Ca^{2+}-Dependent Interactions between Gla and EGF Domains in Human Coagulation Factor IX†

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ABSTRACT: The Ca^{2+}-induced interaction between the Gla and EGF domains of human factor IX was investigated by means of three fragments: 6-kDa Gla, 19-kDa $(EGF)_2$, and 25-kDa Gla–$(EGF)_2$. Size-exclusion chromatography and spectroscopic measurements revealed that the Gla–EGF interaction is rather strong; it can be reconstituted by mixing the 6-kDa and 19-kDa fragments which form a stable 1:1 heterocomplex in the presence of Ca^{2+}. By itself, the 6-kDa Gla self-associates in these conditions. The Gla–EGF interaction can be disrupted in 5 M urea where the compact structure of both domains is preserved. Binding of Ca^{2+} to 19-kDa $(EGF)_2$ occurred with a K_d of 71 μM in the absence and 108 μM in the presence of 5 M urea and stabilized the first EGF domain, increasing its T_m by 12 °C. Addition of Ca^{2+} to the 6-kDa and 25-kDa fragments produced biphasic changes in their fluorescence; the intensity increased slightly at low Ca^{2+} concentration and then decreased in a monotonic manner. In 5 M urea, only the decrease occurred, with apparent K_ds of 0.33 and 0.30 mM for 6-kDa Gla and 25-kDa Gla–$(EGF)_2$, respectively. Thus, in 5 M urea in the presence of Ca^{2+}, the isolated Gla domain has a compact structure and Ca^{2+} binding properties similar to those in the 25-kDa fragment. In the absence of urea, the Gla domain interacts either with itself, when isolated, or with the first EGF domain when present, as in the 6-kDa/19-kDa heterocomplex, in the 25-kDa fragment and presumably intact factor IX.

Coagulation factor IX is a vitamin K-dependent glycoprotein with a modular composition similar to that of three other coagulation proteins: factor VII, factor X, and protein C (Furie & Furie, 1988). All these proteins contain an N-terminal γ-carboxyglutamic acid-rich (Gla) module followed by two epidermal grow factor-like (EGF) and C-terminal serine protease (SP) modules. Factor IX is involved in intrinsic clotting. It is activated into the enzymatic form, factor IXa, by factor XIa or factor VIIa/tissue factor complex by limited proteolysis (Davie *et al.*, 1991). Activated factor IXa in the presence of factor VII and a membrane surface activates factor X to further propagate the clotting cascade (Davie *et al.*, 1991). Calcium ions are required for all of these reactions, whose mechanisms involve numerous interactions between the blood clotting factors, their cofactors, and phospholipid membranes. In the case of factors IX and X, the Gla and EGf modules are known to be involved in such interactions. Numerous studies were performed in order to clarify the functional role of individual modules and to understand the mechanism of blood clotting in detail. A number of fragments containing one or more modules have been prepared from factor IX and other homologous proteins (Astermark *et al.*, 1991; Persson *et al.*, 1991; Ohlin *et al.*, 1990). Among them are a Gla fragment containing only the Gla module, an $(EGF)_2$ fragment containing two EGF modules, and a Gla–$(EGF)_2$ fragment containing all three modules. When such fragments are used for functional studies, the question usually arises about the correspondence of their structure and function to that of the corresponding region of the parent molecule.

The first EGF module of factor IX was chemically synthesized (Huang *et al.*, 1989) and expressed in yeast (Handford *et al.*, 1990). It was shown by NMR that both products were folded similarly to EGF itself and were able to bind Ca^{2+} although with lower affinity than in the whole protein. A more detailed three-dimensional structure of the first EGF module of human factor IX derived from NMR spectroscopy was published recently (Baron *et al.*, 1992). Less is known about the structure of factor IX Gla fragment. By analogy with the Gla module of prothrombin (Soriano-Garcia *et al.*, 1992), it is believed to acquire compact structure only in the presence of Ca^{2+}. However, there are some differences between the structures of the Gla region of factor IX and prothrombin, the latter having an additional disulfide loop encoded by a separate exon (Degen & Davie, 1987). Gla fragments from both proteins have low affinity for Ca^{2+} which seems to increase when the neighboring EGF or kringle module is present (Astermark *et al.*, 1991; Pollock *et al.*, 1988). A similar situation seems to prevail with the Gla fragments of factor X (Persson *et al.*, 1991) and protein C (Ohlin *et al.*, 1990). It was proposed that in these proteins the EGF module influences the structure and Ca^{2+} binding properties of the Gla region and serves as a scaffold for its proper folding (Ohlin *et al.*, 1990; Persson *et al.*, 1991; Astermark *et al.*, 1991). Results obtained with a monoclonal antibody against the Gla region of protein C also suggested that metal binding to a site in the EGF region results in a conformational change in the Gla region and that these two domains interact with each other (Orthner *et al.*, 1989). Recently we have shown that the isolated 6-kDa Gla fragment of human factor IX in the presence of Ca^{2+} is folded independently into a compact domain which, in the 25-kDa Gla–$(EGF)_2$ fragment, is strongly stabilized, presumably by an interaction with the neighboring first EGF module (Vysotchin *et al.*, 1993). Thus, Ca^{2+}-dependent interaction between the Gla and EGF domains in several proteins is well documented and seems to be a general feature of the Gla-containing coagulation proteins. Mean-

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while, the nature of this interaction is still unclear.

The interaction between the Gla and EGF domains seems to be connected with the expression of important functions. It was shown that only the Gla–$(EGF)_2$ fragment but not individual Gla and $(EGF)_2$ fragments were able to inhibit the anticoagulant effect of activated protein C in a clotting assay (Ohlin *et al.*, 1990). It was reported that determinants in both the Gla and first EGF domain contribute to the specific binding to the endothelial cell surface (Ryan *et al.*, 1989). Also, the Gla fragment of prothrombin and factor X require higher concentration of Ca^{2+} than the parent proteins for binding to membranes (Schwalbe *et al.*, 1989). To better understand the mechanism of interaction between the Gla and EGF domains and its consequences, further investigation of their Ca^{2+}-dependent structure and interactions is required. In this paper, we characterized the effect of Ca^{2+} on the stability and interactions of the Gla and EGF domains of human factor IX.

