Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 152–156, January 1992
Biochemistry

Antibody-probed conformational transitions in the protease domain of human factor IX upon calcium binding and zymogen activation: Putative high-affinity Ca^{2+}-binding site in the protease domain

(blood coagulation enzymes/serine proteases/comparative molecular modeling/Ca^{2+}-binding surface loop/factor VIIIa)

S. PAUL BAJAJ*†, ARUN K. SABHARWAL*, JOHN GORKA‡, AND JENS J. BIRKTOFT§

*Departments of Medicine, Biochemistry and Pathology, St. Louis University School of Medicine, St. Louis, MO 63104; and ‡the Howard Hughes Medical Institute and §Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110

Communicated by Carl Frieden, September 27, 1991 (received for review May 6, 1991)

ABSTRACT **The Fab fragment of a monoclonal antibody (mAb) reactive to the N-terminal half (residues 180–310) of the protease domain of human factor IX has been previously shown to inhibit the binding of factor IXa to its cofactor, factor VIIIa. These data suggested that this segment of factor IXa may participate in binding to factor VIIIa. We now report that the binding rate (k_{on}) of the mAb is 3-fold higher in the presence of Ca^{2+} than in its absence for both factors IX and IXa; the half-maximal effect was observed at ≈300 μM Ca^{2+}. Furthermore, the off rate (k_{off}) of the mAb is 10-fold higher for factor IXa than for factor IX with or without Ca^{2+}. Moreover, like the k_{on} for mAb binding, the incorporation of dansyl-Glu-Gly-Arg chloromethyl ketone (dEGR-CK) into factor IXa was ≈3 times faster in the presence of Ca^{2+} than in its absence. Since steric factors govern the k_{on} and the strength of noncovalent interactions governs the k_{off}, the data indicate that the region of factor IX at residues 180–310 undergoes two separate conformational changes before expression of its biologic activity: one upon Ca^{2+} binding and the other upon zymogen activation. Furthermore, the dEGR-CK incorporation data suggest that both conformational changes also affect the active site residues. Analyses of the known three-dimensional structures of serine proteases indicate that in human factor IX a high-affinity Ca^{2+}-binding site may be formed by the carboxyl groups of glutamates 235 and 245 and by the main chain carbonyl oxygens of residues 237 and 240. In support of this conclusion, a synthetic peptide including residues 231–265 was shown to bind Ca^{2+} with a K_d of ≈500 μM. This peptide also bound to the mAb, although with ≈500-fold reduced affinity. Moreover, like factor IX, the peptide bound to the mAb more strongly (≈3-fold) in the presence of Ca^{2+} than in its absence. Thus, it appears that a part of the epitope for the mAb described above is contained in the proposed Ca^{2+}-binding site in the protease domain of human factor IX. This proposed site is analogous to the Ca^{2+}-binding site in trypsin and elastase, and it may be involved in binding factor IXa to factor VIIIa.**

Factor IX is a vitamin K-dependent protein that shares sequence homology with other serine protease zymogens of its class. The gene for human factor IX consists of eight exons that code for a leader sequence of 46 amino acids and a mature protein of 415 amino acids (1). A number of processing events occur during biosynthesis of the functional protein. These include cleavage and removal of the leader polypeptide, γ-carboxylation of the first 12 glutamate residues, partial β-hydroxylation of Asp-64, and glycosylation of one serine and two asparagine residues (1, 2). The resultant factor IX protein circulates in blood as a zymogen of M_r 57,000 (3). Upon activation by factor XIa/Ca^{2+} or by factor VIIa/Ca^{2+}/tissue factor, two peptide bonds (Arg-145–Ala-146 and Arg-180–Val-181) are cleaved to yield a two-chain disulfide-linked serine protease, factor IXa, and a 35-residue activation peptide (3, 4). Factor IXa thus formed converts factor X to factor Xa in the coagulation cascade; for a physiologically significant rate, this reaction requires Ca^{2+}, phospholipid, and factor VIIIa (5, 6).

The genomic structure of factor IX and its amino acid sequence strongly indicate that the protein is organized into several distinct domains (1). These include the N-terminal γ-carboxyglutamic acid (Gla)-rich domain, two consecutive epidermal growth factor (EGF)-like domains, a connecting activation peptide region, and the C-terminal serine protease domain (1). Existing evidence suggests that the Gla and the EGF domain(s) mediate Ca^{2+} and phospholipid binding to the protein (6), while the EGF domain(s) and the protease domain participate in factor VIIIa binding (7–9).

Detailed Ca^{2+}-binding properties of both bovine and human factor IX have been investigated. The cumulative evidence suggests that factor IX contains two high-affinity Gla-independent (10, 11) and several weak Gla-dependent Ca^{2+}-binding sites (12–14). Of the two Gla-independent binding sites, one is located in the first EGF domain of the protein (15). The present studies were undertaken to determine the location of the other high-affinity Ca^{2+}-binding site and its possible role in factor VIIIa binding. A preliminary account of this work has been presented in abstract form (16).

