individuals show immunologically normal levels of factor IX antigen but reduced or negligible levels of factor IX coagulant activity. These variants have been designated cross-reacting material positive. Point mutations and short deletions and additions in the factor IX gene have been identified in over 221 individuals (Giannelli et al., 1990). However, since most of the mutations were analyzed by sequencing the DNA, few characterizations of the mutant proteins have been made. The studies of dysfunctional factor IX have given valuable information on the correlation between structure and function. We have so far characterized five mutant factor IX proteins and identified a single amino acid substitution in each case (Sugimoto et al., 1988, 1989; Sakai et al., 1989; Suehiro et al., 1989; Suehiro et al. 1990). In this paper, we describe a new dysfunctional factor IX found in a hemophilia B patient, in which Gly-311, a highly conserved amino acid residue among serine proteases, is substituted by Glu. This unique mutation results in a complete loss of both coagulant activity and esterase activity.

Materials and Methods

Patient. The patient with factor IX Amagasaki (AMG) has suffered from repeated episodes of ankle and elbow joint bleeding. He was first diagnosed as having hemophilia B when he was 3 years of age, and his family has shown no history of bleeding disorders. He has normal levels of factor IX antigen but no detectable clotting activity. The ox brain prothrombin time was moderately prolonged at 58 s (control 31 s).

Materials. Trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone was obtained from Worthington Biochemical Co., Freehold, NJ. The Cosmosil 5C4-300 column (4.6 × 150 mm) and Chemcosorb 7-ODS-H column (2.1 × 150 mm) were from Nakarai Chemicals, Kyoto, and Chemco Scientific Co., Ltd., Osaka. Normal factor IX and factor IX AMG were purified by immunoaffinity chromatography using two kinds of anti-factor IX monoclonal antibody immobilized on Sepharose 4B or Affi-Gel 10. The characteristics of the two monoclonal antibodies are described elsewhere (Yoshioka

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[1] Abbreviations: RVV-X, the factor X activating enzyme from Russell's viper venom; factor IX AMG, factor IX Amagasaki; Z-Arg-ONb, Z-Arg-*p*-nitrobenzyl ester: SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

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et al., 1985; Sugo et al., 1990). Patient plasma was first concentrated by barium citrate adsorption and elution. The concentrated material was loaded onto a 3A6-Sepharose column, which had been equilibrated in 15 mM sodium citrate, pH 7.8, containing 0.9% NaCl and 10 mM benzamidine hydrochloride. The column was extensively washed with the equilibration buffer containing 2 M NaCl, and then factor IX AMG was eluted with the equilibration buffer containing 3 M NaSCN. The protein fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (Sugimoto et al., 1989). The purified protein had a small amount of a contaminating protein with a molecular mass of about 43 000 daltons. In order to further purify this preparation, the sample was applied to a column of the calcium-dependent anti-factor IX monoclonal antibody immobilized on Affigel-10, equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 5 mM $CaCl_2$. Factor IX AMG was then eluted with 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 10 mM EDTA. The protein fractions were dialyzed against 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl (Suehiro et al., 1990). Factor IX concentration was determined by the amino acid analysis of 24-h acid hydrolysate using a Hitachi 8500 amino acid analyzer (Spackman et al., 1958). Bovine factor VII was purified by Ca^{2+}-dependent immunoaffinity chromatography using murine monoclonal antibody (M31) to bovine factor VII, as reported previously (Higashi et al., 1990). Bovine placenta tissue factor apoprotein was a generous gift from Mr. Shoichi Higashi in our laboratory. The sample gave a single band with an apparent molecular weight of 42 000 on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Protein concentration of tissue factor apoprotein was determined by the dye binding assay of Bradford (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as the standard protein (Bradford, 1976). Human factor VIII was purified from a commercial heat-treated factor VIII concentrate by immunoadsorbent chromatography using a monoclonal antibody to von Willebrand factor followed by aminohexyl-Sepharose chromatography in the presence of potent protease inhibitors (Suehiro et al., 1990). The specific activity of the purified factor VIII was 2700 units/mg and the purification was about 190 000-fold from plasma. Cephalin extracted from rabbit brain was obtained from Sigma. Factor XIa was prepared from human plasma according to the published method (Osterud & Rapaport, 1977). Z-Arg-*p*-nitrobenzyl ester hydrobromide (Z-Arg-ONb) was kindly provided by Dr. K. Takada (Peptide Institute Inc., Protein Research Foundation, Osaka). RVV-X and bovine factor X were purified by published methods (Kisiel et al., 1976; Hashimoto et al., 1985). All other chemicals were of the highest grade commercially available.

