Factor IX Zutphen: a Cys¹⁸ → Arg mutation results in formation of a heterodimer with α_1 -microglobulin and the inability to form a calcium-induced conformation

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Factor IX Zutphen is a variant factor IX molecule isolated from the blood of a patient with severe haemophilia B. The molecular defect in factor IX Zutphen is a Cys¹⁸ → Arg mutation as a result of a T → C transition at residue 6427 of the factor IX gene of the patient. The mutation disrupts the disulphide bond in the Gla-domain between Cys¹⁸ and Cys²³. The remaining free cysteine residue results in the formation of a 95 kDa complex with α_1 -microglobulin through an intermolecular disulphide bond. The same complex circulates at high levels in plasma of carriers of the mutation. The variant molecule has a calcium-binding defect, which is shown not to be caused by incomplete γ -carboxylation. Factor IX Zutphen can not bind to phospholipids and can not be activated by factor XIa or by factor VIIa–tissue factor complex. Two sequential metal ion-dependent conformational transitions (factor IX → factor IX' → factor IX*) have been proposed for

human factor IX [Liebman (1987) *J. Biol. Chem.* **262**, 7605–7612], based upon the metal ion requirements for binding to anti-factor IX:Mg(II) antibodies, which are specific for the factor IX' conformation, and anti-factor IX:Ca(II) antibodies, which are specific for the factor IX* conformation. We used these conformation-specific antibodies, and antibodies raised against a synthetic peptide corresponding to residues 35–50 of human factor IX [anti-factor IX(35–50)] to study the metal ion-induced conformation of factor IX Zutphen. The disruption of the disulphide bond in the Gla-domain, maybe in combination with the complex with α_1 -microglobulin, destabilized the factor IX' conformation. The formation of the factor IX* conformation was prevented independent of the presence of α_1 -microglobulin. The disulphide bond in the Gla-domain is therefore essential for the calcium-dependent conformation and function of factor IX.

INTRODUCTION

Factor IX is the zymogen of a serine protease that plays a key role in the coagulation cascade [1,2]. Factor IXa activates factor X by limited proteolysis in the presence of factor VIIIa, negatively charged phospholipids and Ca²⁺ [3,4]. In the mature factor IX, the first 12 glutamic acid residues have been post-translational modified to γ -carboxyglutamic acid (Gla) residues [5]. Gla residues are involved in the binding of calcium to the N-terminal domain (Gla-domain) of factor IX.

Binding of calcium induces a conformational change in factor IX, which can be monitored with conformation-specific antibodies [6,7]. On the basis of the metal ion selectivity of the binding to these conformation-dependent antibodies, a three-state model has been proposed for this conformational change. The initial conformational transition (factor IX → factor IX') is non-selective for metal ions, such as Ca²⁺, Mg²⁺, Mn²⁺ and Ba²⁺. This conformation can be recognized by anti-factor IX:Mg(II) antibodies. A further conformational transition (factor IX' → factor IX*) is only supported by Ca²⁺ and Sr²⁺ ions, and is necessary for phospholipid binding and activation by factor XIa [7]. The factor IX* conformation can be recognized by anti-factor IX:Ca(II) as well as anti-factor IX:Mg(II) antibodies.

Previously we have described a genetic factor IX variant, factor IX Zutphen, that has an increased electrophoretic mobility in the presence of calcium, when compared with normal factor IX [8], and that does not bind to antibodies which recognize the calcium-dependent conformation of human factor IX [6]. The

variant protein has an increased molecular mass (95 kDa versus 65 kDa), as judged by SDS/PAGE, which is caused by its covalent binding to a protein of approximately 30 kDa through a disulphide bond [9]. Factor IX Zutphen can not be activated by factor XIa, factor Xa/phospholipid or RVV-X (factor X activating enzyme from Russell viper venom), which can explain the severe haemophilia B phenotype of the patient [8].

In this study we further elucidated the relationship between the structural and functional defects of factor IX Zutphen. For this we identified the 30 kDa protein and the molecular defect at the genetic level. We used conformation-specific antibodies to determine how far the mutation and the presence of the complex prevented the formation of the different metal ion-induced conformations. Furthermore, by studying the metal ion dependence of the binding to these antibodies we determined the effect of the mutation and the presence of the complex on the relative stability of the metal-induced conformations. We were able to correlate these data with the observed functional defects of factor IX Zutphen.