MATERIALS AND METHODS

Materials. Human factors IX and XI were isolated according to published procedures (17, 18). Factor XIa was prepared as described (14). ^{125}I-labeled tyrosyl factor IX was prepared by using Bio-Rad Enzymobead reagent as described for prothrombin (19). The radiospecific activity of the preparation was 1.1×10^9 cpm per mg of protein, and it retained >90% of the biologic activity of the unlabeled control. Factor IXa and ^{125}I-labeled factor IXa were prepared as detailed previously (9). Purification of a mouse monoclonal antibody (mAb) that inhibits the interaction of factor IXa with factor VIIIa has also been reported (9). All protein preparations were at least 95% homogenous as judged by SDS gel electrophoresis (20). The active site histidine reagent, dansyl-Glu-Gly-Arg chloromethyl ketone (dEGR-CK) was purchased from Calbiochem. *Staphylococcus* protein A rabbit anti-mouse immunoglobulin suspension was prepared as described (9). ^{125}I-labeled goat anti-mouse IgG (specific activ-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Gla, γ-carboxyglutamic acid; EGF; epidermal growth factor; dEGR-CK, dansyl-Glu-Gly-Arg chloromethyl ketone; mAb, monoclonal antibody.

†To whom reprint requests should be addressed at: Hematology Division, St. Louis University Medical Center, 3635 Vista Avenue at Grand Boulevard, P.O. Box 15250, St. Louis, MO 63110-0250.

ity, 250 μCi/ml; 1 Ci = 37 GBq) was obtained from ICN. The details of the immunosorbent assay for binding of factor IX or factor IXa to the mAb have been described (9).

Incorporation of dEGR-CK into Human Factor IXa. Factor IXa (final concentration, 2 μM) in 0.05 M Tris·HCl/0.15 M NaCl, pH 7.4 (Tris/NaCl) (containing 5 mM Ca^{2+} or 1 mM EDTA) was incubated with 14, 28, or 56 μM dEGR-CK at room temperature. Aliquots of 10 μl were removed at different times and diluted 1:1000 or higher in ice-cold Tris/NaCl/bovine serum albumin (1 mg/ml) buffer. The samples were then immediately assayed for factor IXa activity in a one-stage partial thromboplastin time assay using factor IX-deficient plasma as described (21). Apparent second-order rate constants were obtained by standard techniques (e.g., see Figs. 1 and 2).

Immunologic Dot Blots. The samples (300 μl) in Tris/NaCl buffer containing 30 μg of bovine serum albumin per ml were applied to the nitrocellulose filters, and the filters were incubated in the blocking buffer (5% nonfat dry milk in Tris/NaCl) for 1 h at room temperature on an orbital shaker. The blocking buffer was removed and the filters were incubated overnight in Tris/NaCl buffer containing 1% nonfat dry milk and 5 μg of the mAb per ml. The filters were then washed extensively with Tris/NaCl and incubated for 1 h with ^{125}I-labeled goat anti-mouse IgG (1 μCi/ml) in Tris/NaCl containing 1% nonfat dry milk. The filters were washed extensively with Tris/NaCl buffer, air-dried, and exposed to Kodak X-AR5 film at −70°C with one intensifying screen for 15 d. When mAb binding in the presence of Ca^{2+} was investigated, all buffers contained 5 mM Ca^{2+}. The intensity of each blot was quantitated by using an LKB Ultroscan XL laser densitometer.

Synthesis of Factor IX Peptides. Two peptides corresponding to sequence positions 231–265 (Val-Val-Ala-Gly-Glu-His-Asn-Ile-Glu-Glu-Thr-Glu-His-Thr-Glu-Gln-Lys-Arg-Asn-Val-Ile-Arg-Ile-Ile-Pro-His-His-Asn-Tyr-Asn-Ala-Ala-Ile-Asn-Lys) and 276–307 (Asp-Glu-Pro-Leu-Val-Leu-Asn-Ser-Tyr-Val-Thr-Pro-Ile-Cys-Ile-Ala-Asp-Lys-Glu-Tyr-Thr-Asn-Ile-Phe-Leu-Lys-Phe-Gly-Ser-Gly-Tyr-Val) of human factor IX were made by solid-phase peptide synthesis using the Applied BioSystems Synthesizer (model 431A). The peptides were purified (≥90%) by reverse-phase HPLC as outlined (22). Peptide concentrations were determined by using the molar extinction coefficient of 2390 at 293 nm for tyrosine in 0.1 M NaOH (23).

