Role of Calcium(II) Ions in the Recognition of Coagulation Factors IX and X by IX/X-bp, an Anticoagulant from Snake Venom

Fujio Sekiya, Toshiko Yamashita, and Takashi Morita*

Department of Biochemistry, Meiji College of Pharmacy, Yato-cho, Tanashi, Tokyo 188, Japan

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ABSTRACT: IX/X-bp, an anticoagulant protein isolated from the venom of the habu snake *Trimeresurus flavoviridis*, has a structure homologous to the carbohydrate-recognition domains of C-type (Ca^{2+}-dependent) animal lectins, and it binds to the γ-carboxyglutamic acid (Gla) domains of coagulation factors IX and X in a Ca^{2+}-dependent fashion. In the present study, we elucidated the role of Ca^{2+} ions in this binding. The binding of ^{125}I-labeled IX/X-bp to both coagulation factors required about 1 mM Ca^{2+} ions at pH 7.5. A decrease in the pH to 6.5 had a striking negative effect on the binding, and the Ca^{2+}-requirement curve was shifted rightward. We investigated the binding of Ca^{2+} ions to IX/X-bp directly by equilibrium dialysis and identified two independent binding sites with different affinities. At pH 7.5, the apparent K_d values for these sites were 25 and 200 μM, respectively. When the pH was decreased to 6.5, the affinity of the high-affinity binding site was reduced only slightly but that of the low-affinity site was reduced considerably. Moreover, it was evident from observations of Ca^{2+}-induced changes in the intrinsic fluorescence that IX/X-bp underwent a conformational change upon binding of Ca^{2+} ions. Taking these results together, we propose two features of the role of Ca^{2+} ions in the binding of IX/X-bp to coagulation factors as follows: (i) On the basis of the affinities of IX/X-bp and coagulation factors for Ca^{2+} ions and the effective concentration of Ca^{2+} ions that is required to support the association of these proteins, it appears that IX/X-bp recognizes the conformation of Gla domains with bound Ca^{2+} ions in these factors. (ii) On the basis of the effect of a decrease in pH, it appears that occupation of both IX/X-bp's Ca^{2+}-binding sites is also indispensable.

The carbohydrate-recognition domain (CRD)[1] found in C-type (Ca^{2+}-dependent) animal lectins, such as hepatic lectins and selectins, now seems likely to be a fundamental structural motif that is widely distributed in a variety of proteins [for recent reviews, see Drickamer (1988, 1993), Drickamer and Taylor (1993), and Spiess (1990)]. We previously isolated a protein that includes a CRD-like structure and has anticoagulant activity from the venom of the habu snake *Trimeresurus flavoviridis* (Atoda & Morita, 1989). This protein interacts with coagulation factors IX and X, and so we designated it IX/X-bp (factor IX/factor X-binding protein). IX/X-bp is a heterodimeric protein consisting of two highly homologous subunits of 15 and 14.5 kDa, respectively, that are linked by a disulfide bridge (Atoda et al., 1991; Atoda & Morita, 1993). Each subunit (129 and 123 amino acid residues, respectively) by itself contains, exclusively, the essential structural features of a CRD. Homologues of IX/X-bp with similar activity have also been identified in venoms of other snakes, *Bothrops jararaca* (Sekiya et al., 1993) and *Deinagkistrodon acutus*.[2] In addition, increasing numbers of proteins that are structurally related to IX/X-bp have recently been identified in snake venoms. These new members include botrocetin, which binds von Willebrand factor (Read et al., 1978; Usami et al., 1993); alboaggregin-B (Peng et al., 1991; Yoshida et al., 1993) and echicetin (Peng et al., 1993), which bind platelet glycoprotein Ib; and bothrojaracin, which binds α-thrombin (Zingali et al., 1993). Together, these proteins form a unique and intriguing subfamily in the diverse C-type animal lectin family. All of these proteins consist of two disulfide-linked homologous subunits, and all have a molecular mass of approximately 30 kDa. Despite the high degree of structural similarity, the biological action of each protein is quite distinct. They recognize their respective ligands with a high degree of selectivity, in contrast to the "usual" lectins, whose specificities are determined primarily by simple monosaccharides. Furthermore, the sites in the ligands at which these proteins, including IX/X-bp (Sekiya et al., 1993; Atoda et al., 1994), bind are distinct from the carbohydrate moieties, and thus these proteins are apparently not classifiable as true lectins. Studies with these unique members should provide further insight into the structure and function of CRDs from a novel perspective. Little attention has been paid, however, to these venom proteins, as compared with mammalian CRDs. The goal of our studies is to establish the molecular mechanisms of the interactions between venom CRDs and their ligands.