MATERIALS AND METHODS

Factor IX was purified from a DEAE-Sephadex-adsorbed fraction of human plasma by affinity chromatography on a column of immobilized anti-factor IX monoclonal antibody according to Tharakan et al. (1990) using that same affinity matrix kindly provided by Dr. W. Drohan of this institution.

The 25-kDa fragment containing Gla and two EGF modules was prepared by digestion of factor IX with chymotrypsin. The 6-kDa and 19-kDa fragments consisting of Gla and two EGF modules, respectively, were prepared by digestion of the 25-kDa fragment with elastase. The conditions for the digestion and purification procedure were exactly the same as described earlier (Vysotchin *et al.*, 1993). All fragments were subjected to 10–15 cycles of NH_2-terminal sequence analysis on a Hewlett Packard Model G1000S sequencer. The sequences of the 25-kDa, 19-kDa, and 6-kDa fragments were essentially the same as determined before, including the cleavage before Arg^{116}, in the third disulfide loop of the second EGF domain. However, in the present case, the shortened form of the 6-kDa Gla fragment starting from Leu^{14} did not exceed 10% of the total. The concentrations of the fragments were determined spectrophotometrically using extinction coefficients calculated from the amino acid composition earlier (Vysotchin *et al.*, 1993).

Analytical size-exclusion chromatography measurements were performed at room temperature with a fast protein liquid chromatography system (Pharmacia) on a column of Superdex-75 at flow rate of 1.0 mL/min, monitoring elution by the absorbance at 280 nm. The column was calibrated with standard proteins including bovine serum albumin, carbonic anhydrase, cytochrome *c*, and aprotinin with molecular masses of 66, 29, 12.4, and 6.5 kDa, respectively. Unless otherwise indicated, all experiments were conducted in TBS (0.02 M Tris/0.15 M NaCl, pH 7.4). The buffer and protein solutions used for the Ca^{2+} binding study were first passed over Chelex 100 (Bio-Rad), in order to remove trace metal ion contamination.

Fluorescence measurements were done on an SLM 8000-C fluorometer. The Ca^{2+} and urea titrations were performed at room temperature by continuous addition with a motorized syringe of a concentrated stock solution of the titrant to a stirred solution of the indicated fragment while monitoring the fluorescence intensity at 350 nm with excitation at 280 nm. Both the fluorometer and the syringe–driver were controlled by a computer which automatically corrected the fluorescence intensity for dilution assuming a linear dependence on protein concentration below 0.15 mg/mL. Fluorescence measurements were performed at protein concentrations of 0.1 mg/mL (titration with urea) and 0.1–0.15 mg/mL (titration with Ca^{2+}). Data obtained by titration with Ca^{2+} in 5 M urea were fitted to a binding isotherm of the form:

$$I = 1.0 - (1.0 - I_f)[Ca^{2+}]/(K_d + [Ca^{2+}]) \quad (1)$$

where I is the observed fluorescence normalized to a value of 1.0 in the absence of Ca^{2+}. The parameter I_f represents the final fluorescence at infinite $[Ca^{2+}]$ and was allowed to vary along with the dissociation constant, K_d, during the fitting process.

Circular dichroism measurements were made with a Jasco-500C spectropolarimeter using protein concentrations of 0.6, 2.1, and 1.2 mg/mL for the 6-kDa, 19-kDa, and 25-kDa fragments, respectively, in 0.01- or 0.1-cm path-length cells. Data were expressed as the mean residue ellipticity, $[\theta]$, in units of degrees centimeter squared per decimole. Melting curves were obtained by monitoring the ellipticity at 220 nm for the 6-kDa fragment, at 213 nm for the 19-kDa fragment, and at both wavelengths for the 25-kDa fragment while heating at approximately 1 °C/min with a circulating water bath and jacketed cells.

Differential absorption measurements were made at room temperature with a Perkin-Elmer Model 645 spectrophotometer in matched cuvettes of 1.0-cm path length. Small aliquots of concentrated $CaCl_2$ were added to the sample cell and equivalent volumes of buffer to the reference cell, each containing equal volumes of a solution of 1 mg/mL of the 19-kDa fragment in TBS or TBS with 5 M urea. The magnitude of ΔA, the difference absorption peak at 278 nm, was plotted as a function of $[Ca^{2+}]$, and the normalized data were fitted to eq 2 to obtain a value for the dissociation constant.

$$\Delta A = \Delta A_{max}[Ca^{2+}]/(K_d + [Ca^{2+}]) \quad (2)$$

RESULTS

Characterization of the Ca^{2+}-Induced Interaction between Gla and EGF Domains in the 25-kDa Fragment. Our previous results demonstrated a Ca^{2+}-dependent stabilizing interaction between Gla and EGF domains in human factor IX (Vysotchin *et al.*, 1993) which might be connected with the fluorescence changes observed upon titration of this protein and its fragments with Ca^{2+} (Astermark *et al.*, 1991). To further characterize this interaction, we have checked its resistance toward urea by titrating the 25-kDa Gla–$(EGF)_2$ fragment with this reagent while monitoring fluorescence intensity. The 25-kDa fragment consisting of the Gla and two EGF domains more closely mimics the environment of these domains in the parent protein without the added complexity arising from the serine protease module which has five additional Trp residues. Upon titration with urea, a sigmoidal transition between 2.5 and 4.5 M was observed in the presence of Ca^{2+} while in EDTA such a transition was absent (Figure 1A). The change in fluorescence suggests a change in the exposure of Trp residue(s) and might be connected with disruption of the interaction between the Gla and EGF domains or with their denaturation. To select between these two alternatives, we performed a denaturation study of the 25-kDa Gla–$(EGF)_2$ fragment and its constituent 6-kDa Gla and 19-kDa $(EGF)_2$ fragments in 5 M urea.