Measurements with a Ca^{2+}-Specific Electrode. Calcium ion activity was determined by using a Ca^{2+}-specific electrode and a model 601A/digital Ionalyzer (Orion Research). Titrations of the peptide solutions (500 μM) in 6 ml of buffer were performed by adding small increments (10–20 μl) of 100 mM $CaCl_2$ at room temperature. In these titrations, peptide-bound Ca^{2+} was taken as the difference between the measured free Ca^{2+} concentration and the total added.

Molecular Modeling of Protease Domain of Factor IXa. Structural analysis and molecular graphics model building was done on silicon graphics 4D20G and 4DGT50 systems using the graphics program packages TOM and TURBO-FRODO (24). Molecular dynamics and refinement calculations were performed by using the XPLOR program of Brünger (25) run on the same silicon graphics system. The putative model of the serine protease domain of human factor IXa was constructed by using a knowledge-based comparative model building approach (26). Although the substrate specificity of factor IXa resembles that of trypsin, we selected chymotrypsin (Brookhaven National Laboratory; code 5CHA, subunit B) (27, 28) as the starting model template since the distribution of cysteine bridges in factor IXa is more similar to those in chymotrypsin and fewer insertions are necessary to align the sequences. However, the amino acid sequences of factor IXa in those regions of its protease domain that are predominantly responsible for its primary substrate specificity are more similar to the corresponding sequences in trypsin and kallikrein. Therefore, the structures of trypsin and kallikrein (codes 2PTC and 2KAI) were used as templates for this part of the factor IXa protease domain. Similarly, the structures of trypsin and elastase (codes 2PTC and 3EST) provided the templates for the factor IXa region near the putative calcium binding site.

RESULTS

Binding of the mAb to factor IX or IXa was measured by an immunosorbent technique described earlier (9). The observed first-order rate constants for the reaction of factor IX plus EDTA or Ca^{2+} (Fig. 1 *A* and *B*) and factor IXa plus EDTA or Ca^{2+} (Fig. 2 *A* and *B*) were plotted as a function of mAb concentration (Fig. 1 *C* and *D* and Fig. 2 *C* and *D*). The slopes and the *y* intercepts of these plots yielded the association (k_{on}) and the dissociation (k_{off}) rate constants, respectively. The rate constants are listed in Table 1. The k_{off} is ≈10-fold higher for factor IXa than for factor IX in the absence or presence of saturating concentrations of Ca^{2+}. However, the k_{on} is ≈3-fold higher in the presence of Ca^{2+} than in its absence and is the same for both factors IX and IXa. Similarly, the incorporation of dEGR-CK into factor IXa was ≈3-fold higher in the presence of Ca^{2+} than in its absence (Table 1).

Since the spatial arrangement of the atoms (steric factors) governs the approach (i.e., k_{on}) of interacting species and the strength of the noncovalent bonds formed governs the dissociation (k_{off}) of the complex into individual reactants, our rate data indicate that factor IX must go through two separate conformational changes in the residue 180–310 region of the protease domain: one upon Ca^{2+} binding and the other upon conversion to factor IXa. Based on the dEGR-CK incorpo-

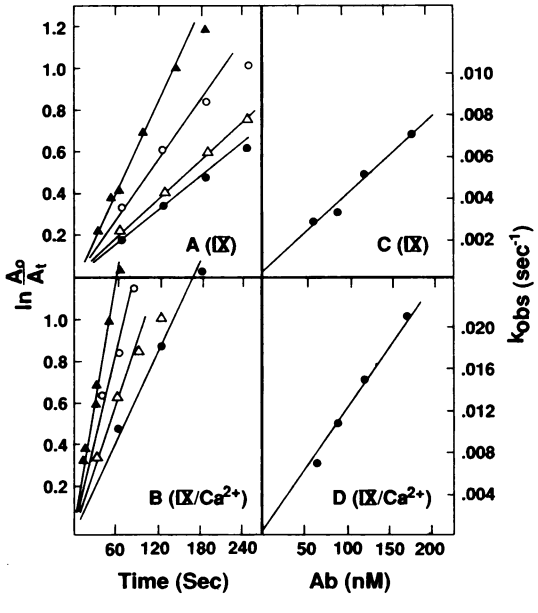

FIG. 1. Effect of saturating concentrations of Ca^{2+} on the association (k_{on}) and dissociation (k_{off}) rate constants for the interaction of factor IX with the mAb. First-order kinetic plots obtained in the presence of 1 mM EDTA are shown in *A* and those obtained in the presence of 5 mM Ca^{2+} are shown in *B*. For both *A* and *B*, the concentration of ^{125}I-labeled factor IX used was 11.4 nM. The concentrations of mAb were as follows: ●, 57 nM; △, 85.5 nM; ○, 114 nM; ▲, 171 nM. A_o, initial concentration of ^{125}I-labeled factor IX; A_t, unreacted factor IX at a given time. (*C*) Observed first-order rate constants plotted against mAb concentration for the reactions in 1 mM EDTA. (*D*) Observed first-order rate constants plotted against mAb concentration for the reactions in 5 mM Ca^{2+}.