IX/X-bp forms a complex with both the zymogen and the activated forms of factors IX and X with unit stoichiometry. The binding specificity of IX/X-bp is very strict, and little, if any, binding to other γ-carboxyglutamic acid (Gla)-containing coagulation proteins such as prothrombin was detected (Atoda & Morita, 1989). As we showed recently, IX/X-bp binds to the N-terminal Gla domains of coagulation factors; it recognized the isolated Gla domain fragments but

* To whom correspondence should be addressed. Telephone/Fax: +81-424-21-0429.

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[1] Abbreviations: CRD, carbohydrate-recognition domain; Gla, γ-carboxyglutamic acid; TBS, Tris-buffered saline (20 mM Tris-HCl, 140 mM NaCl, pH 7.5); MBS, MES-buffered saline [20 mM 2-(*N*-morpholino)ethanesulfonic acid−NaOH, 140 mM NaCl, pH 6.5].

[2] H. Atoda, and T. Morita, unpublished observation.

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not the Gla-domainless proteins (Atoda et al., 1994). This interaction with the Gla domain leads to the blockade of the association between the coagulation factors and their respective cofactors, with resultant interference in blood coagulation. The binding is absolutely dependent on Ca^{2+} ions (Sekiya et al., 1993; Atoda et al., 1994). Since the Gla domains are the major Ca^{2+}-binding sites of these factors, it seems likely that IX/X-bp should recognize the Ca^{2+}-bound conformation of the Gla domains; i.e., Ca^{2+} ions might be required to support the binding to coagulation factors. Alternatively, in view of the C-type lectin-like structure of IX/X-bp, it also seems likely that Ca^{2+} ions might bind to IX/X-bp and change its conformation, thereby promoting the association of these proteins. In the present study, we examined the role of Ca^{2+} ions in the interaction of IX/X-bp and coagulation factors in an attempt to clarify these issues.

EXPERIMENTAL PROCEDURES

Materials. Bovine factors IX and X were purified as described previously (Hashimoto et al., 1985), and were homogeneous as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Radiochemicals were from Du Pont/New England Nuclear. Gels for chromatography were from Pharmacia.

Isolation of IX/X-bp. The original procedure for isolation included affinity chromatography on immobilized factor X or gel filtration (Atoda & Morita, 1989), but we are now able to prepare this protein more easily in large quantities by ion-exchange chromatography, as follows. Crude *T. flavoviridis* venom (The Japan Snake Institute, Gunma, Japan) was dissolved in (500 mg/10 mL) and dialyzed against 10 mM sodium phosphate, pH 6.8. Insoluble materials were removed by centrifugation, and the solution was applied to a column of S-Sepharose Fast Flow (1.6 × 35 cm) preequilibrated with the same buffer. After the column was washed with the same buffer (approximately 2 column volumes), it was developed with a linear gradient of NaCl in the buffer (0−0.15 M; 300 mL each). IX/X-bp was eluted immediately after the start of elution with gradient as a sharp, symmetrical peak. This fraction (~90% pure at this stage) was collected, dialyzed against 50 mM Tris-HCl, pH 8.0, and applied to a column of Q-Sepharose Fast Flow (1.6 × 30 cm) preequilibrated with the same buffer. The column was washed extensively with the initial buffer and then eluted with a linear gradient of NaCl (0−0.2 M; 150 mL each). IX/X-bp was eluted in 0.15 M NaCl, and the preparation was apparently homogeneous. All isolation procedures were performed at 4 °C. The protein obtained (approximately 20 mg) was dialyzed against appropriate buffers and stored at either 4 °C or −80 °C.