EXPERIMENTAL

Materials

Protein A-Sepharose and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). Goat anti-(rabbit IgG)–horseradish peroxidase (HRP) conjugate and prestained molecular mass standards were purchased from Bio-Rad (Richmond, CA, U.S.A.). Anti-[human α_1 -microglobulin (α_1 -M)]–HRP con-

Abbreviations used: α_1 M, α_1 -microglobulin; DTT, dithiothreitol; factor IX-AcN, Cys-carboxyamidomethylated normal factor IX; Fmoc, [(9-fluorenylmethyl)-oxy]carbonyl; Gla, γ -carboxyglutamic acid; HRP, horseradish peroxidase; OPD, o-phenylenediamine; OVA, chicken egg ovalbumin; PEG, polyethylene glycol; PVDF, polyvinylidene difluoride; SPDP, N-succinimidyl-3-(2-pyridyldithio)propionate; TCA, trichloroacetic acid; TEA, triethanolamine; TMB, 3,3',4,4'-tetramethylbenzidine.

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jugate was from Serotec (Bicester, U.K.). Human factor XIa was from Enzyme Research Laboratories (Swansea, U.K.). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was from Millipore (Bedford, U.S.A.).

Identification of the mutation

All of the coding regions and their flanking regions, the promoter region and the 3'-untranslated region of the factor IX gene of the patient with factor IX Zutphen were analysed, using PCR and direct sequencing of the amplified regions as described previously [10].

Proteins

Normal factor IX and factor IX Zutphen were isolated from (patient) plasma, essentially according to previously published methods, consisting of barium citrate precipitation [11], ammonium sulphate precipitation [12] and affinity chromatography on a non-Ca(II)-dependent monoclonal antibody (E12)-Sephacrose 4B column. Bound factor IX was eluted from the column with 0.1 M glycine (pH 2.5). Both normal (65 kDa) and variant factor IX (95 kDa) were more than 95% pure, as analysed by SDS/PAGE. Cys-carboxyamidomethylated normal factor IX (factor IX-AcN) was prepared by reducing normal factor IX with dithiothreitol (DTT) and carboxyamidomethylating the cysteine residues with iodoacetamide, following a standard procedure [13]. The 30 kDa polypeptide was isolated by separation from factor IX Zutphen, isolated from the plasma of a carrier of the mutation (containing both normal factor IX and factor IX Zutphen). After treatment with 20 mM DTT in 20 mM sodium citrate containing 0.9% NaCl and 10 mM benzamidine for 60 min at 37 °C, factor IX was precipitated by addition of 80 μ l of 1 M BaCl₂/ml. After centrifugation, the supernatant, which contained only one (30 kDa) band on reduced SDS/PAGE, was dialysed extensively against 10% acetic acid and lyophilized.

Anti-factor IX:Ca(II), anti-factor IX:Mg(II) and non-Ca(II)-dependent antibodies against human factor IX were fractionated by affinity purification from rabbit anti-factor IX serum as described previously [6, 7]. The synthetic peptide factor IX(34–50) was synthesized on a solid-phase peptide synthesizer, using standard [(9-fluorenylmethyl)oxy]carbonyl (Fmoc) chemistry (Department of Immunohaematology, University Hospital, Leiden, The Netherlands). The peptide was conjugated to rabbit serum albumin by glutaraldehyde and used to immunize a New Zealand White rabbit following standard protocols [14]. Anti-factor IX(34–50) antibodies were isolated from rabbit serum by affinity chromatography on Protein A-Sepharose 4B [14]. Molar concentrations of factor IX preparations were calculated either directly from the protein content, using a molecular mass of 57 kDa and an A_{280} of 13.3 [15], or from the antigen concentration determined in an ELISA using non-Ca(II)-dependent antibodies and a calibrated standard.

Protein analysis

The N-terminal sequence of the 30 kDa polypeptide was determined at the gas-phase sequencer facility (Department of Medical Biochemistry, State University of Leiden, Leiden, The Netherlands) which is supported by N.W.O. through the Netherlands Foundation for Chemical Research (S.O.N.). Gla analysis was performed according to Kuwada and Katayama [16], at the Department of Biochemistry of the State University of Maastricht by courtesy of Dr. C. Vermeer. The number of Gla residues per mol of protein was calculated from the Gla/Glu

ratio, in the case of factor IX Zutphen corrected for the number of Glu residues in α_1 M.