Heating of the 6-kDa Gla fragment in the presence of 5 M urea and Ca^{2+} while monitoring the change of ellipticity at 220 nm revealed a signmoidal denaturation transition with a

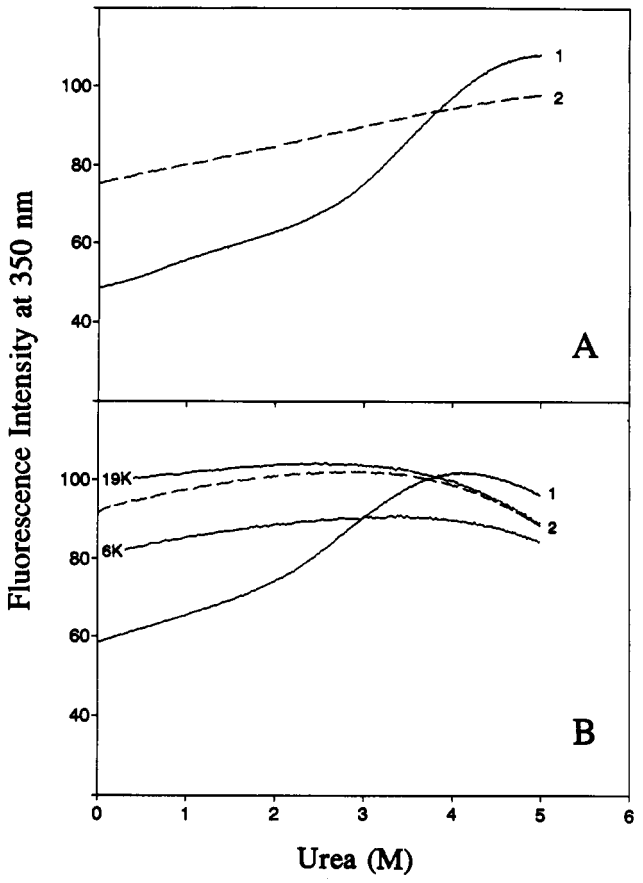

FIGURE 1: Titration with urea of 25-kDa, 6-kDa, 19-kDa, and a 1:1 mixture of the latter two fragments in TBS containing 1 mM Ca^{2+} or 1 mM EDTA. Panel A represents the relative change in fluorescence intensity at 350 nm of the 25-kDa fragment in 1 mM Ca^{2+} (solid curve 1) or 1 mM EDTA (dashed curve 2) upon titration with urea. Panel B represents the change in fluorescence of a 1:1 mixture of the 6- and 19-kDa fragments in the presence of 1 mM Ca^{2+} (solid curve 1) or 1 mM EDTA (dashed curve 2). Titration of the individual 6-kDa and 19-kDa fragments in the presence of 1 mM Ca^{2+} is shown by the solid curves denoted 6K and 19K, respectively.

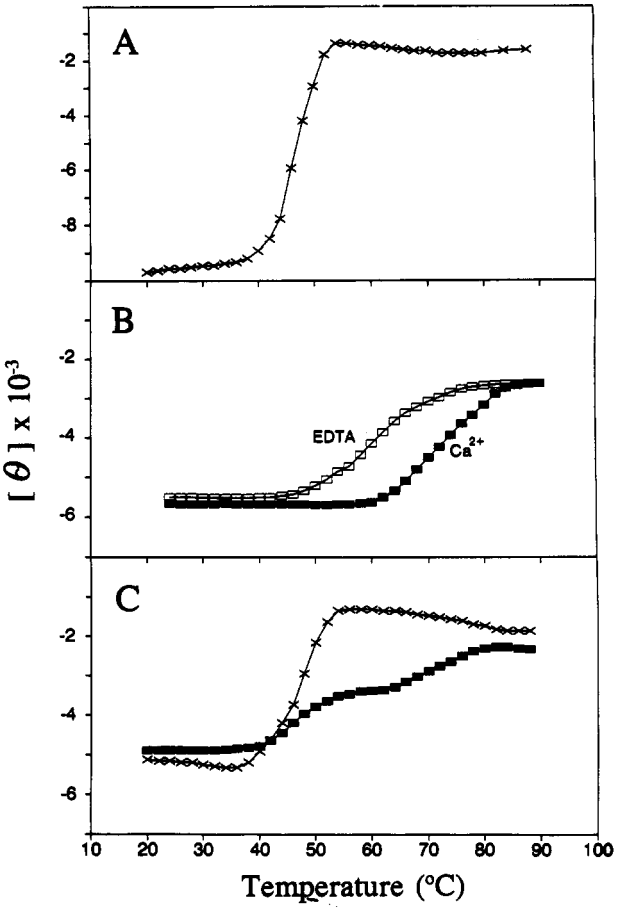

FIGURE 2: Melting of the 6-kDa, 19-kDa, and 25-kDa fragments in TBS in the presence of 5 M urea and, except where noted, 5 mM Ca^{2+}. Panel A represents melting of the 6-kDa fragment detected while monitoring the change in ellipticity at 220 nm. Panel B represents melting of the 19-kDa fragment in the presence of 5 mM Ca^{2+} (filled rectangles) or 2 mM EDTA (open rectangles) while monitoring ellipticity at 213 nm. Panel C represents changes in ellipticity at 220 nm (×) or at 213 nm (filled rectangles) of the 25-kDa fragment upon heating.

temperature midpoint at 46 °C (Figure 2A), indicating that the compact structure of the Ca^{2+}-loaded 6-kDa Gla fragment is preserved. The 19-kDa $(EGF)_2$ fragment also exhibited a sigmoidal denaturation transition under these conditions with a T_m at 72 °C, registered as a change in ellipticity at 213 nm (Figure 2B), suggesting that its compact structure is also preserved. Figure 2C presents melting curves of the 25-kDa Gla–$(EGF)_2$ fragment in Ca^{2+} and 5 M urea detected by the change in ellipticity both at 220 and at 213 nm. The former wavelength registers preferentially the change in α-helical content which favors the Gla domain while the latter is more sensitive to the β-structure, thus favoring the EGF domain-(s). The 220-nm curve exhibits a well-expressed denaturation transition whose T_m coincides with that of the 6-kDa Gla fragment alone. The curve obtained at 213 nm is biphasic. The first phase corresponds to the transition seen at 220 nm assigned to the Gla domain. In addition, there is a second higher temperature transition whose T_m near 72 °C coincides with that of the 19-kDa $(EGF)_2$ fragment alone under the same conditions (panel B). These results indicate that the compact structures of the Gla and first EGF domains in the 25-kDa fragment are also preserved in 5 M urea, confirming that the sigmoidal transition observed upon titration of the 25-kDa fragment with urea (Figure 1A) can be attributed to the disruption of the intramolecular interaction between the Gla and EGF domains without unfolding of either. The fact that in 5 M urea the T_m of the Gla domain alone (Figure 2A) coincides with that of the Gla domain in the 25-kDa fragment (Figure 2C, first transition) provides additional evidence for the loss of that interaction in 25 kDa; i.e., the Gla domain is no longer stabilized by the EGF as it is in the absence of urea (Vysotchin *et al.*, 1993).