Binding Assay with ^{125}I-IX/X-bp. IX/X-bp was labeled with Na^{125}I by use of IODOBEADS (Pierce) in accordance with the manufacturer's instructions. The specific activity of the labeled protein was in the range of (1.0−2.0) × 10^6 cpm/μg. Binding assays were conducted as follows, except where otherwise indicated. Wells of breakable microtiter plates (Labsystems, Finland) were coated with a solution of 10 μg/mL factor IX or X in 50 μL of 20 mM Tris-HCl, 140 mM NaCl, pH 7.5 (TBS), at 4 °C overnight, and the remaining nonspecific binding sites were blocked by incubation with 1% bovine serum albumin (essentially fatty acid-free; Sigma) in TBS for 1 h. Each well was incubated with approximately 50 000 cpm of ^{125}I-IX/X-bp (approximately 0.5 μg/mL) in 50 μL of TBS (or some other buffer as indicated) containing an appropriate concentration of $CaCl_2$ and 1 μg/mL albumin for 30 min at 37 °C. Wells were then washed twice with 200 μL of the buffer that contained the same concentration of $CaCl_2$, cut into pieces, and counted for bound radioactivity in a γ-counter.

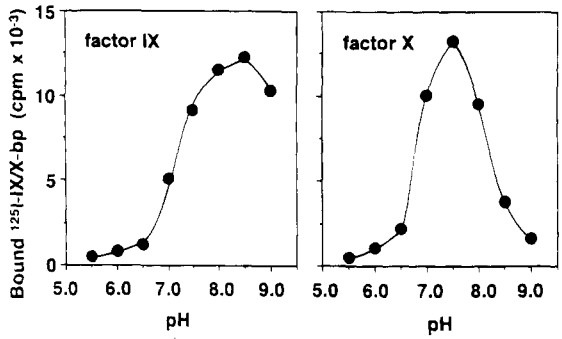

FIGURE 1: Effects of pH on the binding of IX/X-bp to factors IX and X. Binding of ^{125}I-IX/X-bp to solid-phase factors IX (left) and X (right) was investigated. The media used were 50 mM Tris buffer adjusted to the indicated pH with acetic acid, plus 100 mM NaCl and 5 mM $CaCl_2$. Total radioactivity was 30 000 cpm/well. Other conditions are described in detail under Experimental Procedures.

Equilibrium Dialysis. This was conducted at ambient temperature in a microvolume dialyzer with 250-μL cells (Hoffer Scientific Instrument, San Francisco, CA). Dialysis membranes were pretreated with a boiled solution of 0.1 M $NaHCO_3$/2% EDTA and washed extensively with metal-free water prior to use. A 200-μL aliquot of a solution of $CaCl_2$ containing $^{45}Ca^{2+}$ as a tracer (1 000 000 dpm/cell) was dialyzed against 200 μL of IX/X-bp solution (1.2 μg/mL; 40 μM) for 20 h with constant rotation. Protein-bound Ca^{2+} was quantified by liquid scintillation counting. The buffers and protein solutions used were freed from any possible contamination by metal ions either by passage through a column of Chelex 100 (Bio-Rad) or by dialysis against a suspension of Chelex 100 (1 g/L) in the appropriate buffer. Prior to use, protein solutions were also passed through a membrane filter (22-μm pores) to remove any aggregates.

Ca^{2+}-Induced Changes in Fluorescence. A 800-μL aliquot of IX/X-bp solution (10 μg/mL), free of metal ions, was placed in a micro-quartz cell. Calcium chloride solution at an appropriate concentration was added serially in small aliquots, and the fluorescence spectrum was recorded after each addition with excitation at 280 nm in a spectrofluorometer (Model FP-777; JASCO, Tokyo, Japan). The fluorescence intensity was corrected for changes in volume.

RESULTS

It has been shown that, in general, the recognition of ligands by C-type lectins has a characteristic dependence on pH; considerable binding is observed under neutral and basic conditions, but binding is much weakened below pH 6.5 (Drickamer, 1988). As a first step in our analysis of the nature of the binding to factors IX and X of IX/X-bp, we investigated the dependence on pH of such binding. IX/X-bp was labeled with ^{125}I, and its binding to factors IX and X immobilized on microtiter plates was examined in the presence of 5 mM Ca^{2+} ions. As is shown in Figure 1, binding of IX/X-bp to both proteins is apparent at neutral