SDS/PAGE and Western blotting

SDS/PAGE was performed according to Laemmli [17]. Protein bands were visualized by silver staining according to Morrissey [18] or transferred to PVDF using a semi-dry blotting system (Pharmacia, Uppsala, Sweden). Proteins were detected with specific antibodies, conjugated to HRP. HRP-activity was visualized, using a chemiluminescence kit (Boehringer, Mannheim, Germany).

Activation of factor IX

Factor IX was activated by factor XIa by adding 5 μ l of factor XIa (10 μ g/ml) to a final volume of 200 μ l of factor IX (100 nM) in 50 mM triethanolamine (TEA) (pH 8.0), 150 mM NaCl, 20 mM CaCl₂, 0.1% polyethylene glycol (PEG) 4000. At distinct time points, 20 μ l samples were added to an equal volume of SDS-sample buffer containing 40 mM EDTA and analysed by SDS/PAGE and Western-blotting.

ELISA

PVC microtitre plates (ICN) were coated with non-Ca(II)-dependent antibodies (4 μ g/ml, 100 μ l/well) in coating buffer [0.1 M NaHCO₃ (pH 9.0), 0.5 M NaCl, 0.025% NaN₃] for 14 h at 25 °C. Plates were washed with 50 mM TEA (pH 7.5), 150 mM NaCl, 0.1% Tween-20 (ELISA-buffer) prior to the sample incubation. Samples were diluted in ELISA-buffer and incubated for 2 h (100 μ l/well). Bound factor IX was detected after incubation with non-Ca(II)-dependent anti-factor IX–HRP conjugate for 1.5 h (100 μ l/well). Plates were washed five times with ELISA-buffer between incubations. The HRP activity was detected with H₂O₂ and 3,3',4,4'-tetramethylbenzidine (TMB) and quantified by measuring the A_{450} after stopping the reaction with H₂SO₄ [19]. The relative amount of factor IX– α_1 M complex in plasma was determined in a sandwich ELISA analogous to the factor IX ELISA, but using anti- α_1 M–HRP as the conjugate.

Binding to anti-factor IX:Ca(II), anti-factor IX:Mg(II) or phospholipids

Metal ion-dependent antibodies or isolated human brain phospholipids [20], were coated as for the factor IX ELISA. Proteins were serially diluted in duplicate in ELISA-buffer, containing 0.1% chicken egg ovalbumin (OVA) and 20 mM CaCl₂. Incubation and washing procedures were as for the ELISA. Bound protein was eluted with 100 μ l/well ELISA-buffer containing 0.1% OVA and 10 mM EDTA for 2 h and quantified in a factor IX ELISA, using the same protein for the standard line. Dissociation constants and number of binding sites were calculated by least-square fitting of the data to the quadratic binding equation.

Metal ion dependence of the binding of factor IX to anti-factor IX:Mg(II) antibodies

Anti-factor IX:Mg(II) antibodies were coated to microtitre plates as for the factor IX ELISA. To remove contaminating metal ions, 50 mM TEA (pH 7.5)/150 mM NaCl was pretreated with Chelex before adding 0.1% Tween and 0.1% OVA (Chelex-buffer). Fixed concentrations of factor IX in Chelex-buffer were incubated at different metal ion concentrations. For standard curves, the factor IX concentration was varied in the presence of

20 mM MgCl₂ or CaCl₂. Further procedures were as for ELISA, except that the washing and incubation buffer contained 20 mM CaCl₂. The relative amount of factor IX able to bind to the antibodies was derived from interpolation in the standard curves.

Metal ion dependence of the binding of factor IX to anti-factor IX(34–50) antibodies

Normal or patient plasma (1/100) were incubated with anti-factor IX(34–50) IgG (65 µg/ml) in Chelex-buffer (see above), in which the concentrations of CaCl₂ or MgCl₂ were varied. Complexes between factor IX and anti-factor IX(34–50) were determined in a sandwich ELISA. A monoclonal antibody (B5) that had equal affinity for free factor IX and factor IX–anti-factor IX(34–50) complexes, was used for the coating. Bound factor IX–anti-factor IX(34–50) complexes were detected with goat anti-(rabbit IgG)–HRP. The further procedure was as for ELISA, except that the washing and incubation buffer contained 10 mM EDTA. The relative concentration of factor IX–anti-factor IX(34–50) complex at different metal ion concentrations was calculated as the ratio between the A_{450} , measured in the presence of metal ions, and the A_{450} , measured in the presence of 10 mM EDTA.