In addition, size-exclusion chromatography of the 25-kDa fragment in the presence of Ca^{2+} and EDTA indicated a significantly higher elution volume of the Ca^{2+}-loaded form of the 25-kDa fragment, suggesting a more compact structure than that of the fragment in the presence of EDTA (Figure 3A). This difference is essentially abolished in 5 M urea (Figure 3B). The slightly different elution volume under these conditions may be related to changes in the column parameters caused by urea. These experiments are consistent with the idea that in the presence of Ca^{2+} the Gla domain forms a compact structure which, in the absence of urea, interacts with the neighboring EGF domain(s) and reduces the Stokes radius of the 25-kDa fragment.

Characterization of the Ca^{2+}-Induced Interaction between the 6-kDa and 19-kDa Fragments. To check if this interaction could be detected with the isolated domains as in the case of the SP-N and SP-C domains of the SP module of factor IX (Vysotchin *et al.*, 1993), we performed fluorescence and size-

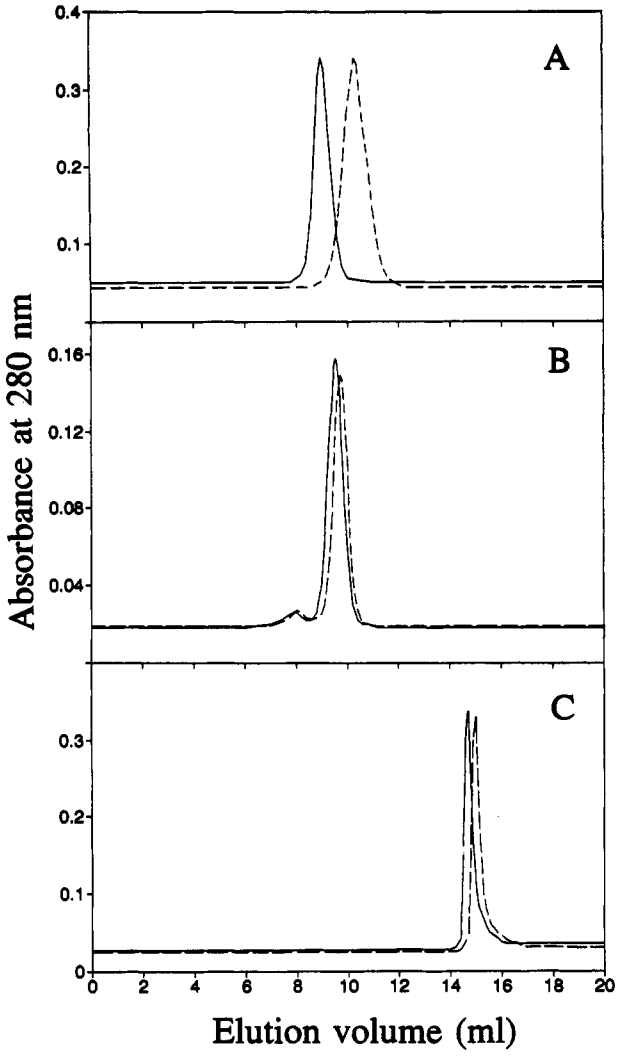

FIGURE 3: Size-exclusion chromatography of the 25-kDa fragment on Superdex-75 in TBS (panel A) and in TBS with 5 M urea (panel B) and of the 6-kDa fragment in TBS with 5 M urea (panel C), all in the presence of 5 mM Ca^{2+} (dashed lines) or 2 mM EDTA (solid lines).

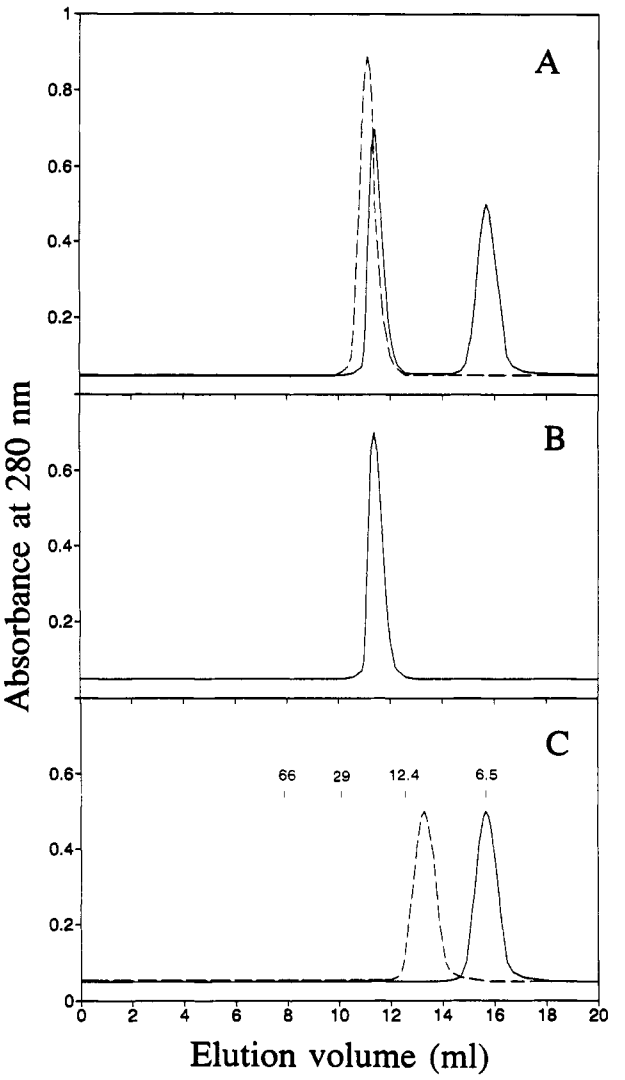

FIGURE 4: Interaction of the 6-kDa and 19-kDa fragments in the presence of Ca^{2+} as determined by analytical size-exclusion chromatography on a Superdex-75 column in TBS. Panel A, 1:1 mixture of 6-kDa and 19-kDa fragments; panel B, 19-kDa fragment alone; panel C, 6-kDa fragment alone. Experiments were performed in 5 mM Ca^{2+} (dashed lines) or 2 mM EDTA (solid lines). The elution positions of protein markers of the indicated mass in kilodaltons are shown in panel C.