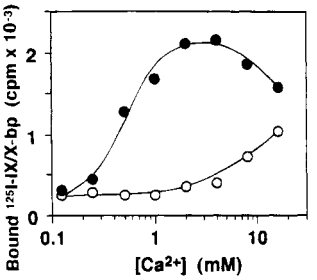

FIGURE 2: Dependence on Ca^{2+} ions of the IX/X-bp binding to factor IX at pH 6.5 and pH 7.5. Binding of ^{125}I-IX/X-bp to factor IX was examined in the presence of the indicated concentrations of Ca^{2+} ions at pH 7.5 (closed circles) and pH 6.5 (open circles). The media used were TBS or 20 mM MES−NaOH, 140 mM NaCl, pH 6.5 (MBS). Total radioactivity was 40 000 cpm/well.

and slightly alkaline pH. However, below pH 7.0, the binding dramatically reduced. The optimal pH for the binding to factor IX was 8.0−8.5, and that for binding to factor X was 7.5. Above pH 8.0, the binding properties with the two coagulation factors were different; the binding to factor X declined immediately as the pH was increased, while the binding to factor IX was unchanged at higher pH. This difference must reflect a difference between these two factors, but we have no explanation for this phenomenon at this time. Nevertheless, it was clear that the affinity of IX/X-bp for both coagulation factors was much weakened under acidic conditions, as is the case for other C-type lectins and their ligands.

In order to characterize the striking effect of acidic pH in greater detail, we chose to compare binding at pH 6.5 and pH 7.5. The effect of varying Ca^{2+} concentration on the binding of ^{125}I-IX/X-bp to solid-phase factors IX and X was investigated. Figure 2 shows the results with factor IX, and those for factor X were essentially the same (data not shown). At pH 7.5, the binding required physiological concentrations of Ca^{2+} ions; half-maximal binding and maximal binding were observed at 0.6 and 3 mM Ca^{2+} ions, respectively. At pH 6.5, a rightward shift of the Ca^{2+}-requirement curve was clearly seen. At least a 10-hold higher concentration was necessary, and the binding did not reach the level that was obtained at pH 7.5 with saturating concentrations of Ca^{2+} ions under our experimental conditions (up to 30 mM Ca^{2+}). Apparently, the affinity for Ca^{2+} ions was reduced at low pH. Such a reduction in affinity should be attributable to IX/X-bp, since the Ca^{2+}-binding properties of factor IX were reported to be essentially unchanged under these conditions (Amphlett et al., 1978). It seems likely, therefore, that IX/X-bp itself is capable of binding Ca^{2+} ions, and that formation of a complex with Ca^{2+} ions is a prerequisite for the recognition of coagulation factors.

In order to examine Ca^{2+} binding to IX/X-bp directly, we performed equilibrium dialysis. IX/X-bp was shown to be capable of binding two Ca^{2+} ions per molecule (Figure 3). Scatchard analyses show that the two binding sites were independent and had different affinities. At pH 7.5, the K_d value for the high-affinity binding site was 25 ± 12 μM (mean ± SE, n = 5) and that for the low-affinity site was 202 ± 110 μM. At pH 6.5, the affinity of the high-affinity site decreased only slightly (K_d = 50 ± 19 μM), whereas that of the low-affinity site was greatly diminished (K_d ~ 2 mM). Note that the K_d value given here for the low-affinity Ca^{2+}-binding site at pH 6.5 is an approximation obtained