RESULTS AND DISCUSSION

Characterization of the factor IX Zutphen molecule

Factor IX Zutphen is a genetic variant of human coagulation factor IX, which has a strongly reduced specific activity (<4% factor IX activity; 65% factor IX antigen) [8,9]. The only molecular defect which could be identified in the factor IX gene of the patient was a T → C nucleotide transition in exon II at position 6427 (numbering according to Yoshitake et al. [21]). This transition changes the codon for amino acid residue 18 from TGT in the normal gene to CGT in the factor IX Zutphen gene and predicts the replacement of Cys¹⁸ in normal factor IX by Arg in the factor IX Zutphen protein. When it was isolated from plasma, the apparent molecular mass of factor IX Zutphen (± 95 kDa, Figure 1, lanes 2) is increased by ± 30 kDa when compared with normal factor IX (± 65 kDa; Figure 1, lanes 1). After reduction, factor IX Zutphen (lanes 4) shows a band of identical apparent molecular mass as normal factor IX (lanes 3), but also a band of ± 30 kDa. This polypeptide, that does not react with anti-factor IX antibodies, is apparently linked to factor IX Zutphen through a disulphide bond. The 30 kDa polypeptide was separated from factor IX Zutphen by barium citrate adsorption after reduction of the isolated heteroduplex (Figure 1, lanes 5). N-terminal sequencing of the peptide allowed the identification of residues 8–18 of the polypeptide as DNIQVQENF(Y)I. This information was used for a database search, performed at the NCBI using the BLAST network service [22]. Only one single perfect match was found with residues 8–18 of α_1 M [23], also called protein HC [24]. The identity of the 30 kDa polypeptide as α_1 M was confirmed by immunoblotting (Figure 1c, lanes 2, 4 and 5).

α_1 M is a low-molecular-mass plasma glycoprotein, that can be detected in various body fluids as free monomers that carry a brown chromophore and as a 1:1 complex with IgA [25]. α_1 M has three cysteine residues, two of which are involved in an intramolecular disulphide bond. The remaining cysteine is involved in a non-reducible covalent bond between IgA or the chromophore, which is also present in the complex with IgA [26]. As judged by the absorbance spectrum between 280 nm and 340 nm (results not shown), the factor IX Zutphen– α_1 M complex does not contain such a chromophore.

Figure 1 SDS/PAGE analysis of factor IX Zutphen

Protein samples (0.2 µg) were separated by 5–15% gradient SDS/PAGE and silver-stained for protein (a) or blotted on to PVDF membranes which were subsequently incubated with non-Ca(II)-dependent anti-(human factor IX)–HRP (b) or with anti- α_1 M–HRP (c). HRP activity was visualized by chemiluminescence. Samples in lanes 3 and 4 were reduced prior to application to the gel. Lanes 1 and 3, normal factor IX; lanes 2 and 4, factor IX Zutphen; lane 5, 30 kDa peptide isolated as described in the Experimental section. Abbreviations: FIX_Z, high-molecular-mass form of factor IX Zutphen; FIX_N, normal-molecular-mass form of factor IX; degr., thrombin-cleaved degradation product of factor IX [40]; 30 kDa, peptide complexed with factor IX Zutphen.

The factor IX Zutphen– α_1 M complex results directly from the Cys¹⁸ → Arg mutation

Six carriers and four non-carriers from the family of the patient

Figure 2 Sandwich ELISA for factor IX- α_1 M complex

Serial dilutions of patient plasma (■), carrier plasma (△), pooled normal plasma (▲) and isolated factor IX Zutphen (□) were incubated in microtitre plates, coated with non-Ca(II)-dependent anti-(human factor IX) antibodies. 80% of the factor IX in the plasma of the carrier is variant protein, due to lyonization. Bound factor IX- α_1 M complexes were detected with anti- α_1 M-HRP conjugate.

[8] were screened by immunoblotting with the anti- α_1 M antibody for the presence of an identical complex in their plasma. The complex could be detected in the barium citrate eluate of the plasma of each carrier, whereas it was absent in the eluates of non-carriers (results not shown). The co-segregation of the mutation with the presence of the complex in the barium citrate eluates shows that this complex results from the point mutation and not from another pathological origin.