Cleavage of Factor IX AMG by Factor VIIa–Tissue Factor Complex or RVV-X. Samples of purified normal factor IX and factor IX AMG (9.5 μg each) were activated to the IXaβ form by incubating for 3 h at 37 °C with factor VIIa–tissue factor complex at an enzyme/substrate ratio of 1:100 (mol/mol) in 170 μL of 50 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl, 5 mM $CaCl_2$, and rabbit brain cephalin. The IXaα form was obtained by incubating either normal factor IX or factor IX AMG (9.5 μg each) with RVV-X at an enzyme/substrate ratio of 1:20 (w/w) in 170 μL of 50 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl and 8.5 mM $CaCl_2$ for 3 h at 37 °C. Reaction mixtures were activated completely as judged by SDS–polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970). The gel was stained with Coomassie Brilliant Blue R-250.

Tryptic Peptide Mapping by High-Performance Liquid Chromatography (HPLC). Normal factor IX (8.5 nmol) and factor IX AMG (7.5 nmol) were reduced and S-pyridylethylated (Miyata et al., 1987). The S-pyridylethylated proteins were digested with trypsin (E/S = 1:50 w/w) in 50 mM Tris-HCl, pH 8.0, containing 2 M urea for 16 h at 37 °C. Each digest was chromatographed by reversed-phase HPLC using a Cosmosil 5C4-300 column (Sakai et al., 1989; Sugimoto et al., 1989). Peptides were eluted with a linear gradient of 0–32% acetonitrile in 0.1% trifluoroacetic acid for 90 min followed by a gradient of 32–64% acetonitrile in 0.1% trifluoroacetic acid for 30 min. The flow rate was 0.5 mL/min. The effluent was monitored by measuring absorbance at 214 nm. Rechromatography was performed on a Chemcosorb 7-ODS-H column equilibrated with 10 mM ammonium formate buffer, pH 6.5. A linear gradient of 0–40% acetonitrile in 30 min at a flow rate of 0.5 mL/min was used to elute the peptides (Miyata et al., 1982).

Amino Acid Composition and Sequence Analysis. Peptides were hydrolyzed in 5.7 M HCl containing 1% phenol under reduced pressure for 20 h at 110 °C. The hydrolysates were analyzed by reversed-phase HPLC of the phenylthiocarbamoyl derivatives using the PICO-TAG system (Waters, Millipore, Milford, MA) (Bidlingmeyer et al., 1984). Amino acid sequence was analyzed with an Applied Biosystems Model 477A gas-phase sequencer (Hewick et al., 1981), connected to a 120A phenylthiohydantoin (PTH) analyzer.

DNA Preparation. High molecular weight DNA was obtained by standard techniques (Maniatis et al., 1982) from citrated whole blood. The DNA was desalted by ethanol precipitation and redissolved in water.

Enzymatic Amplification of DNA and Sequence Analysis. A 489 base pair segment of exon VIII of factor IX gene was amplified by the polymerase chain reaction. Two primers, PCR5h2 5′-GCCAATTAGGTCAGTGGTCC-3′ [30 730–30 749; numbering by Yoshitake et al. (1985)] and PCR3h1 5′-CCACTATCTCCTTGACATGAATCTC-3′ (31 218–31 194), were synthesized with an Applied Biosystems DNA synthesizer. One microgram of genomic DNA was amplified with 50 pmol of each primer and 2 units of DNA polymerase from *Thermus thermophilus* (Toyobo Co., Osaka, Japan) (Saiki et al., 1988). The samples were subjected to 35 cycles consisting of a 1-min denaturation period at 94 °C, a 1-min annealing period at 55 °C, and a 2-min extension period at 72 °C. The amplified DNA was electrophoresed on 10% polyacrylamide gel in 69 mM Tris–borate buffer, pH 8.0, containing 2 mM EDTA. The segment of the gel containing the amplified region was cut out and extracted. The DNA sample was then desalted by spin dialysis on a Centricon 30 (Amicon) and freeze-dried. The amplified 489 base pair fragment was sequenced directly using the published method 3 (Wong et al., 1987) using an endo-labeled sequencing primer of 5′-ATTAATAAGTACAACCATGA-3′ (30 908–30 927).