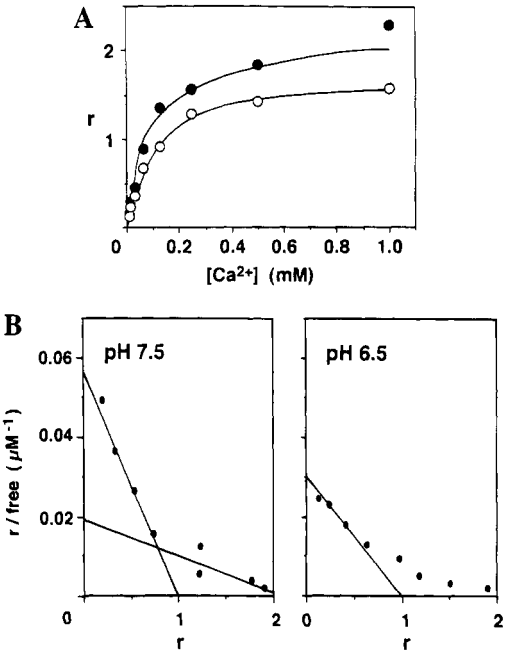

FIGURE 3: Binding of Ca^{2+} ions to IX/X-bp. Binding of Ca^{2+} ions to IX/X-bp was examined by equilibrium dialysis as described under Experimental Procedures. (A) Direct plots of the number of moles of Ca^{2+} bound per mole of protein (r) at pH 7.5 (in TBS; closed circles) and pH 6.5 (in MBS; open circles) as a function of the concentration of free Ca^{2+} ions. (B) Scatchard plots of the data in (A): left, pH 7.5; right, pH 6.5. Note that the line corresponding to the low-affinity site at pH 6.5 cannot be drawn with confidence because the affinity was so weak that the data include large standard errors, and these errors are further amplified when data are plotted here, making interpretation difficult.

directly from the point in the graph of r versus Ca^{2+} concentration, which represents half-saturation of the low-affinity binding site (i.e., r = 1.5). IX/X-bp forms aggregates at relatively high concentrations (>3 mg/mL), and thus it is difficult to achieve concentrations sufficient for an unequivocal determination of the K_d for such a low-affinity association. It is, nonetheless, apparent that the affinity for Ca^{2+} ions of the low-affinity site was greatly decreased at lower pH. This change corresponds to the increase in the required Ca^{2+} concentration for the binding of this protein to coagulation factors at low pH. We also investigated the effects of other polyvalent cations on $^{45}Ca^{2+}$ binding to IX/X-bp. We found that Sr^{2+}, Ba^{2+}, Mn^{2+}, and La^{3+} ions could totally replace the bound Ca^{2+} ions but, by contrast, Mg^{2+} ions at high concentrations (up to 10 mM) could not (data not shown).

The binding of Ca^{2+} ions to IX/X-bp was also demonstrated by observations of the Ca^{2+}-induced changes in the intrinsic fluorescence of IX/X-bp. Addition of Ca^{2+} ions augmented the fluorescence, as shown in Figure 4A. The fluorescence emission spectra were recorded after excitation at 280 nm, and, thus, the fluorescence should represent the microenvironment of a certain tryptophan residue(s) in IX/X-bp. Binding of Ca^{2+} ions to IX/X-bp appears to promote its conformational rearrangement in such a way that the microenvironment around the relevant tryptophan residue(s) is perturbed. Double-reciprocal plots of the data from this experiment gave straight lines (Figure 4B), indicating that only a single class of Ca^{2+}-binding sites was detectable by this method. The estimated K_d values for Ca^{2+} ions were 23 ± 3 and 45 ± 13 μM (mean ± SE, n = 6) at pH 7.5 and

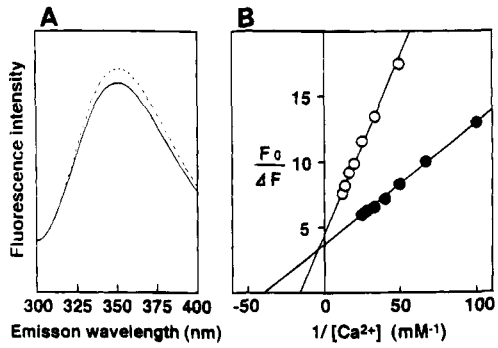

FIGURE 4: Changes in the intrinsic fluorescence of IX/X-bp induced by Ca^{2+} ions. (A) Fluorescence emission spectra of IX/X-bp in the absence (solid line) and presence (dotted line) of 50 μM Ca^{2+} ions at pH 7.5. Excitation was at 280 nm. (B) Double-reciprocal plot of Ca^{2+}-induced changes in the fluorescence of IX/X-bp. The ratio of the increment in emission intensity at 340 nm, corrected for the volume change (ΔF), to the emission intensity in the absence of Ca^{2+} ions (F_0) was obtained at each Ca^{2+} concentration. The reciprocal of $\Delta F/F_0$ was plotted against the reciprocal of the concentration of Ca^{2+} ions. Closed circles, pH 7.5 (in TBS); open circles, pH 6.5 (in MBS).

6.5, respectively. The change in fluorescence appeared to represent the binding of Ca^{2+} ions to high-affinity binding sites.