exclusion experiments with the 6-kDa and 19-kDa fragments and their 1:1 mixture. Titration of such a mixture with urea in the presence of Ca^{2+} produced a dose-dependent sigmoidal change in the intrinsic fluorescence intensity between 2.5 and 4.5 M urea similar to that observed with the 25-kDa fragment (Figure 1B, curve 1); this change was absent in EDTA (curve 2). No such change was observed with the individual 6-kDa and 19-kDa fragments; their fluorescence in Ca^{2+} (curves 6K and 19K) followed the same way as that of the mixture in EDTA. This suggests that the Ca^{2+}-dependent interaction between the Gla and EGF domain(s) is reconstituted in the 1:1 mixture of the individual fragments and can be disrupted with urea.

Size-exclusion chromatography experiments were performed in order to directly test the formation of a heterocomplex between the 6-kDa Gla and 19-kDa $(EGF)_2$ fragments. As shown in Figure 4, in the presence of EDTA (solid profiles) a 1:1 mixture of these fragments eluted in two peaks (panel A) corresponding to those of the individual components under identical conditions (panels B and C). In the presence of Ca^{2+} (dashed profile), the mixture elutes as a single peak slightly ahead of the position of the 19-kDa fragment alone, indicating formation of a heterocomplex (panel A). Essentially all of the applied material was recovered in this peak, and analysis by SDS–PAGE confirmed the presence of both components. The complex is apparently of high affinity, resulting in very little trailing in the equimolar mixture. This made it possible to estimate the stoichiometry of the heterocomplex by application of well-defined mixtures of the 6-kDa and 19-kDa fragments as presented in Figure 5. Increasing the proportion of the 19-kDa fragment in the mixture caused a steady decrease of the intensity of the peak corresponding to the 6-kDa fragment while increasing that of the complex. A plot of the peak area of the 6-kDa fragment versus the molar ratio in the mixture revealed the formation of an equimolar complex between the 6-kDa Gla and 19-kDa $(EGF)_2$ fragments in the presence of Ca^{2+} (Figure 5C, inset). This together with the similarity of titration curves of the Ca^{2+}-loaded 25-kDa fragment and the mixture of the 6-kDa and 19-kDa fragments (Figure 1) suggests that interaction between the separated Gla and EGF domains occurs in the same manner as in the 25-kDa fragment.

It was also found that the 6-kDa Gla fragment by itself elutes much earlier in the presence of Ca^{2+} than in the presence of EDTA (Figure 4C), suggesting Ca^{2+}-dependent self-

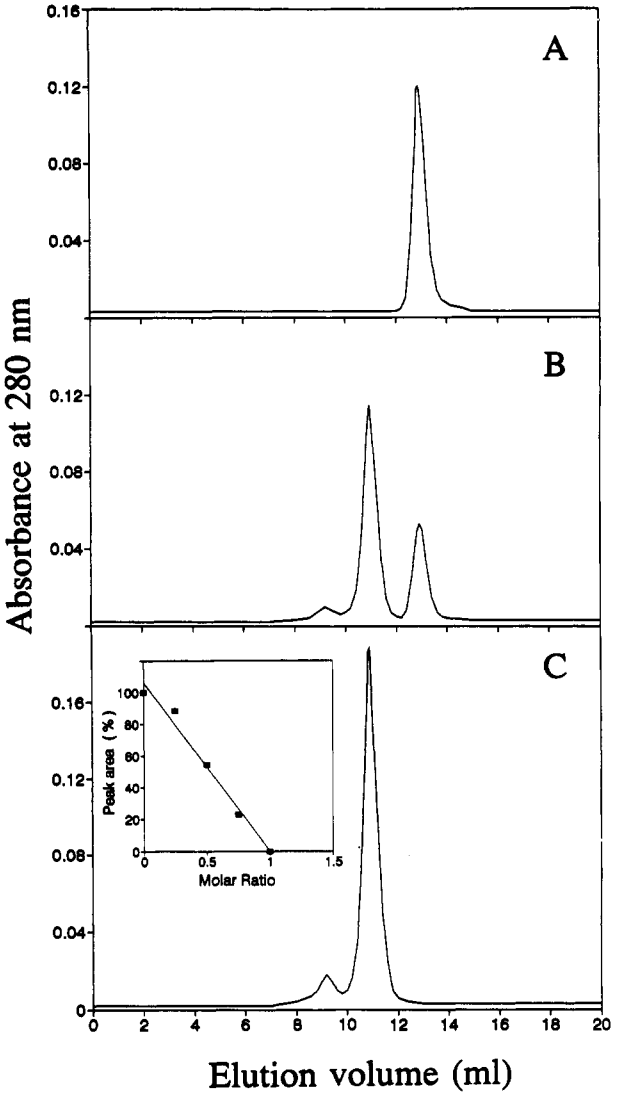

FIGURE 5: Stoichiometry of association of 6-kDa and 19-kDa fragments. Size-exclusion chromatography of 6-kDa fragment alone (A) and mixed with 0.5 (B) and 1.0 (C) molar equiv of the 19-kDa fragment. The inset in panel C plots the decrease in area of the 6-kDa fragment peak vs the molar ratio of the 19-kDa to 6-kDa fragments. The amount of 6-kDa fragment applied was constant in all cases.

association. Comparison of the elution volumes of the 6-kDa Gla fragment in Ca^{2+} and EDTA with those of proteins of known molecular mass in this range is consistent with formation of a dimer.[1] Interestingly, the Gla fragment fails to self-associate in the presence of 5 M urea where its elution volume in the presence of Ca^{2+} differs only slightly from that in EDTA (Figure 3C). The small differences between these two forms are consistent with the fact that in urea it still forms a Ca^{2+}-induced compact structure (Figure 2A,B), reducing slightly the Stokes radius.