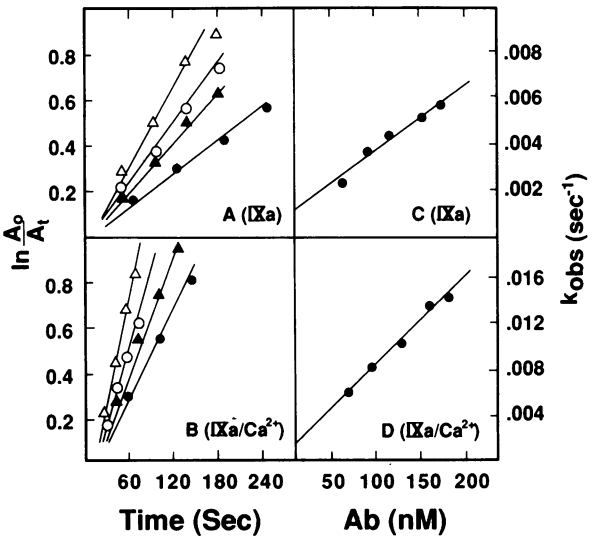

FIG. 2. Effect of saturating concentrations of Ca^{2+} on the association (k_{on}) and dissociation (k_{off}) rate constants for the interaction of factor IXa with the mAb. First-order kinetic plots obtained in the presence of 1 mM EDTA are shown in *A* and those obtained in the presence of 5 mM Ca^{2+} are shown in *B*. For both *A* and *B*, the concentration of ^{125}I-labeled factor IXa used was 11.4 nM. The concentrations of mAb were as follows: ●, 57 nM; ▲, 85.5 nM; ○, 114 nM; △, 171 nM. (*C*) Observed first-order rate constants plotted against mAb concentration for the reactions in 1 mM EDTA. (*D*) Observed first-order rate constants plotted against mAb concentration for the reactions in 5 mM Ca^{2+}.

ration data, the binding of Ca^{2+} also appears to affect the active site residues. If, as has been implicated in previous studies (9), the protease domain of factor IXa contains a binding site for factor VIIIa, then the observed conformational changes during Ca^{2+} binding and zymogen activation are a prerequisite for satisfactory binding of factor VIIIa to factor IXa.

Next, we investigated the dependence of observed first-order rate constants for the binding of factors IX and IXa to the mAb as a function of Ca^{2+} concentration (Fig. 3). The half-maximal increase in the observed rate constant for factor IX was at 300 μM Ca^{2+} (Fig. 3*A*) and for factor IXa it was at 250 μM Ca^{2+} (Fig. 3*B*). Thus, the Ca^{2+}-induced conformational change noted involving residues 180–310 is due to the occupancy of a high-affinity Ca^{2+}-binding site(s) in human factor IX.

The mAb is known to be directed against residues 180–310 of factor IX (9, 29). Additional information as to which residues in factor IX participate in binding to the mAb may be obtained from an analysis of a putative molecular model of the protease domain of factor IX. First, residues 192–199, 207–211, 216–220, 228–231, 233–234, 269–273, 288–290, and

Table 1. Association (k_{on}) and dissociation (k_{off}) rate constants for binding of factor IX or factor IXa to the mAb in the presence of Ca^{2+} or EDTA

Protein	k_{on}, $M^{-1}\cdot sec^{-1}$	k_{off}, sec^{-1}	Rate constant, for dEGR-CK incorporation, $M^{-1}\cdot min^{-1}$
Factor IX/EDTA	4×10^4	1.9×10^{-4}	—
Factor IX/Ca^{2+}	1.2×10^5	1.7×10^{-4}	—
Factor IXa/EDTA	3.1×10^4	1.5×10^{-3}	5.1×10^3
Factor IXa/Ca^{2+}	1.0×10^5	2.0×10^{-3}	1.4×10^4

The apparent second-order rate constants for incorporation of dEGR-CK into human factor IXa were obtained in the presence of 5 mM Ca^{2+} or 1 mM EDTA.

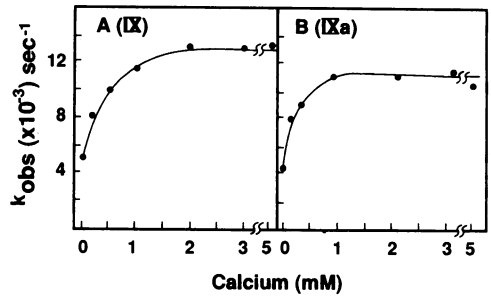

FIG. 3. Reaction of factor IX or IXa with the mAb at different concentrations of Ca^{2+}. Observed first-order rate constants for factor IX (*A*) and for factor IXa (*B*) are plotted against various concentrations of Ca^{2+}. ^{125}I-labeled factor IX (or ^{125}I-labeled factor IXa) concentration was 11.4 nM and the mAb binding site concentration was 114 nM. Ca^{2+} concentrations were as indicated.