Analysis of Factor X Activation by Factor IXa and IXa AMG. Factor X activation kinetics by normal factor IXa and factor IXa AMG were evaluated in a two-stage assay using the factor Xa specific chromogenic substrate S-2222 (Kabivitrum AB, Sweden). One hundred microliters of purified factor IX (100 μg/mL) was incubated with 10 μL of factor XIa and 10 μL of 50 mM $CaCl_2$ at 37 °C for 24 h. Then 60 μL of the reaction mixture was incubated with 60 μL of various concentrations of factor X, 10 μL of factor VIII (100 units/mL), 5 μL of phospholipids (Platelin) (0.72 mL/mL), and 13.5 μL of 50 mM $CaCl_2$. After 30 min, the reaction was terminated by the addition of 15 μL of 0.1 M EDTA. A

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          1        10        20        30        40        50        60        70        80
HFIX  VVGGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRIIPHHNYN
BTRY  IVGGYTCGANTVPYQVSLNS-GYHFCGGSLINSQWVVSAAHCYKSGIQVRLGEDNINV--VEGNEQFISASKSIVHPSYN

                 90        100       110       120       130       140       150
HFIX  AAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNIFLKFGSGYVSGWGRVFHKG-RSALVLQYLRVPLVDRATCLRS
BTRY  S--NTLNNDIMLIKLKSAASLNSRVASISLPTSCASAG----TQCLISGWGNTKSSGTSYPDVLKCLKAPILSDSSCKSA

          160       170       180       190       200       210       220       230  235
HFIX  TKFTIYNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEKTKLT
BTRY  YPGQITSNMFCAGYLEGGKDSCQGDSGGPVVCS----GKLQGIVSWGSGCAQKNKPGVYTKVCNYVSWIKQTIASN
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FIGURE 1: Amino acid sequence alignment of the serine protease domain of human factor IXa (HFIX) and bovine trypsin (BTRY). The amino acid sequences of human factor IXa and bovine trypsin were taken from references (McGraw et al., 1985; Yoshitake et al., 1985; Marquart et al., 1983). The numbers in parentheses used in this paper are based on this figure. Deletions are indicated by dashes. (*) indicates the active site triads, and (#) indicates the residue which has been substituted by glutamic acid in factor IX AMG.

sample (100 μL) of the reaction mixture was then removed and added to a cuvette containing 800 μL of 0.05 M Tris-HCl buffer, pH 8.3, containing 0.15 M NaCl and 100 μM S-2222. After 5 min, 100 μL of 20% acetic acid was added to stop the reaction, and then the absorbance was measured at 405 nm using a spectrophotometer.

Esterase Activity of Factor IXa and Factor IXa AMG. Esterase activity was measured using Z-Arg-ONb as the substrate. The assay mixture containing 3.3 μg of either factor IXaβ, IXaα, IXaβ AMG, or IXaα AMG and 4 mM Z-Arg-ONb was incubated in 50 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl and 5 mM $CaCl_2$ at 37 °C in a total volume of 400 μL. After 20 min, the reaction was stopped by the addition of 100 μL of 20% acetic acid, and then a sample (10 μL) was injected into a reversed-phase HPLC column, Cosmosil 5C18 (4.6 × 50 mm), at a flow rate of 0.5 mL/min. The reaction product, *p*-nitrobenzyl alcohol, was separated from the substrate, Z-Arg-ONb, using an isocratic system of 0.1% trifluoroacetic acid containing 45% acetonitrile. The effluent was monitored at 280 nm, and *p*-nitrobenzyl alcohol was quantitated.

Structural Modeling. The modeling procedure used to predict the tertiary structure of the protease domain of factor IXa was described previously (Cool et al., 1985; Geddes et al., 1989; Bajaj et al., 1990). The high-resolution structure of bovine pancreatic trypsin (Marquart et al., 1983) was used as the basis for the modeling of factor IX and factor IX AMG. An initial model was constructed using coordinates from the polypeptide chain backbone of trypsin and the amino acid side chains from the conserved amino acids in the primary structures of both trypsin and factor IX. The nonhomologous side chains of factor IXa were then positioned as follows: The three-dimensional structure of the factor IXa side chain was obtained from a standard amino acid group dictionary obtained from the X-ray diffraction analyses of amino acid. The side chain in trypsin was then replaced by the corresponding side chain found in factor IX, and the maximal number of conformational torsional angles from the original trypsin side chain were conserved when the factor IXa side chain was positioned.