The presence of the complex was also demonstrated directly in plasma of the patient and one carrier of the defect (individual IV-4 in the original pedigree [8]), using a specific sandwich ELISA (Figure 2). The isolated factor IX Zutphen was used as a control. This shows that the complex is not an artifact of the barium citrate precipitation technique. The low, but significant, amount of the complex that can be detected in normal plasma (Figure 2) is in agreement with the observation of a trace amount of a 95 kDa band that reacts with anti-factor IX antibodies as well as anti- α_1 M antibodies in immunopurified preparations of normal factor IX (results not shown).

Mutations which introduce free cysteine residues in factor IX normally give rise to very low antigen levels [27]. Apparently, factor IX Zutphen is rescued from intra- or extracellular degradation by the formation of a complex with α_1 M. The reason why factor IX Zutphen forms a heterodimer with α_1 M and not with albumin, like some of the anti-thrombin and fibrinogen variants that contain a free cysteine residue [28–32], is not obvious. However, α_1 M shares a remarkable homology in post-translational processing with the vitamin K-dependent clotting factors with respect to the presence of N-linked and O-linked carbohydrates, proteolytic cleavage at a dibasic site [33–35] and γ -carboxylation [36]. This suggests that α_1 M and factor IX are localized in the same intracellular compartments. It would therefore be interesting to know whether a factor IX- α_1 M complex does exist for the Cys²³ → Arg and Cys²³ → Tyr factor IX variants [27], in which the disulphide loop in the Gla-domain is affected also.

Figure 3 Binding of factor IX species to immobilized anti-factor IX:Ca(II) (a) and anti-factor IX:Mg(II) antibodies (b)

Antibodies were coated to microtitre plates and incubated with normal factor IX (■) and factor IX Zutphen (□) in the presence of 20 mM Ca²⁺. Plotted is the amount of bound factor IX, determined after elution with EDTA, as a function of the added amount of factor IX. To be able to compare the number of antigen binding sites for normal factor IX and factor IX Zutphen, they were incubated on the same plate. The results of one such experiment are shown.

Gla-analysis

Factor IX Zutphen and normal factor IX were subjected to Gla-analysis. For both proteins, the calculated number of Gla-residues per mol was 11. This shows that the reported defective calcium-induced conformation [6,8] is not caused by an impaired γ -carboxylation. Furthermore this shows that the presence of the disulphide bond in the Gla-domain is no prerequisite for a fully carboxylated protein, as claimed by some authors [37].

Factor IX Zutphen shows decreased binding to conformation-specific antibodies

To determine how the mutation in factor IX Zutphen affected the calcium-induced conformation, we studied its binding to anti-factor IX:Ca(II) and anti-factor IX:Mg(II) antibodies. The dissociation constant (K_d) for the binding of normal factor IX to immobilized anti-factor IX:Ca(II) (Figure 3a) and anti-factor IX:Mg(II) antibodies (Figure 3b) was determined in three

Figure 4 Western blot with conformation-specific antibodies

Samples (0.1 μ g) of normal factor IX (lanes 1 and 3), factor IX Zutphen (lanes 2 and 4) and factor IX-AcN (lane 5) were separated on 5–15% gradient SDS/PAGE gels, and blotted on to PVDF membranes. Samples in lanes 3 and 4 were reduced by 50 mM DTT prior to application on the gel. Factor IX was detected with 0.1 μ g/ml anti-factor IX:Ca(II) antibodies (a) or anti-factor IX:Mg(II) antibodies (b).

independent experiments as 4.0 ± 0.5 nM and 1.1 ± 0.2 nM respectively. The number of antigen binding sites ranged between 0.1 and 0.4 pmol per well in different experiments. No measurable binding of factor IX Zutphen to the anti-factor IX:Ca(II) antibodies could be observed (Figure 3a).

Although no complete saturation of binding of factor IX Zutphen to the anti-factor IX:Mg(II) antibodies could be accomplished, the K_d could be estimated as 45 ± 15 nM (mean of three independent experiments) (Figure 3b). The number of antigen binding sites for factor IX Zutphen was only $60 \pm 5\%$ of those for normal factor IX, indicating that only a fraction of the anti-factor IX:Mg(II) antibodies is able to recognize the factor IX' conformation of factor IX Zutphen. These data suggest that factor IX Zutphen can partially form the factor IX' conformation, but can not form the factor IX* conformation. However, the interpretation is complicated by the presence of the α_1 M peptide, which might interfere with antibody binding.

