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Comparison of Lipid Binding and Kinetic Properties of Normal, Variant, and γ-Carboxyglutamic Acid Modified Human Factor IX and Factor IX_a†

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Received May 16, 1985

ABSTRACT: The abilities of normal and three abnormal factor IX_a molecules to activate factor X and to bind to phospholipid membranes have been compared to define the contributions of protein–lipid interactions and factor IX_a light chain–heavy chain interactions to the functioning of this protein. The abnormal proteins studied had altered amino acid residues in their light chains. The heavy-chain regions, containing the active site serine and histidine residues, were normal in the abnormal proteins on the basis of titration by antithrombin III. The binding constants (K_d) for normal (N), variant [Chapel Hill (CH) and Alabama (AL)], and γ-carboxyglutamic acid (Gla) modified (MOD) factors IX and IX_a to phosphatidylserine (PS)/phosphatidylcholine (PC) small, unilamellar vesicles (SUV) were measured by 90° light scattering. The K_d values for factor IX_N binding were quite sensitive to the PS content of the membrane but less sensitive to Ca^{2+} concentrations between 0.5 and 10 mM. The zymogen and activated forms of both normal and abnormal factor IX bound with similar affinities to PS/PC (30/70) SUV. In the cases of factor IX_{aN} and factor IX_{aAL}, but not factor IX_{aCH} or factor IX_{aMOD}, irreversible changes in scattering intensity suggested protein-induced vesicle fusion. Since the activation peptide is not released from factor IX_{aCH}, the normal interaction of factor IX_a with a membrane must require the release of the activation peptide and the presence of intact Gla residues. The rate of factor X activation by normal and abnormal factor IX_a was obtained by using a chromogenic substrate for factor X_a in the presence of PS/PC (30/70) SUV and 5 mM Ca^{2+}. A comparison of the relative amount of surface-bound factor IX_a (calculated with the measured K_d values) and the relative rates of factor X activation indicated that only in the case of factor IX_{aMOD} did decreased lipid binding account for decreased activity of the abnormal proteins. Because the structural alterations of the variant proteins are in the light chain and because decreased lipid affinity did not account for decreased activity, results suggest that the proper functioning of factor IX_a must entail interactions between the light and heavy chains on the phospholipid surface.

Human factor IX is a vitamin K dependent glycoprotein that, during the process of blood coagulation, is activated in the presence of Ca^{2+} by factor XI_a and/or factor VII_a and tissue factor (Bajaj et al., 1983). In turn, activated factor IX (factor IX_a)[1] catalyzes the activation of factor X, with Ca^{2+}, factor $VIII_a$, and a negatively charged phospholipid surface serving as cofactors (van Dieijen et al., 1981). The complete activation of human factor IX to factor IX_a involves the cleavage of two specific peptide bonds (see Figure 1). These two proteolytic events result in the release of an activation peptide and in the appearance of light and heavy peptide chains linked by a disulfide bridge (Fujikawa et al., 1974; Østerud

† This work was in part supported by USPHS Grants GM 32707 (B.R.L.), HL 32656 (M.J.G.), HL 06350 (M.J.G. and H.R.R.), and HL 07149-07 (D.M.M.).

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[1] Abbreviations: factor IX_a, activated factor IX; factor IX_{CH}, factor $IX_{Chapel\ Hill}$; factor IX_{AL}, factor $IX_{Alabama}$; factor IX_{MOD}, Gla-modified factor IX; factor IX_N, normal factor IX; PS, bovine brain phosphatidylserine; PC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; Gla, γ-carboxyglutamic acid; SUV, small unilamellar vesicles; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol); TEA, triethanolamine; Tos-Gly-Pro-Arg-NA, N^{α}-*p*-tosylglycyl-L-prolyl-L-arginine-*p*-nitroanilide.