The insertions and deletion found in factor IXa were positioned on the basis of the strong tertiary structural homology known to exist among chymotrypsin, elastase, and trypsin. Fragments 20–26 of 1SGC and 171–179 of 2TGD (codes based on the PDB database) were used to position the insertions at residues 21 and 59–60, respectively (numbering based on Figure 1). Fragments 6–14 and 171–184 of 3SGB positioned residues 82–83 and 119–122. The insertion at 193–196 and the deletion at 138 in Figure 1 were placed on fragments 135–141 and 193–204 of 1EST, respectively.

To obtain the best overall structural model, the full set of factor IXa protease domain coordinates were subjected to structural energy optimization. A mutant factor IXa protease domain with Gly → Glu exchange was treated similarly.

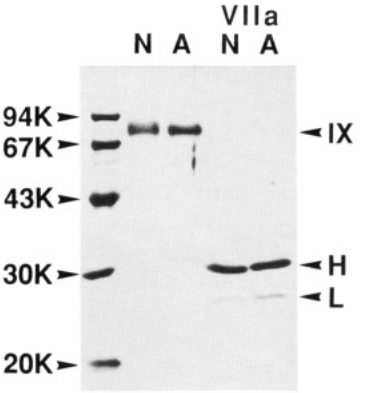

FIGURE 2: SDS–polyacrylamide gel electrophoresis of normal factor IX and factor IX AMG activated by factor VIIa–tissue factor. Factor IX (N) or factor IX AMG (A) were incubated with for 3 h at 37 °C with factor VIIa–tissue factor complex at an enzyme/substrate ratio of 1:100 (mol/mol), and the digest was analyzed by the electrophoresis under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250.

RESULTS

Cleavage of Factor IX AMG by Factor VIIa–Tissue Factor Complex. Factor IX AMG was purified from the patient's plasma by barium citrate adsorption followed by immunoaffinity chromatography. A single band was observed under reduced and nonreduced conditions on SDS–polyacrylamide gel electrophoresis and showed the same migration as that of normal factor IX (Figure 2). In the presence of Ca^{2+} and phospholipids, bovine factor VIIa–tissue factor complex converted factor IX AMG to the two-chain factor IXaβ form. This was also seen when normal factor IX was used, and the activation rate was essentially the same as that of factor IX AMG (data not shown), indicating that proteolytic events of factor IX AMG by factor VIIa–tissue factor are indistinguishable from those of normal factor IX.

Tryptic Peptide Mapping of Factor IX AMG. To elucidate the structural difference between factor IX and factor IX AMG, samples were reduced, S-pyridylethylated, and digested with trypsin. The resulting peptides were separated on a reversed-phase C4 column. The results are shown in Figure 3. The chromatogram of the tryptic peptides derived from the abnormal factor IX was virtually the same as that of the normal control. Due to the similarity between the chromatographs, the peaks were rechromatographed on a C18 column using the 10 mM ammonium formate buffer–acetonitrile system, and the aberrant peptide, T29′, was found to elute 4 min faster than the normal peptide T29. Amino acid compositions of all the tryptic peptides isolated from the abnormal factor IX were then analyzed and found to cover 90% of the amino acid sequence of factor IX. The amino acid composition of the peptide T29′ showed one less glycine and one more glutamic acid than that of the peptide, T29, isolated from

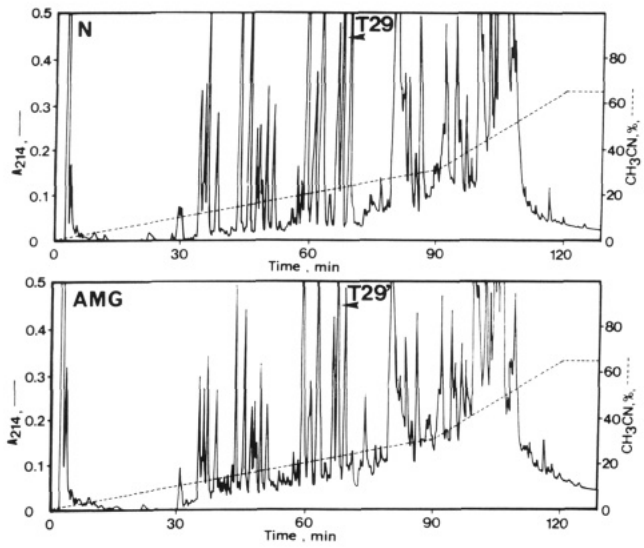

FIGURE 3: Peptide mapping of the tryptic digest of normal factor IX (N) and factor IX AMG (AMG). Each digest was injected onto a Cosmosil 5C4-300 column (4.6 × 150 mm) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile.