307–310 are not located on the surface of the protein. Thus, these residues are not expected to be involved in direct interactions with the mAb. Second, the activation of factor IX to factor IXa is not impaired by this mAb (9). This observation eliminates residues 181–190, if one assumes that the conformation of factor IX near the activation site resembles that of trypsinogen and chymotrypsinogen. Third, the inhibition of factor IXa by antithrombin III is not affected by this mAb (9). Using the structure of ovalbumin (30) as a guide and following the general principles observed in the structures of complexes of the proteases and their inhibitors, a putative model of factor IXa–antithrombin III was constructed (J.J.B., unpublished data). From this model, it was inferred that residues 200–206, 221–227, and 261–266 of factor IXa would be in contact with antithrombin III and not involved in mAb binding. The above considerations thus leave residue segments 231–265 and 276–307 of factor IX as the most plausible candidates for containing the epitope recognized by the mAb.

The residue 231–265 peptide was found to contain one Ca^{2+}-binding site (Fig. 4). In the standard Tris/NaCl buffer, the peptide bound Ca^{2+} with positive cooperativity. However, this cooperativity was abolished when Ca^{2+}-binding measurements were made in 1.0 M NaCl. A simple explanation for this observation may be that, at a low salt concentration, the peptide exists as a dimer, which binds Ca^{2+} with reduced affinity. And upon binding of one atom of Ca^{2+}, the dimeric peptide dissociates into the monomeric form, which binds Ca^{2+} with high affinity. A K_d value of 500 μM for the

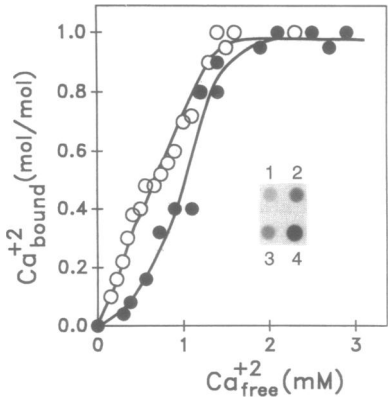

FIG. 4. Binding of Ca^{2+} to the peptide corresponding to residues 231–265 of human factor IX. The buffer used was either 0.05 M Tris·HCl/0.15 M NaCl, pH 7.5 (●) or 0.05 M Tris·HCl/1.0 M NaCl, pH 7.5 (○). (*Inset*) Immunodot blot analysis of various samples: 1, 30 pmol (120 ng) of residue 231–265 peptide in 1 mM EDTA; 2, 0.2 pmol (12 ng) of human factor IX in 1 mM EDTA; 3, 30 pmol of residue 231–265 peptide in 5 mM Ca^{2+}; 4, 0.2 pmol of factor IX in 5 mM Ca^{2+}.