Therefore, factor IX Zutphen was separated from α_1 M by SDS/PAGE under reducing conditions (Figure 4). This procedure still allowed normal factor IX (lane 3) to be recognized by the anti-factor IX:Ca(II) (Figure 4a) and anti-factor IX:Mg(II) (Figure 4b) antibodies, although the bands are less intense than those of non-reduced samples (lane 1). The separation of α_1 M from factor IX Zutphen by reduction (Figure 4, lane 4) did not result in any recognition by the anti-factor IX:Ca(II) antibodies but resulted in a strong increase in the binding of anti-factor IX:Mg(II) antibodies. Also, in contrast to anti-factor IX:Ca(II) antibodies, anti-factor IX:Mg(II) antibodies still do recognize factor IX-AcN (lane 5), in which all disulphide bonds of factor IX are irreversibly disrupted. The latter shows that at least one

Figure 5 Metal ion dependence of the binding to anti-factor IX:Mg(II) antibodies

(a) CaCl₂ concentration dependence of the binding of normal factor IX (50 pM; $\blacksquare$) and factor IX Zutphen (300 pM; $\square$) to anti-factor IX:Mg(II) antibodies. (b) MgCl₂ concentration dependence of the binding of normal factor IX (50 pM; $\blacksquare$) and factor IX Zutphen (300 pM; $\square$) to anti-factor IX:Mg(II) antibodies. The relative amount of factor IX able to bind to anti-factor IX:Mg(II) (i.e. factor IX') in the presence of different concentrations of Ca²⁺ or Mg²⁺ was determined as indicated in the Experimental section.

disulphide bond is required for the factor IX* conformation, but that no disulphide bonds are required for the formation of the factor IX' conformation.

In factor IX Zutphen, the disulphide bond in the Gla-domain is the only disulphide bond that cannot reform after reduction and transfer to the membrane. Consequently, the inability of factor IX Zutphen to bind to the anti-factor IX:Ca(II) antibodies results from the inability to form the factor IX* conformation as a result of the absence of the disulphide bond in the Gla domain between Cys¹⁸ and Cys²³. The reduced affinity and avidity of the anti-factor IX:Mg(II) antibodies for factor IX Zutphen can, however, at least in part be explained by steric hindrance from α_1 M (Figure 4). Therefore, the factor IX' conformation may still be formed in factor IX Zutphen.

The metal ion-induced conformation of factor IX Zutphen is destabilized

To determine the contribution of the disulphide bond to the formation of the factor IX' conformation, the metal ion dependence of the conformational change, as recognized by the anti-factor IX:Mg(II) antibodies, was studied (Figure 5). At

Figure 6 Binding of factor IX species to anti-factor IX(35–50) antibodies in the presence of calcium or EDTA

Dilutions of pooled normal plasma (■, ●) or patient plasma (□, ○) were incubated with anti-factor IX(35–50) antibodies (65 µg/ml) in the presence of 20 mM CaCl₂ (●, ○) or 10 mM EDTA (■, □). Complexes between factor IX and anti-factor IX(35–50) were detected as described in the Experimental section.

different metal ion concentrations, the amount of factor IX able to bind to the anti-factor IX:Mg(II) antibodies was determined relative to the amount in the presence of 20 mM metal ion (either Mg²⁺ or Ca²⁺). For normal factor IX the EC₅₀ was 0.10 mM for Ca²⁺ (Figure 5a) and 0.30 mM for Mg²⁺ (Figure 5b). Saturation of the conformational change was achieved at concentrations higher than 0.5 mM Ca²⁺ or Mg²⁺. For factor IX Zutphen the EC₅₀ was > 5 mM for both Ca²⁺ (Figure 5a) and Mg²⁺ (Figure 5b). Since no plateau was reached at 20 mM Ca²⁺ or Mg²⁺, factor IX Zutphen may not fully express the factor IX' conformation at these metal ion concentrations, which may hamper the accurate determination of the EC₅₀. Nonetheless, these data show that the factor IX' conformation is less stable in factor IX Zutphen than in normal factor IX.