Table I: Amino Acid Composition of Aberrant Tryptic Peptide T29′ Derived from S-Pyridylethylated Factor IX Amagasaki[a]

amino acid	residues per molecule: 370 pmol of peptide T29′	350 pmol of peptide T29′	normal peptide T29
Glu	1.02 (1)	0.95 (1)	0
Ser	1.62 (2)	1.99 (2)	2
Gly	2.94 (3)	3.10 (3)	4
Arg	1.12 (1)	0.91 (1)	1
Tyr	0.84 (1)	0.86 (1)	1
Val	1.00 (1)	0.95 (1)	1
Phe	0.82 (1)	0.89 (1)	1
Trp	nd[b] (1)	nd[b] (1)	1
total	11	11	11
position[c]			302–312

[a] Amino acid analyses were performed in duplicate, and values in parentheses were taken from the sequence analysis shown in Table II. [b] nd, not determined. [c] The position of peptide T29 was referenced (McGraw et al., 1985; Yoshitake et al., 1985).

Table II: Amino Acid Sequence of Aberrant Peptide T29′ Derived from S-Pyridylethylated Factor IX Amagasaki

cycle	position[a]	PTH-amino acid	pmol	normal peptide T29[a]
1	302	Phe	808	Phe
2	303	Gly	558	Gly
3	304	Ser	292	Ser
4	305	Gly	500	Gly
5	306	Tyr	347	Tyr
6	307	Val	482	Val
7	308	Ser	143	Ser
8	309	Gly	369	Gly
9	310	Trp	229	Trp
10	311	Glu	128	Gly
11	312	Arg	56	Arg

[a] Amino acid sequence and position from Phe-302 to Arg-312 of normal factor IX was referenced (McGraw et al., 1985; Yoshitake et al., 1985).

normal factor IX (Table I). The difference was confirmed by sequence analysis. As shown in Table II, peptide T29′ differed by a single amino acid at position 311, where a glycine in the normal was replaced by a glutamic acid in the abnormal.

DNA Sequence Analysis of Factor IX AMG. DNA sequence analysis of the amplified region of a normal subject

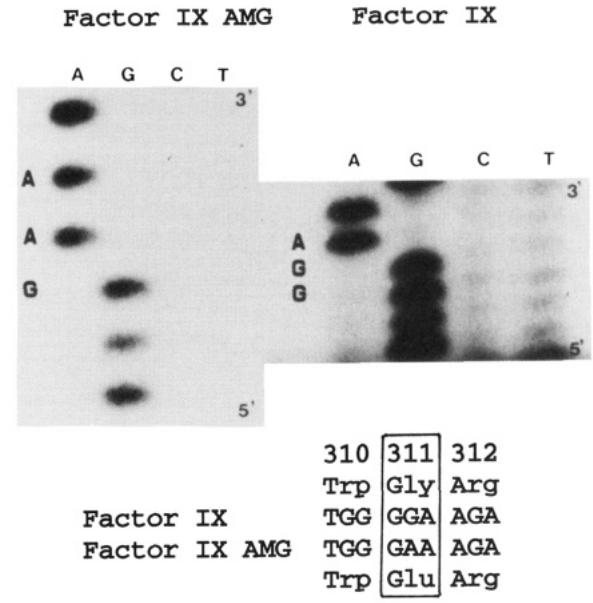

FIGURE 4: Partial nucleotide sequence of the coding strand of exon VIII of the gene for human factor IX. Sequences from the factor IX AMG gene (left) and the normal gene (right) are shown. The cDNA and the predicted amino acid sequences of normal factor IX and factor IX AMG are shown at the bottom.