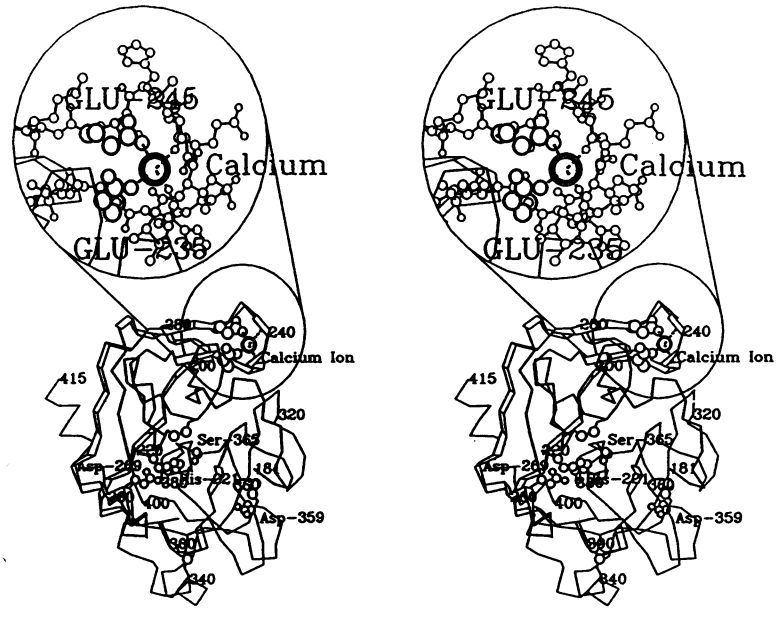

FIG. 5. Stereo diagram depicting a putative model of the protease domain of human factor IXa. A Cα model and a few selected side chains are shown. These include the charge relay system residues: Asp-269[102], His-221[57], and Ser-365[195], and the residue Asp-359[189] located in the substrate binding pocket. Also included in the figure are the Ca^{2+} and the two glutamic acid residues that are proposed to act as calcium ligands. The part of the Cα model that includes the two peptide segments is shown as a ribbon, and the remaining part of the 181[15]–310[141] segment is shown in heavy lines. The last half of the protease domain is shown in thin lines. The three cysteine bridges are shown as two balls connected with thin lines to the Cα model. The N and C termini and every 20th residue are labeled at their Cα positions. The large circle shows a magnified image of the environment around the calcium ion. All atoms in residues 232[67]–248[83] are shown as ball and stick models. Ligand bonds between the calcium ion and the carbonyl oxygens of residues 237[72] and 240[75] and the carboxylates of Glu-235[70] and Glu-245[80] are shown as dotted lines. The view of the model is the one commonly used for presentation of α-chymotrypsin (27).

peptide–Ca^{2+} interaction was calculated in the presence of 1.0 M NaCl. This peptide was also found to bind to the mAb, although with ≈500-fold reduced affinity (Fig. 4 *Inset*). Moreover, like factor IX, this peptide also bound to the mAb more strongly (≈3-fold) in the presence of Ca^{2+} than in its absence (Fig. 4 *Inset*). The residue 276–307 peptide was found to bind neither Ca^{2+} nor the mAb.

DISCUSSION

The data presented in the current study clearly establish the existence of a high-affinity Ca^{2+}-binding site in the residue 231–265 segment of the protease domain of factor IX. Furthermore, a determinant(s) for the mAb is also located in this segment of the protein. Thus, the mAb is reactive to a Ca^{2+}-sensitive epitope; this concept is strongly supported by our data presented in Figs. 1–3.

Two serine proteases of known three-dimensional structures—namely, trypsin and elastase—contain Ca^{2+}-binding sites (31, 32). In both structures the Ca^{2+}-binding site is contained in a surface loop that is formed by residues 234[69]–246[81].¶ The residues in this loop connect two adjacent strands in the antiparallel β-sheet structure that forms the first domain in the chymotrypsin-like proteases. All the calcium ligands are provided by residues in this loop. They are the side chain carboxyl oxygens of Glu-235[70] and Glu-245[80] and the main chain carbonyl oxygens of residues 237[72] and 240[75]. Additional calcium ligands, in elastase, are the side chain of residue 239[74] and a solvent molecule, while in trypsin they are the two solvent molecules. The above surface loop, referred to as the calcium binding loop, is further stabilized by hydrogen bonds between the main chain amide groups of residues 242[77] and 243[78] and the carboxyl group of residue 245[80].

The serine protease domain of factor IX is homologous to trypsin and elastase. Furthermore, the residue 231–265 peptide of factor IX contains the above-described Ca^{2+}-binding surface loop in trypsin and elastase. This peptide in the present study (Fig. 4) was found to bind one calcium ion with high affinity. Furthermore, the amino acid sequence of the putative calcium binding loop in the protease domain of factor IX could be incorporated into the three-dimensional structure of trypsin or elastase without any serious difficulty. The only problem occurs at His-243[Gly-78], which in most reported trypsin structures assumes a conformation compatible with only a glycine residue. However, in a recent report (33) an alternative conformation has been described that permits any residue at position 243[78]. The difference between the two trypsin structures is the orientation of the peptide bond between residues 243[78] and 244[79]. From these considerations, we predict that a Ca^{2+}-binding site in the protease domain of factor IX is formed by the carboxyl groups of glutamates 235[70] and 245[80] and the main chain carbonyl oxygens of residues 237[72] and 240[75]. A putative model for the protease domain of human factor IXa, which includes the Ca^{2+}-binding site, is shown in Fig. 5.

A characteristic feature of all proteases in which it has been demonstrated that the above-described surface loop binds calcium is an abundance of acidic and uncharged polar residues and a concomitant absence of lysine and arginine residues. In addition to directly donating ligands to the calcium ion, the acidic residues will create an electronegative field that would attract the positively charged metal ion. On the other hand, basic residues would create an electropositive field that would have the opposite effect in attracting the calcium ion. Thus, trypsin contains four acidic and three polar residues, and elastase contains two acidic and seven polar residues (31, 32). Similarly, human factor IX contains five acidic and five polar residues in this loop (1). Human

¶For comparison, the factor IX amino acid numbering system has been used. The numbers in brackets refer to the chymotrypsinogen numbering system.

factor VII contains six acidic, including Glu-235[70] and Glu-245[80], and three polar residues (34), and on the basis of these considerations we predict that it contains a Ca^{2+}-binding site in this loop. Human protein C has glutamates at positions 235[70] and 245[80] but contains two arginines as well as two bulky tryptophans in this loop (35). Although factor X has glutamate at position 245[80], it has aspartate (instead of glutamate) at position 235[70] and an arginine at position 236[71] (36). On this basis, we predict that both factor X and protein C do not contain a Ca^{2+}-binding site in this loop. Our prediction is consistent with the experimental data, since Gla domainless protein C or factor X contains only one Ca^{2+}-binding site, which in all probability is located in the EGF-1 domain of each of these proteins (refs. 37 and 38; A.K.S., unpublished data). Similarly, thrombin has lysine (39) at position 235[70] and thus is predicted not to have a Ca^{2+}-binding site in this loop; this prediction is also consistent with our earlier experimental data (40).