Binding of Ca^{2+} to Gla and EGF Domains. It is well established that Ca^{2+} binds to the Gla and first EGF domains of factor IX (Mórita *et al.*, 1984; Handford *et al.*, 1990; Huang *et al.*, 1989; Furie & Furie, 1988). This binding causes changes in the intrinsic fluorescence parameters of the parent factor IX and some of its fragments (Morita *et al.*, 1984; Astermark *et al.*, 1991). In agreement with Astermark *et al.* (1991), the fluorescence of our 19-kDa fragment was not affected by Ca^{2+}. However, the absorption spectrum showed Ca^{2+}-induced perturbations indicating changes in the environment of tyrosine residues. As shown in Figure 6, the absorption difference

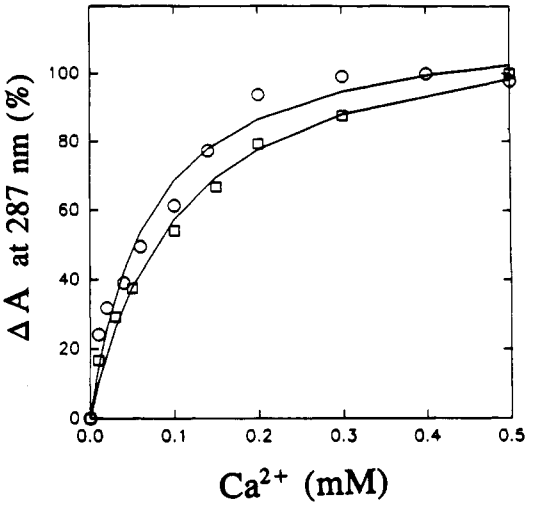

FIGURE 6: Titration of the 19-kDa fragment with Ca^{2+} in TBS without (circles) and with 5 M urea (squares). The solid curves represent best fits of the dta to eq 2 with dissociation constants of 71 and 108 μM, respectively. The 100% level corresponds to an absorbance change (ΔA) of 0.08.

(ΔA) at 278 nm increased in a dose-dependent manner upon addition of Ca^{2+}, both in the absence and in the presence of 5 M urea, with K_d values of 71 and 108 μM, respectively. Thus, not only the compact structure but also the Ca^{2+} binding properties of the 19-kDa $(EGF)_2$ fragment are minimally affected by the presence of the denaturing agent. This is consistent with the fact that in 5 M urea, the presence of Ca^{2+} strongly stabilizes the 19-kDa fragment, shifting its T_m by 12 °C (Figure 2C).

The fluorescence of the 25- and 6-kDa fragments is very sensitive to metal ions (Figure 7A,B, respectively). When titrating with Ca^{2+}, the fluorescence intensity first increases slightly in the region below 0.1 mM and then shows a large decrease. The initial rise persists to higher concentration in the case of the 6-kDa Gla fragment. Similar titration curves were observed with the corresponding fragments of factor X (Persson *et al.*, 1991), protein C (Ohlin *et al.*, 1990), and bovine factor IX (Astermark *et al.*, 1991). The shape of the curves suggests a complex process in which Ca^{2+} binding sites of different affinity have different effects on the environment of the tryptophan residue(s). Included among these changes can be expected at least two processes: (1) the formation of compact structure by the Gla domain and (2) association of the Gla domain, with itself in the case of the 6-kDa fragment and with the EGF domain(s) in the case of the 25-kDa fragment.

The dependence of the self-association of the 6-kDa fragment on $[Ca^{2+}]$ was examined by size-exclusion chromatography as in Figure 2C, equilibrating the column with buffers containing different concentrations of the metal ion and determining the proportion of material in the two peaks corresponding to the monomer and dimer. It was found that in 0.1 mM Ca^{2+} there is only one peak whose elution volume

[1] Strictly speaking, the possibility of a larger complex cannot be excluded if it were to interact with the column. Whatever its size, it seems clear that the complex is homogeneous since it elutes in a sharp peak. Some degree of self-association must occur since Ca^{2+}-induced interaction of monomers with the column would have the opposite effect, i.e., retardation. Because the size of the self-association complex is not critical for the main conclusions of this study, we will assume that it is a dimer, consistent with its elution position relative to standard proteins and with what was reported by others for the Gla fragment of prothrombin based on similar methods (Pollock *et al.*, 1988).

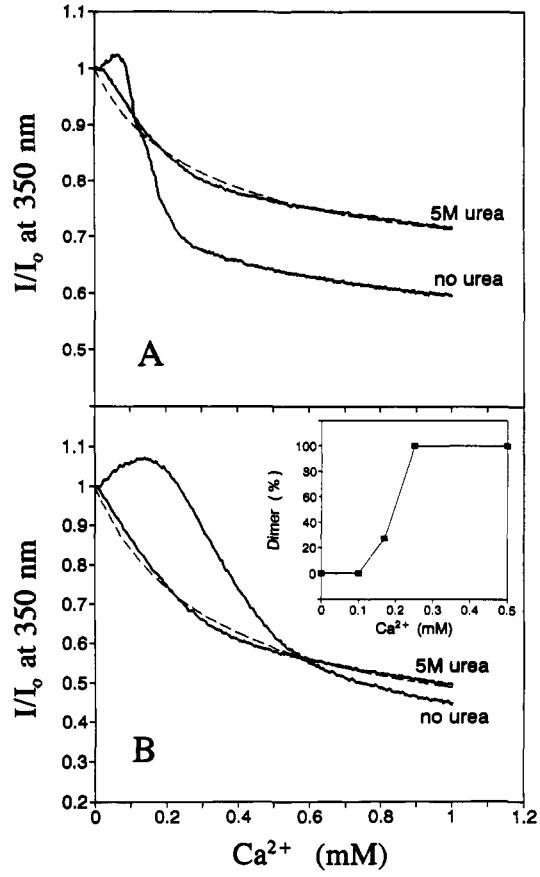

FIGURE 7: Titration of 25-kDa (panel A) and 6-kDa (panel B) fragments with Ca^{2+} while monitoring the fluorescence intensity at 350 nm in TBS ± 5 M urea as indicated. The dashed lines represent theoretical best fits of eq 1 to the data obtained in urea to give apparent K_d values of 0.30 mM for the 25-kDa fragment and 0.33 mM for 6-kDa fragment. The corresponding values obtained for the fraction of initial fluorescence intensity at infinite [Ca^{2+}] are 0.63 and 0.32. The inset panel B plots the percentage of Gla dimer *vs* Ca^{2+} concentration determined by size-exclusion chromatography as in Figure 4C (see text).