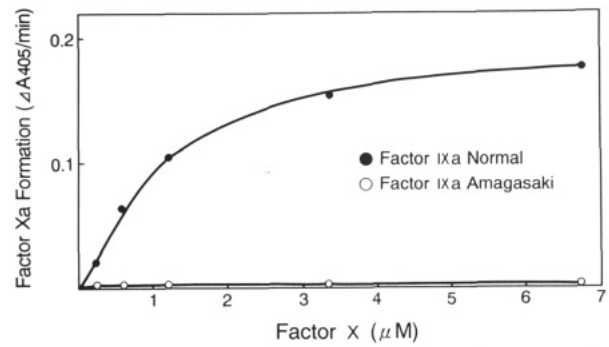

FIGURE 5: Activation of factor X by factor IXa and factor IXa AMG. Coupled chromogenic assay for factor IXa was performed using S-2222. ●, nomal factor IXa; ○, factor IXa AMG.

[nucleotides 30729–31218; numbering by Yoshitake et al. (1985)] was identical to the gene sequence (Yoshitake et al., 1985). On the other hand, the sequence from the subject with hemophilia B AMG showed a point mutation of guanine to adenine at position 31053 (Figure 4). This mutation changes the glycine to a glutamic acid at position 311 in the catalytic domain of the molecule and supports the protein-based data.

Factor X Activation by Factor IXa AMG. The abilities of normal factor IXa and factor IXa AMG to activate factor X were examined. Normal factor IXa and factor IXa AMG were incubated with factor X in the presence of factor VIII, phospholipids, and $CaCl_2$, after being activated by partially purified factor XIa. The resulting factor Xa formation was assessed by amidolytic assay using S-2222 as a factor Xa substrate. As shown in Figure 5, factor IXa AMG showed a complete loss of the ability to activate factor X, while normal factor IXa exhibited a hyperbolic relationship with increasing concentration of factor X.

Esterase Activity of Factor IXa AMG. Esterase activity of factor IXa was measured by using Z-Arg-ONb as a substrate. We are aware that Z-Arg-ONb is a poor substrate for factor IXa. However, since Z-Arg can interact through its positively charged arginine side chain with the negatively charged side chain of Asp-359 (primary substrate binding site) of factor IXa, we reasoned that this is a suitable substrate to

Table III: Specific Activity of Factor IXa toward Z-Arg-ONb[a]

activator and protein	sp act. [nmol of *p*-nitrobenzyl alcohol/(min·pmol of factor IX)]
factor VIIa–tissue factor complex	
normal factor IX	0.095[b]
factor IX AMG	0.001[b]
RVV-X	
normal factor IX	0.067[c]
factor IX AMG	0.003[c]

[a] In each case, the concentrations of factor IXa and Z-Arg-ONb is 157 nM and 4 mM, respectively. [b] Mean value of three experiments. [c] Mean value of two experiments.

evaluate whether or not the catalytic triad and primary substrate binding site in the variant IXa molecule is functional. The normal and abnormal factors IX were activated by either factor VIIa–tissue factor complex or RVV-X, and the resulting factors IXaα and IXaβ were assayed. In all experiments, the concentration of factor IXa was 157 nM. Normal factor IXa expressed a significant amount of hydrolysis of Z-Arg-ONb, as shown in Table III. However, factor IXa AMG showed almost a basal level of esterase activity. These data indicated that the conformational state of the catalytic triad or primary substrate binding site in factor IX AMG has been changed and it no longer hydrolyzed the small synthetic substrate.

Discussion

We have identified the molecular basis of the mutation in factor IX AMG. The patient's plasma contained immunologically normal levels of factor IX with no coagulant activity. Factor IX AMG has been purified by using a Ca^{2+}-dependent monoclonal antibody coupled to Affi-Gel, indicating that the Ca^{2+} binding conformation is intact, since another mutant factor IX having a Gla-21 → Lys substitution did not bind (unpublished results). The cleavage of factor IX AMG by factor VIIa–tissue factor complex was normal, giving rise to a two-chain form, so the cleavage site and the recognition site(s) for factor VIIa–tissue factor were not altered. However, the resulting factor IXa AMG showed a complete lack of the ability to activate factor X in the presence of factor VIII, Ca^{2+}, and phospholipids. It also showed negligible or no esterase activity for the small synthetic substrate, Z-Arg-ONb. These data showed that the conformation of the catalytic triad or primary substrate binding site in factor IX AMG had been altered. Structural analyses of the mutant protein from the patient indicated that a single amino acid substitution of Gly-311 for Glu occurred. This substitution was also confirmed in the patient's chromosomal DNA where a G-to-A exchange was seen at the middle position of the codon for Gly-311. Although additional amino acid substitutions in factor IX AMG cannot be ruled out, this Gly-to-Glu replacement can render the mutant protein inactive for the following reasons.