Guinea pig factor IX has lysine at position 235[70] instead of the glutamic acid found in other species (41). Thus, it is predicted that it will not have a Ca^{2+}-binding site in this loop. Moreover, as has been reported for thrombin, which also has lysine at position 235 (42), an ionic bond between Lys-235[70] and Glu-245[80] may stabilize an active conformation in this region of guinea pig factor IX; this could replace the function of Ca^{2+}, which is thought to bridge Glu-235[70] and Glu-245[80] residues.

Recently, several investigators have attempted to investigate the functional role of this surface loop in coagulation proteases and have inferred that this loop may serve as part of a binding site for protein cofactors in these proteases. For factor IXa, this protein cofactor is factor VIIIa, for factor Xa it is factor Va, for factor VIIa it is tissue factor, and for thrombin it is thrombomodulin (5, 6). The observations that factor VIIIa binding to factor IXa is blocked by the Fab fragment of a mAb that binds to the peptide containing the calcium binding loop and that binding of the antibody to both the peptide and factor IX is stimulated by Ca^{2+} suggest that this loop region is likely to be involved in factor VIIIa binding (9). Similarly, in preliminary studies, peptides from factor Xa corresponding to residues containing the loop region have been reported to prevent the interaction of factors Va and Xa (43); this suggests that the loop region of the protease domain of factor Xa contains at least a part of the binding site for factor Va. Furthermore, Fair and coworkers (44) and Kisiel and coworkers (45) have provided initial evidence that factor VII residues corresponding to this surface loop including the N-terminal adjacent residues (220[55]–243[78]) are involved in binding of factor VII to tissue factor. Finally, it has recently been reported that in thrombin residues 238[73] and 240[75] are involved in its interaction with thrombomodulin (46). Thus, an overall conclusion to be reached from these observations is that in all of these coagulation proteases a part of the binding site for the requisite cofactor is contained in this surface loop common to all proteases.

We thank Dr. K. G. Mann for useful discussions and Steve Maki for technical assistance. The study was supported by Grants HL 36365 and 30572 (Project 5) to S.P.B. and the Lucille P. Markey Charitable Trust supporting the Markey Center for Research in Molecular Biology of Human Disease at Washington University to J.J.B. A.K.S. is supported by Training Grant HL 07050. S.P.B. is an American Heart Association-Bristol Myers Thrombosis Grant Recipient.

1. Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W. & Kurachi, K. (1985) *Biochemistry* **24**, 3736–3750.
2. Nishimura, K., Kawabata, S., Kisiel, W., Hase, S., Ikenaka, T., Takao, T., Shimonishi, Y. & Iwanaga, S. (1989) *J. Biol. Chem.* **264**, 20320–20325.
3. DiScipio, R. G., Kurachi, K. & Davie, E. W. (1978) *J. Clin. Invest.* **61**, 1528–1538.
4. Bajaj, S. P., Rapaport, S. I. & Russell, W. A. (1983) *Biochemistry* **22**, 4047–4053.
5. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P. & Krishnaswamy, S. (1990) *Blood* **76**, 1–16.
6. Furie, B. & Furie, B. C. (1988) *Cell* **53**, 505–518.
7. Rees, D. J. G., Jones, I. M., Handford, P. A., Walter, S. J., Esnouf, M. P., Smith, K. J. & Brownlee, G. G. (1988) *EMBO J.* **7**, 2053–2061.
8. Huber, P., Ben-Tal, O., Gilbert, G. E., Furie, B. C. & Furie, B. (1990) *Circulation* **82**, Suppl. 3, 1449 (abstr.).
9. Bajaj, S. P., Rapaport, S. I. & Maki, S. L. (1985) *J. Biol. Chem.* **260**, 11574–11580.
10. Morita, T. & Kisiel, W. (1985) *Biochem. Biophys. Res. Commun.* **133**, 417–422.
11. Morita, T., Isaacs, B. S., Esmon, C. T. & Johnson, A. E. (1984) *J. Biol. Chem.* **259**, 5698–5704, and erratum (1985) **260**, 2583.
12. Amphlett, G. W., Byrne, R. & Castellino, F. J. (1978) *J. Biol. Chem.* **253**, 6774–6779.
13. Amphlett, G. W., Kisiel, W. & Castellino, F. J. (1981) *Arch. Biochem. Biophys.* **208**, 576–585.
14. Bajaj, S. P. (1982) *J. Biol. Chem.* **257**, 4127–4132.
15. Handford, P. A., Baron, M., Mayhew, M., Willis, A., Beesley, T., Brownlee, G. G. & Campbell, I. D. (1990) *EMBO J.* **9**, 475–480.
16. Bajaj, S. P., Maki, S. L. & Birktoft, J. J. (1990) *Circulation* **82**, Suppl. 3, 1450 (abstr.).
17. Bajaj, S. P., Rapaport, S. I. & Prodanos, C. (1981) *Prep. Biochem.* **11**, 397–412.
18. Kurachi, K. & Davie, E. W. (1977) *Biochemistry* **16**, 5831–5839.
19. Bajaj, S. P., Rapaport, S. I., Prodanos, C. & Russell, W. A. (1981) *Blood* **58**, 886–891.
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
21. Spitzer, S. G., Kuppuswamy, M. N., Saini, R., Kasper, C. K., Birktoft, J. J. & Bajaj, S. P. (1990) *Blood* **76**, 1530–1537.
22. Gorka, J., McCourt, D. W. & Schwartz, B. D. (1989) *Pep. Res.* **2**, 376–380.
23. Goodwin, T. W. & Morton, R. A. (1946) *Biochem. J.* **40**, 628–632.
24. Cambillau, C. & Horjales, E. (1987) *J. Mol. Graph.* **5**, 174–177.
25. Brünger, A. T. (1988) *J. Mol. Biol.* **203**, 803–816.
26. Bajaj, S. P., Spitzer, S. G., Welsh, W. J., Warn-Cramer, B. J., Kasper, C. K. & Birktoft, J. J. (1990) *J. Biol. Chem.* **265**, 2956–2961.
27. Birktoft, J. J. & Blow, D. M. (1972) *J. Mol. Biol.* **68**, 187–240.
28. Blevins, R. A. & Tulinsky, A. (1985) *J. Biol. Chem.* **260**, 4264–4275.
29. Frazier, D., Smith, K. J., Cheung, W.-F., Ware, J., Lin, S.-W., Thompson, A. R., Reisner, H., Bajaj, S. P. & Stafford, D. W. (1989) *Blood* **74**, 971–977.
30. Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. G. & Carrell, R. W. (1990) *Nature (London)* **347**, 99–102.
31. Bode, W. & Schwager, P. (1975) *FEBS Lett.* **56**, 139–143.
32. Meyer, E., Cole, G., Radhakrishnan, R. & Epp, O. (1988) *Acta Crystallogr. Sec. B: Struct. Crystallogr.* **44**, 26–39.
33. Mangel, W. F., Singer, P. T., Cyr, D. M., Umland, T. C., Toledo, D. L., Stroud, R. M., Pflugrath, J. W. & Sweet, R. M. (1990) *Biochemistry* **29**, 8351–8357.
34. Thim, L., Bjoern, S., Christensen, M., Nicolaisen, E. M., Lund-Hansen, T., Pedersen, A. H. & Hedner, U. (1988) *Biochemistry* **27**, 7785–7793.
35. Foster, D. & Davie, E. W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4766–4770.
36. Leytus, S. P., Foster, D. C., Kurachi, K. & Davie, E. W. (1986) *Biochemistry* **25**, 5098–5102.
37. Sugo, T., Björk, I., Holmgren, A. & Stenflo, J. (1984) *J. Biol. Chem.* **259**, 5705–5710.
38. Johnson, A. E., Esmon, N. L., Laue, T. M. & Esmon, C. T. (1983) *J. Biol. Chem.* **258**, 5554–5560.
39. Mann, K. G., Elion, J., Butkowski, R. J., Downing, M. & Nesheim, M. E. (1981) *Methods Enzymol.* **80**, 286–302.
40. Bajaj, S. P., Butkowski, R. J. & Mann, K. G. (1975) *J. Biol. Chem.* **250**, 2150–2156.
41. Sarkar, G., Koeberl, D. D. & Sommer, S. S. (1990) *Genomics* **6**, 133–143.
42. Bode, W., Mayer, I., Baumann, U., Huber, R., Stone, S. R. & Hofsteenge, J. (1989) *EMBO J.* **8**, 3467–3475.
43. Chattopadhyay, A. & Fair, D. S. (1989) *Blood* **74**, 291 (abstr. 1102).
44. Kumar, A., Blumenthal, D. K. & Fair, D. S. (1989) *Blood* **74**, 95 (abstr. 352).
45. Wildgoose, P., Kazim, A. L. & Kisiel, W. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7290–7294.
46. Wu, Q. Y., Sheehan, J. P., Tsiang, M., Lentz, S. R., Birktoft, J. J. & Sadler, J. E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6775–6779.