corresponds to the monomeric form. At 0.25 mM Ca^{2+}, the fragment was eluted in a single peak corresponding to the dimer. At intermediate concentration of Ca^{2+} (0.17 mM), there were two well-resolved peaks with elution volumes corresponding to those of the monomer and the dimer, indicating that the equilibrium is not rapid on the time scale of chromatography (15 min). A plot of the peak area of the monomer versus Ca^{2+} concentration (Figure 7B, inset) indicates that dimerization is cooperative with respect to [Ca^{2+}], there being no dimer at 0.1 mM and 100% dimer at 0.25 mM. The lag in dimer formation occurs in the region of the small increase in fluorescence and is complete when the large quenching effect is just beginning. Thus, the latter effect cannot be due to dimerization *per se*, but must reflect additional conformational changes with continued loading of Ca^{2+} irrespective of the state of association.

In the presence of 5 M urea, the titration curves of both the 25-kDa and the 6-kDa fragments became monophasic, without the initial increase at low Ca^{2+} concentration (Figure 7). This suggests that the factors responsible for the increase of fluorescence at low [Ca^{2+}] were eliminated by urea, which, as shown above, prevents self-association of the 6-kDa fragment and intramolecular association of the Gla and EGF domains in the 25-kDa fragment. The two curves are very similar. The dashed curves represent the best fits of the data to a single class of sites with apparent K_ds of 0.30 and 0.33 mM for the 25- and 6-kDa fragments, respectively. There is a small consistent deviation between the experimental and theoretical curves suggestive of some cooperativity in the binding to both fragments. It seems that under these conditions, the 6-kDa Gla fragment and the Gla domain in the 25-kDa fragment bind Ca^{2+} in the same manner, at least as manifested by changes in fluorescence; i.e., the structure and Ca^{2+} binding properties of the Gla domain of factor IX are preserved when isolated.

DISCUSSION

The results presented here provide direct evidence for a Ca^{2+}-dependent interaction between the Gla and EGF domains in human factor IX and afford some insight into the nature of that interaction. Size-exclusion chromatography and spectroscopic measurements with three fragments, 6-kDa Gla, 19-kDa $(EGF)_2$, and 25-kDa Gla–$(EGF)_2$, revealed that the interaction is rather strong. It can be reconstituted when the Gla and EGF domains are isolated and subsequently mixed together. In this case, the 6-kDa Gla and 19-kDa $(EGF)_2$ fragments interact with each other to form a 1:1 heterocomplex. This most likely involves the first EGF domain since the second one, in both the 19- and 25-kDa fragments, is cleaved and devoid of meltable structure (Vysotchin *et al.*, 1993). The intramolecular interaction within the 25-kDa fragment is very similar to the intermolecular one between the isolated domains. Neither is formed in 2 mM EDTA or 5 M urea. The former removes Ca^{2+} and essentially destroys the compact structure of the Gla domain (Vysotchin *et al.*, 1993), preventing formation of the complex. The effect of urea is different. Titration of those complexes with this reagent produced similar sigmoidal fluorescence-detected transitions between 2.5 and 4.5 M. These transitions were not due to denaturation of individual domains because all transitions connected with the melting of Gla and EGF were preserved in 5 M urea. Rather, they reflect disruption of the interaction between the urea-stable Gla and EGF domains.

In the presence of Ca^{2+} the 6-kDa Gla fragment self-associates, probably forming a dimer. Urea prevents this self-association without the loss of compact structure. The affinity of the Gla fragment for the $(EGF)_2$ fragment is much higher than for itself. This explains why the 25-kDa fragment remains monomeric in Ca^{2+}; its Gla domain, rather than self-associate, forms an intramolecular heterocomplex analogous to the intermolecular complex between the 6- and 19-kDa fragments. It should be noted that the isolated Gla domain of prothrombin also has a tendency to dimerize (Pollock *et al.*, 1988). Earlier work revealed that fragment 1 of prothrombin (Gla plus kringle domains) undergoes dimerization at millimolar levels of Ca^{2+} (Prendergast & Mann, 1977; Jackson *et al.*, 1979). Evidence for heterodimer formation between fragment 1 and factor X has been presented by Harlos et al. (1992), and these authors suggested that Gla-mediated heterodimer formation might serve a functional role in coagulation. The present results highlight the propensity of the Gla domain of factor IX, like that of prothrombin, to interact with itself or neighboring structures.

Our experiments revealed that not only the compact structure of the first EGF domain but also its Ca^{2+} binding properties are preserved in 5 M urea. At neutral pH in the absence of denaturing reagents, melting of the EGF domains of factor IX occurs at an inconveniently high temperature (Vysotchin *et al.*, 1993). The use of 5 M urea shifted the melting of the first EGF, the only domain in the 19-kDa fragment that retains compact structure, to lower temperature,

allowing the effect of Ca^{2+} to be observed. The T_m was elevated by 12 °C in the presence of Ca^{2+} (Figure 4C), indicating a strong Ca^{2+}-induced stabilization. This observation reinforces the assignment of the Ca^{2+}-induced absorbance changes in the 19-kDa $(EGF)_2$ fragment to the first EGF domain. The dissociation constant increased slightly from 71 to 108 μM in 5 M urea. The former value is 3–5-fold lower than that reported for the first EGF modules of factor IX that had been chemically synthesized (Huang *et al.*, 1991) or expressed in yeast (Handford *et al.*, 1990) (K_d = 0.4 and 0.2–0.3 mM, respectively) but comparable with the value determined for the high-affinity site(s) in Gla-domainless factor IX (K_d = 52.85 μM) (Morita *et al.*, 1984; Morita & Kisiel, 1985). The reason for such a discrepancy is not clear, but one should take into account that the proteolytically obtained fragment was folded *in vivo* in a native environment and subjected to all posttranslational modifications while the synthetic and recombinant EGF domains contained no β-hydroxylated aspartic acid or O-linked carbohydrate (Nishimura *et al.*, 1992). Also, the 19-kDa fragment, in contrast to synthetic and recombinant EGF, contains a neighboring second EGF module with which it interacts in the parent molecule (Vysotchin *et al.*, 1993). Although the second EGF in the fragment has an internal cleavage and is devoid of meltable structure (Vysotchin *et al.*, 1993), its side chains could conceivably influence the Ca^{2+} binding properties of the first one.