DISCUSSION

Numerous proteins that contain the CRD-like structure have been isolated from mammalian tissues as well as from snake venoms and various invertebrates (Drickamer, 1988, 1993; Spiess, 1990). CRDs consist of approximately 130 amino acid residues, of which about 20 are invariant or well conserved (Drickamer, 1988; Spiess, 1990). Most such mammalian proteins are integrated in membranes or are tightly associated with the extracellular matrix; they have relatively large structural elements in addition to CRDs; and some are composed of multiple subunits. For example, mammalian asialoglycoprotein receptor (hepatic lectin) is a heterohexameric protein consisting of two different but very similar polypeptides. Each of the polypeptides has a C-terminal CRD region and a rather long non-CRD extension that includes transmembrane and intracellular regions (Spiess, 1990; Drickamer & Taylor, 1993). Although the physiological significance of these mammalian proteins has been recognized and many researchers have undertaken extensive investigations, the abundance of non-CRD regions and the low solubility of these proteins in aqueous solutions, as well as the difficulties encountered in preparing them in large quantities, have all hampered efforts to elucidate structure/function relationships of these proteins. By contrast, the structures of IX/X-bp and related venom proteins are rather simple; the subunits consist exclusively of the essential elements of the CRD. IX/X-bp is a soluble protein without any posttranslationally modified amino acid residues (Atoda et al., 1991). It can be easily obtained by conventional chromatographic procedures in large quantities (see Experimental Procedures), and, most important of all, its binding sites on coagulation factors have been localized to small segments, the Gla domains (Atoda et al., 1994). Because of these advantageous features of IX/X-bp, we believe that it should provide an excellent model system for gaining insights into the general nature of the binding of CRDs to their ligands. It is noteworthy that CRD-like structures are being identified in more and more proteins (Drickamer, 1993) and, furthermore, the functions of such structures are not restricted to the recognition of carbohydrates [one apparent example is the recently cloned phospholipase A_2 receptor (Ishizaki et al., 1994)]. These observations suggest that the CRD-like structure is a fundamental motif in proteins that has evolved from a single ancestral gene and now exhibits very divergent features. Moreover, increasing numbers of proteins that are structurally related to IX/X-bp and have interesting biological activities have recently been isolated from snake venoms (e.g., botrocetin) (Read et al., 1978; Peng et al., 1991, 1993; Yoshida et al., 1993; Zingali et al., 1993). Thus, evaluation of the binding properties of IX/X-bp should also contribute to our understanding of the unique pharmacological actions of these venom proteins.

In the present study, we showed that IX/X-bp has two Ca^{2+}-binding sites per molecule and that IX/X-bp undergoes a conformational change upon binding of Ca^{2+} ions, as was apparent from the increase of intrinsic fluorescence caused by Ca^{2+} ions. This result is consistent with our crystallographic data (Mizuno et al., 1991). IX/X-bp forms a crystal only when Ca^{2+} ions are present, an indication that it adopts a loose, amorphous conformation and a rigid, ordered conformation in the absence and presence of Ca^{2+} ions, respectively. Moreover, preliminary data from our ongoing X-ray diffraction studies of IX/X-bp crystals indicate that one metal ion is incorporated into each subunit of IX/X-bp; thus, there are two ions per molecule.[3] There are reports about the Ca^{2+}-binding properties of CRDs in mammalian proteins (e.g., Andersen et al., 1982; Loeb & Drickamer, 1988; Geng et al., 1991), but ours is the first evidence for the binding of Ca^{2+} ions to a CRD-like protein from venom. All the mammalian CRDs examined to date have two or more Ca^{2+}-binding sites per CRD. Thus, there is a major difference between mammalian proteins and IX/X-bp, which has only one binding site per subunit. Furthermore, the amino acid residues that act as Ca^{2+} ligands in CRD modules derived from mannose-binding protein (Weis et al., 1991, 1992) and E-selectin (Graves et al., 1994), which were identified by X-ray crystallography, are conserved in the primary structures of neither of the IX/X-bp subunits. Thus, studies with IX/X-bp should give us an additional opportunity to clarify the structural requirements for Ca^{2+}-binding proteins. In this regard, it is of interest to note that the affinity of IX/X-bp for Ca^{2+} ions has a unique pH-dependency; a decrease in pH, from 7.5 to 6.5, gives a dramatic negative effect on the binding of Ca^{2+} ions to the low-affinity binding site. This range of pH is close to the typical K_a value of the side chain of His residue. It may be possible, therefore, that a certain His residue(s) in the IX/X-bp molecule participates in the recognition of Ca^{2+} ions. IX/X-bp has two His residues in the A chain (positions 12 and 38) and four residues in the B chain (positions 12, 34, 38, and 61) (Atoda et al., 1991), and none of them are conserved in the primary structures of mannose-binding protein and E-selectin. Furthermore, the corresponding positions in mannose-binding protein or E-selectin of IX/X-bp's N-terminal halves, at which all the His residues are present, are distal from the putative metal-binding sites of these proteins, which are located in the C-terminal halves