(1) Koeberl et al. have analyzed a hemophilia B patient (HB26) who showed a 3% coagulant activity and identified a Gly-to-Arg substitution at position 311 by sequencing the patient's DNA after it was amplified by the polymerase chain reaction method (Koeberl et al., 1989). This mutation site that Koeberl found is the same site that we have identified in factor IX AMG. Unfortunately, they did not describe anything about the antigen level in the plasma and did not characterize the abnormal factor IX protein. However, these data support our finding that an amino acid mutation at position 311 is responsible for the lack of the coagulant activity.

(2) Gly-311 (equivalent to Gly-142 in α-chymotrypsin) in the catalytic domain of factor IX is highly conserved in other serine proteases including trypsin, elastase, chymotrypsin, the blood coagulation factors, and the complement factors. We can infer that it might be responsible for maintaining the conformation of the active site triad or primary substrate binding site. We have constructed structural models based on the coordinates of bovine trypsin and the serine protease domains of both factor IXa and factor IXa AMG to estimate the effect of the Gly-311-to-Glu mutation. There are several publications using a similar methodology to show the effect of changing a single amino acid on the predicted three-dimensional structure of factor IX (Geddes et al., 1989; Bajaj et al., 1990; Evans et al., 1989). Figure 6A shows a model view of the environment surrounding residue Gly-311 (131)[2] in factor IXa. Examination of the three-dimensional structures of normal and abnormal factor IXa predicts that the presence of the bulky side chain of glutamic acid at position 311 (131) would physically displace several amino acids. Contacts would occur between the glutamic acid residue and Val-181 (1), Ser-319 (139), Ala-320 (140), Gln-362 (182), and Asp-364 (184). Furthermore, the model predicts that a hydrogen bond would form between the γ-carboxyl group of the side chain of the glutamic acid and the α-amino group of Val-181 (1), located at the amino terminus of the heavy chain of factor IXa. It is known that the positive charge of the amino terminus in the case of chymotrypsin activation is very important in forming the substrate binding site by means of the ionic interaction between it and the side chain of Asp-194. It is reasonable to assume that the Gly-311 → Glu mutation would affect this hydrogen bond which is formed between Val-181 (1) and Asp-364 (184) and this would in turn cause the binding site not to form properly. The lack of both the ability to activate factor X and esterase activity in factor IX AMG can be explained by the Gly-311 → Glu exchange, which would disrupt the specific conformational changes in the active site environment that accompany the conversion of the zymogen to the enzyme.

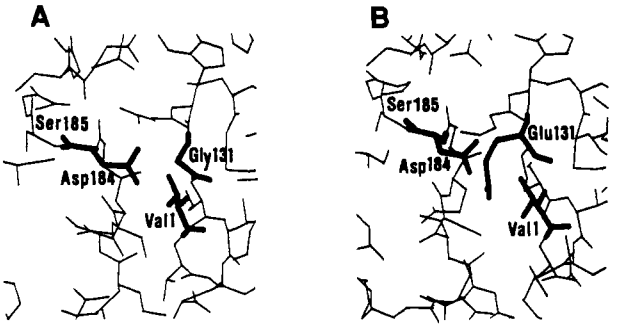

FIGURE 6: Model views showing the environment of residue 311 in factor IXa and factor IXa AMG. (A) Putative model of the environment of residue 311 (131) in factor IXa. Val-181 (1) and Asp-364 (184) must be hydrogen-bonded. (B) Putative model of the environment of residue 311 (131) in factor IXa AMG. The side chain of Glu-311 (131) looks like it is disturbing the hydrogen bond between Val-181 (1) and Asp-364 (184). Gly-311 (131), Val-181 (1), Asp-364 (184), and Glu-311 (131) are shown as thick lines. Ser-365 (185) is one of the residues of the catalytic triad.

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[2] For comparison, the factor IX amino acid numbering system has been used. The numbers in parentheses are based on Figure 1.