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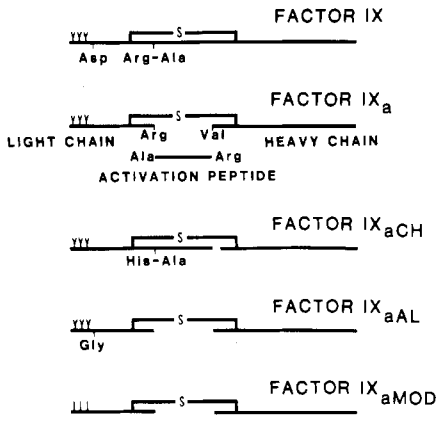

FIGURE 1: Schematic structures of normal factor IX and factor IX_a and abnormal factor IX_a. Normal activation of human factor IX involves two cleavages (Arg_{145}–Ala_{146} and Arg_{180}–Val_{181}; Braunstein et al., 1981) and the release of an activation peptide. In factor IX_{aCH}, only the Arg_{180}–Val_{181} bond is cleaved due to an amino acid substitution of His for Arg_{145} (Noyes et al., 1983), and the activation peptide is not released. In factor IX_{aAL}, an amino acid substitution occurs at position 47 (Gly for Asp; Davis et al., 1984). In factor IX_{aMOD}, an average of 3 of the 12 Gla residues per molecule (shown as Y) are chemically modified (Wright et al., 1984) to γ-methylglutamic acid [shown as (|)].

et al., 1978). The heavy chain of factor IX_a contains the active site serine and histidine residues (DiScipio et al., 1978). The light chain contains 12 γ-carboxyglutamic acid (Gla) residues; this implicates the light-chain region of the protein in binding to phospholipid membrane. The Gla residues are also required for the Ca^{2+}-dependent conformational change necessary for full expression of factor IX_a coagulant activity (Morita et al., 1984; Straight et al., 1985). Whether this conformational change is needed for enhanced substrate (facotr X) or cofactor (factor $VIII_a$) affinity or for improved phospholipid binding is unclear, as is the nature of the structural/functional interactions between the light and heavy chains.

At least three abnormal factor IX molecules have been characterized recently (Figure 1). These molecules are structurally altered with respect to the light-chain regions and have decreased coagulent activity (Straight et al., 1985; Noyes et al., 1983; Griffith et al., 1985a; Braunstein et al., 1981; Chung et al., 1978). Two of these proteins [factor $IX_{Chapel\ Hill}$ (factor IX_{CH}) and factor $IX_{Alabama}$ (factor IX_{AL})] are abnormal gene products, which are isolated from the plasma of hemophilia B patients who exhibit normal levels of factor IX antigen but decreased factor IX clotting activity. The third abnormal factor IX molecule [Gla-modified factor IX (factor IX_{MOD}); Figure 1] is prepared by chemical modification of an average of three Gla residues per molecule and has approximately 5% coagulant activity after the modification (Straight et al., 1985). In all of these abnormal proteins, the active site serine and histidine residues are normal as determined by titration with antithrombin III and by the ability of the proteins to catalyze factor X activation in the presence of polylysine. It has been suggested (Griffith et al., 1985a; Straight et al., 1985), therefore, that the decreased activities of the proteins may be due to abnormal or decreased binding to charged membrane surfaces.

In this paper, we have measured and compared the lipid binding and kinetic properties of normal factor IX_a to those of three abnormal factor IX_a molecules. Only in the case of Gla-modified factor IX_a did the measured decrease in the rate of factor X activation correspond to a decrease in the amount of factor IX_a bound to the phospholipid. We conclude that the light chain of factor IX_a must influence the proper functioning of the heavy chain of the protein on the phospholipid surface.

Materials and Methods

Poly(L-lysine) (M_r 65 000) and morpholine were perchased from Sigma (St. Louis, MO). Formaldehyde (37% w/w) was purchased from Mallinkrodt (McGraw Park, IL). N^{α}-*p*-Tosylglycyl-L-prolyl-L-arginine-*p*-nitroanilide (Tos-Gly-Pro-Arg-NA) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Poly(ethylene glycol) (PEG, M_r 6000–8000) and triethanolamine (TEA) were purchased from Fisher Scientific Co. (Raleigh, NC). All chemicals, including 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES) and ethylenediaminetetraacetic acid (EDTA), were ACS reagent grade or the best available grade; all solvents were HPLC grade.

Bovine brain phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (PC), and 1,2-dioleoyl-3-*sn*-phosphatidylglycerol were purchased from Avanti Biochemicals (Birmingham, AL). Lipids were judged to be greater than 98% pure on the basis of thin-layer chromatography on silica gel GHL impregnated with 0.01 M dipotassium oxalate (Analtech, Newark, DE) and eluted with chloroform/methanol/water (65/25/4 v/v/v). Phospholipids were stored under an argon atmosphere in chloroform or chloroform/methanol (1/1) at −70 °C at concentrations between 15 and 32 mM.