Binding of Ca^{2+} to factor IX and its fragments is accompanied by changes in fluorescence intensity (Morita *et al.*, 1984; Astermark *et al.*, 1991). The changes observed here with the 25-kDa fragment are qualitatively very similar to those seen by Astermark *et al.* (1991) with the corresponding bovine fragment; the fluorescence increases slightly at low $[Ca^{2+}]$ and decreases substantially at higher concentration. We found that the initial increase is abolished in 5 M urea where Ca^{2+} binding persists but the domain–domain interactions are disrupted. This suggests that the initial increase may be connected with domain–domain interactions induced by filling of high-affinity site(s), possibly including that on the first EGF domain. The decrease in fluorescence that follows at higher $[Ca^{2+}]$ (or from the beginning in 5 M urea) would then be attributed to acquisition of additional structure by Gla with continued loading of Ca^{2+}. As shown previously, decarboxylation of Gla residues abolished the decrease in fluorescence while preserving the increase (Astermark *et al.*, 1991).

The 6-kDa Gla fragment also exhibits an increase in fluorescence at low $[Ca^{2+}]$ which obviously cannot be connected with the high-affinity site on EGF. Thus, the first sites to be filled in isolated Gla have an effect on the Trp^{42} fluorescence which mimics that seen with the 25-kDa fragment. This happens in the region of Ca^{2+} concentration where self-association occurs and is abolished in 5 M urea, suggesting that it arises from Ca^{2+}-induced interactions of Gla with itself, interactions that have an effect on the Trp^{42} environment that is analogous to those caused by hetero-interactions within the 25-kDa fragment. The large decrease in fluorescence that accompanies the continued addition of Ca^{2+} to the Gla fragment occurs at almost 10-fold lower concentration of the metal ion than was reported for the bovine fragment (Astermark *et al.*, 1991). This is surprising since the amino acid sequences of the Gla domains of bovine and human factor IX are identical (Soriano-Garcia *et al.*, 1989). However, one should take into account that our 6-kDa fragment was generated with a different enzyme [elastase *vs* lysyl endopeptidase (Astermark *et al.*, 1991)] and should contain two additional residues at the C-terminus, Gln^{44} and Tyr^{45}. The latter was proposed to be critical for binding of the Gla to membranes (Schwalbe *et al.*, 1989). Perhaps these two residues also could alter the concentration of Ca^{2+} required to quench the fluorescence of nearby Trp^{42}.

Trp^{42} probably accounts for all of the fluorescence changes seen with the 25-kDa fragment as well (Soriano-Garcia *et al.*, 1992; Astermark *et al.*, 1991). The only other Trp is sandwiched between two disulfide bonds in the first EGF domain, and its fluorescence was not perturbed by titration of the 19-kDa $(EGF)_2$ fragment with Ca^{2+}. Its constant contribution to the total fluorescence in the 25-kDa fragment explains the lower percent quenching compared to 6-kDa Gla (Figure 7). This is most evident in 5 M urea where the two fragments are otherwise indistinguishable with respect to their response to Ca^{2+}. The conformation of the Ca^{2+}-loaded Gla domain in these two fragments also appears to be the same in 5 M urea since the T_m of the transition in the 6-kDa fragment and that of the corresponding transition in the 25-kDa fragment are the same (Figure 2).

Although Ca^{2+} strongly stabilizes the first EGF domain, the only Ca^{2+}-induced conformational changes detected by NMR (Huang *et al.*, 1991) involved the N-terminal tail of the synthetic analogue (Tyr^{45}–Gln^{50}) which encompasses the border with the Gla domain (Asp^{47}–Gly^{48}). This region also contains the sites of two mutations that are associated with moderate to severe hemophilia with little or no loss of antigen [Gln^{50} → Pro in factor IX New London (Lozier *et al.*, 1990) and Asp^{47} → Gly in factor IX Alabama (McCord *et al.*, 1990)]. The structural basis for the decreased activity of these mutants is not clear, but their location strongly suggests that their main consequence might be to perturb the above-described interaction between the Gla and EGF domains. In this context, it is of interest that both mutations occur naturally in protein Z which has a modular composition identical to factor IX (Sejima *et al.*, 1990). The small increase in fluorescence that is uniformly caused by low $[Ca^{2+}]$ in Gla–$(EGF)_2$ fragments of factors IX and X and protein C, as well as the Gla–(EGF) fragment of factor X, is conspicuously absent in the Gla–(EGF) fragment of protein Z (Persson & Stenflo, 1992). This rise in fluorescence was proposed above to be connected with the Ca^{2+}-induced interaction between the Gla and EGF domains, suggesting that such interaction is reduced or absent in protein Z. Since it has now been established that this interaction stabilizes the Gla domain in factor IX (Vysotchin *et al.*, 1993) (and present work), one could predict the absence of such a stabilizing effect in protein Z as well as factors IX Alabama and New London.

The main conclusions of this paper are summarized in Figure 8. Ca^{2+}, which induces compact structure in the Gla domain (Vysotchin *et al.*, 1993), also binds to the first EGF domain of factor IX, strongly stabilizing its structure. This generates interacting surfaces on both domains, allowing an interaction

FIGURE 8: Schematic representation of Ca^{2+}-induced conformational changes in the Gla and first EGF domain of human factor IX. The interfacial surfaces between domains are denoted by cross-hatches. The Trp (W) residue in the Gla domain responsible for Ca^{2+} induced fluorescence changes is shown by the circle. The increased shading from left to right reflects the increased stability of the domains due to binding of Ca^{2+} and interaction between domains. This interaction can be disrupted in 5 M urea without unfolding of the domains.

between them which further stabilizes the Gla domain (Vysotchin *et al.*, 1993), and may induce additional conformational changes important for function. Addition of 5 M urea disrupts the interaction without destroying the compact structure or Ca^{2+} binding properties of either domain. Urea also prevents association of the domains and simplifies the otherwise complex fluorescence changes that occur upon titration with Ca^{2+}.

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