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References

Bajaj, S. P., Spitzer, S. G., Welsh, W. J., Warn-Cramer, B. J., Kasper, C. K., & Birktoft, J. J. (1990) *J. Biol. Chem. 265*, 2956–2961.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cool, D. E., Edgell, C. J. S., Louie, G. V., Zoller, M. J., Brayer, G. D., & MacGillivray, R. T. A. (1985) *J. Biol. Chem. 260*, 13666–13676.

Evans, J. P., Brinkhous, K. M., Brayer, G. D., Reisner, H. M., & High, K. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 10095–10099.

Furie, B., & Furie, B. C. (1988) *Cell 53*, 505–518.

Geddes, V. A., Le Bonniec, B. F., Louie, G. V., Brayer, G. D., Thompson, A. R., & MacGillivray, R. T. A. (1989) *J. Biol. Chem. 264*, 4689–4697.

Giannelli, F., Green, P. M., High, K. A., Lozier, J. N., Lillcrap, D. P., Ludwig, M., Olek, K., Reitsma, P. H., Goossens, M., Yoshioka, A., Sommer, S., & Brownlee, G. G. (1990) *Nucleic Acids Res. 18*, 4053–4059.

Hashimoto, N., Morita, T., & Iwanaga, S. (1985) *J. Biochem. 97*, 1347–1355.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Higashi, S., Kawabata, S., Nishimura, H., Funasaki, H., Ohyama, S., Miyamoto, S., Funatsu, A., & Iwanaga, S. (1990) *J. Biochem. 108*, 654–662.

Kisiel, W., Hermodson, M. A., & Davie, E. W. (1976) *Biochemistry 15*, 4901–4906.

Koeberl, D. D., Bottema, C. D. K., Buerstedde, J. M., & Sommer, S. S. (1989) *Am. J. Hum. Genet. 45*, 448–457.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marquart, M., Walter, J., Deisenhofer, J., Bode, W., & Huber, R. (1983) *Acta Crystallogr., Sect. B B39*, 480.

McGraw, R. A., Davis, L. M., Noyes, C. M., Lundblad, R. L., Roberts, H. R., Graham, J. B., & Stafford, D. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2847–2851.

Miyata, T., Iwanaga, S., Sakata, Y., & Aoki, N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6132–6136.

Miyata, T., Morita, T., Inomoto, T., Kawauchi, S., Shirakami, A., & Iwanaga, S. (1987) *Biochemistry 26*, 1117–1122.

Osterud, B., & Rapaport, S. I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5260–5264.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science 239*, 487–491.

Sakai, T., Yoshioka, A., Yamamoto, K., Niinomi, K., Fujimura, Y., Fukui, H., Miyata, T., & Iwanaga, S. (1989) *J. Biochem. 105*, 756–759.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.

Suehiro, K., Kawabata, S., Miyata, T., Takeya, H., Takamatsu, J., Ogata, K., Kamiya, T., Saito, H., Niho, Y., & Iwanaga, S. (1989) *J. Biol. Chem. 264*, 21257–21265.

Suehiro, K., Miyata, T., Takeya, H., Takamatsu, J., Saito, H., Murakawa, M., Okamura, T., Niho, Y., & Iwanaga, S. (1990) *Thromb. Res. 60*, 311–320.

Sugimoto, M., Miyata, T., Kawabata, S., Yoshioka, A., Fukui, H., Takahashi, H., & Iwanaga, S. (1988) *J. Biochem. 104*, 878–880.

Sugimoto, M., Miyata, T., Kawabata, S., Yoshioka, A., Fukui, H., & Iwanaga, S. (1989) *Br. J. Haematol. 72*, 216–221.

Sugo, T., Mizuguchi, J., Kamikubo, Y., & Matsuda, M. (1990) *Thromb. Res. 58*, 603–614.

Thompson, A. R. (1986) *Blood 67*, 565–572.

Yoshioka, A., Giddings, J. C., Thomas, J. E., Fujimura, Y., & Bloom, A. L. (1985) *Br. J. Haematol. 59*, 265–275.

Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W., & Kurachi, K. (1985) *Biochemistry 24*, 3736–3750.

Wong, C., Dowling, C. E., Saiki, R. K., Higuchi, R. G., Erlich, H. A., & Kazazian, H. H., Jr. (1987) *Nature 330*, 384–386.