[3] H. Mizuno, H. Atoda, and T. Morita, unpublished observation.

of the CRDs, not only in the primary structures but also in the tertiary structures (Weis et al., 1992; Graves et al., 1994).

Among vitamin K-dependent coagulation proteins, factors IX and X (and also factor VII and protein C) have very similar structures; each of them is composed of, from the N- to the C-terminal, a Gla domain, a tandem repeat of two epidermal growth factor-like domains, and an activation peptide followed by a serine protease catalytic module (Furie & Furie, 1988). It has been established that these proteins have multiple Ca^{2+}-binding sites. The Gla domains are the major binding sites, and epidermal growth factor-like domains bind a Ca^{2+} ion with higher affinities ($K_d \sim 0.1$ mM) (Stenflo, 1991). In addition, it was shown recently that the catalytic module of factor IX is also capable of binding Ca^{2+} ions, just like chymotrypsin (Bajaj et al., 1992). The Ca^{2+}-binding and other metal-binding properties of these factors have been studied in detail, with emphasis on the Gla domains. For example, in factor IX, Amphlett et al. (1978) reported the presence of 2 classes of binding sites, with K_d values of 0.1 and 1.3 mM, and a total of 13 bound Ca^{2+} ions, while Bajaj (1982) reported that there are 16 Ca^{2+}-binding sites with average K_d values of 0.6 mM. Taken together, various results indicate that factor IX binds 10 or more Ca^{2+} ions in its Gla domain with average K_d values of approximately 0.5 mM although the precise numbers of binding sites and K_d values seem to depend on the experimental conditions. Similar considerations apply to binding of Ca^{2+} ions to the Gla domain of factor X [for a review, see Jackson (1984)].

IX/X-bp recognizes the Gla domains of factors IX and X, as we have recently shown (Atoda et al., 1994), and at pH 7.5 the half-maximal binding occurs at 0.6 mM Ca^{2+} ions (Figure 2). This concentration of Ca^{2+} ions is similar to the average K_d values of the Gla domains of the coagulation factors (i.e., about half of a population of factor IX or X molecules is associated with Ca^{2+} ions at this concentration), while both Ca^{2+}-binding sites of IX/X-bp should be already filled under these conditions (see Figure 3A). It appears that IX/X-bp recognizes the conformations of Gla domains with bound Ca^{2+} ions in factors IX and X. Furthermore, the experiments conducted at pH 6.5 suggest that the IX/X-bp's two Ca^{2+}-binding sites also must be filled to ensure binding to coagulation factors since the Ca^{2+}-binding property of factor IX is essentially the same at pH 6.5 and pH 7.5 (Amphlett et al., 1978; this feature is the major reason that we chose these two pH values), whereas the affinity of the low-affinity site of IX/X-bp is very much weakened at pH 6.5 and the required Ca^{2+} concentration for the binding of IX/X-bp to coagulation factors is increased by a factor of approximately 10 (this study).

In conclusion, we have shown here that (i) IX/X-bp recognizes Ca^{2+}-bound conformations of Gla domains in factors IX and X, (ii) IX/X-bp has two independent Ca^{2+}-binding sites, and (iii) occupation of both sites by Ca^{2+} ions and, presumably, subsequent conformational rearrangement are also essential for the recognition of coagulation factors. At present, it is still unclear how Ca^{2+} ions mediate the association of IX/X-bp and coagulation factors: do they act as "bridges" between the two proteins, or do they alter the conformations of these proteins so as to make "a lock and key"? This issue requires further investigation, and our ongoing X-ray crystallographic approach appears promising in this regard. Nonetheless, the information presented here should be valuable for the application of this interesting protein as a convenient tool in the elucidation of structures and functions of coagulation factors as well as for the exploration of the complex mechanisms of the coagulation cascade which involves various protein/protein and protein/metal interactions.

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