The presence of the α₁M peptide does not interfere with the binding of anti-factor IX(34–50) antibodies to factor IX Zutphen, as can be judged from the identical dose–response curves for normal factor IX and factor IX Zutphen (Figure 6). Similar to what was reported for antibodies raised against a synthetic peptide corresponding to residues 28–46 of human factor IX [38], anti-factor IX(34–50) antibodies bind to factor IX only in the absence of metal ions. In the presence of saturating concentrations of Ca²⁺ or Mg²⁺ the binding of the antibody to normal factor IX as well as to factor IX Zutphen is equally strongly decreased (Figure 6). The metal ion-induced internalization of residues 34–50 in normal factor IX and factor IX Zutphen can therefore be compared unambiguously. Similar to what was observed for the induction of the factor IX' conformation (Figure 5), lower concentrations of Ca²⁺ (EC₅₀ = 1.4 mM) than Mg²⁺ (EC₅₀ = 2.9 mM) were needed to inhibit the binding of anti-factor IX(34–50) to normal factor IX. The binding of factor IX Zutphen to anti-factor IX(34–50) antibodies was inhibited only by much higher concentrations of Ca²⁺ (EC₅₀ = 7.2 mM) or Mg²⁺ (EC₅₀ = 8.8 mM).

In conclusion, the metal ion-induced conformation, as monitored with both the anti-factor IX:Mg(II) and anti-factor IX(34–50) antibodies, is less stable in factor IX Zutphen than in normal factor IX. This destabilization can be caused by the

Figure 7 Cleavage pattern of factor IX species by factor XIa

Normal factor IX (a) and factor IX Zutphen (b) were activated by factor XIa in the presence of 20 mM calcium. After indicated time intervals, 0.2 µg samples were taken, reduced, separated on SDS/12%–PAGE gels and transferred to PVDF membranes. Factor IX was detected with non-Ca(II)-dependent antibodies.

disruption of the disulphide bond, as well as by the formation of the disulphide bond with α₁M, which may force the Gla domain in to a non-native conformation.

Functional defects of factor IX Zutphen

Factor IX Zutphen showed no detectable binding for immobilized phospholipids (apparent $K_d \geq 1 \mu\text{M}$), whereas normal factor IX had an apparent K_d of $0.15 \pm 0.02 \mu\text{M}$ (results not shown). This is in agreement with the requirement of the factor IX* conformation for this process [7].

Fab-fragments of anti-factor IX:Ca(II) antibodies inhibit the activation of factor IX by factor XIa, but still allow the formation of factor IXα by cleavage at residue Arg¹⁴⁵ [7]. The cleavage at Arg¹⁴⁵ by factor XIa is less metal ion specific and can be supported by Mg²⁺ ions [39]; results not shown). This would mean that a correct factor IX' conformation would allow the (slow) cleavage at Arg¹⁴⁵. Therefore the pattern of the cleavage of factor IX Zutphen by factor XIa was studied under conditions that factor IX Zutphen forms the factor IX' conformation. To be able to detect even minimal cleavage, we used enzyme:substrate ratios (1:30, w/w) that converted normal factor IX into IXa almost completely within 10 min (Figure 7a). Under the same conditions, no cleavage of factor IX Zutphen could be detected

(Figure 7b). The band corresponding to the heavy chain of factor IX α was already present in the starting material. Although factor IX Zutphen may not fully express the factor IX' conformation at 20 mM CaCl₂, we still would expect to detect cleavage of the protein fraction that did express the factor IX' conformation. The absence of any detectable cleavage suggests that steric hindrance from α_1 M prevents the cleavage at Arg¹⁴⁵. Interestingly, factor IX-AcN was slowly cleaved only at Arg¹⁴⁵ by factor XIa in the presence of 20 mM Ca²⁺ (results not shown). This indicates that complete reduction of factor IX, including the disulphide bond in the Gla domain, still allows the cleavage at Arg¹⁴⁵.

The functional defects of factor IX Zutphen therefore result from the combined effects of the Cys¹⁸ → Arg mutation: the disruption of the disulphide bond in the Gla domain prevents the formation of the factor IX* conformation, and consequently prevents phospholipid binding and cleavage at Arg¹⁸⁰ by factor XIa, whereas the formation of the complex with α_1 M results in steric hindrance which prevents the cleavage at Arg¹⁴⁵ by factor XIa.

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