Vesicles. Small, unilamellar vesicles (SUV) of PS/PC were prepared above their phase transition in 100 mM NaCl, 10 mM TES, 0.02% NaN_3, and 25 μM EDTA, pH 7.4. Lipid mixtures were sonicated in a Heat Systems Model 350 Cell Disruptor Cup Horn and fractionated by centrifugation (Lentz et al., 1980, 1982). Large, unilamellar vesicles (LUV) were prepared by reverse-phase evaporation as previously described (Szoka et al., 1978) and extruded through 0.4- and 0.2-μm Nucleopore (Pleasanton, CA) polycarbonate membranes (Szoka et al., 1980). Unless otherwise stated, SUV were used in all experiments. Total phosphate concentrations were determined by the assay of Chen et al. (1956).

Proteins. Factor IX (normal and abnormal) and factor X were isolated from human plasma as described previously (Griffith et al., 1985a). Protein concentrations were determined spectrophotometrically at 280 nm with extinction coefficient values of 1.33 and 1.16 mL mg^{-1} cm^{-1} for factor IX and factor X, respectively (DiScipio et al., 1977). Human antithrombin III was isolated from barium citrate adsorbed plasma by heparin–agarose affinity chromatography as described previously (Griffith et al., 1985b). Antithrombin III concentration was determined spectrophotometrically at 280 nm with an extinction coefficient value of 0.61 mL mg^{-1} cm^{-1} and a M_r value of 65 000 (Miller-Andersson et al., 1974). One mole of purified antithrombin III was required to inactivate 1 mol of human α-thrombin in the presence or absence of heparin (Rosenberg & Damus, 1973).

Factor IX Activation. Normal and abnormal factor IX were activated by incubation with partially purified human factor IX_a that was covalently coupled to agarose (Sepharose 4B; Pharmacia) (Braunstein et al., 1981; Griffith et al., 1985a). The clotting activities of solutions containing activated factor IX were determined as described previously (Griffith et al., 1985a). The clotting activities of activated factors IX_{CH} and IX_{AL} were 18% and 15%, respectively, of the clotting activity of normal factor IX_a.

Factor IX_a Active Site Measurement. Factor IX_a active site concentration was determined as described previously (Griffith et al., 1985a). In brief, the rate of factor X activation by factor IX_a [preincubated with heparin (0.01 mg/mL) and with

varying amounts of antithrombin III] was monitored by measuring the rate of change in absorbance at 400 nm (Tos-Gly-Pro-Arg-NA hydrolysis) in the presence of polylsine. The amount of antithrombin III required to inhibit 100% of the activated factor IX was determined graphically. By assuming that 1 mol of factor IX_a is inhibited by 1 mol of antithrombin III, the concentration of factor IX_a active sites was calculated.

Chemical Modification of Factor IX and Factor IX_a. Partial modification of the Gla residues of factor IX and factor IX_a was performed by treatment with a mixture of formaldehyde and morpholine as described previously (Straight et al., 1985). Approximately 3 mol of Gla/mol of protein was modified to γ-methyleneglutamic acid under the conditions used. Solutions were exhaustively dialyzed against buffer to remove traces of formaldehyde and morpholine prior to determining phospholipid binding affinities.

Factor X Activation by Factor IX_a in the Presence of Ca^{2+} and PS/PC SUV. Factor IX_a (normal or abnormal; final concentration of 40 nM) was added to a solution containing 0.5 μM factor X, 50 μM phospholipid [PS/PC (30/70) SUV], 5.0 mM Ca^{2+}, 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG, and 0.3 M Tos-Gly-Pro-Arg-NA in a 1.0-cm path-length cuvette. The cuvette was placed in a Hewlett-Packard 8451A diode array spectrophotometer, and the change in absorbance at 400 nm (hydrolysis of Tos-Gly-Pro-Arg-NA) was monitored as a function of time at room temperature. Factor X activation rates per mole of factor IX_a were calculated as described by Griffith et al. (1985a) using the factor IX_a active site concentration.

Binding Measurements. Ninety-degree light scattering measurements (Nelsestuen & Lim, 1977) were performed on an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL) at 37 °C with excitation and emission slits set at 1 and 4 nm, respectively. Prior to light scattering measurements, the buffer (0.10 M NaCl, 0.01 M TES, 25 μM EDTA, 0.02% NaN_3, pH 7.4) was filtered through 0.22-μm GS-type filters (Millipore Corp., Bedford, MA). Factor IX or factor IX_a (~1 mg/mL) was added in sequential aliquots with stirring to a cuvette containing 20–50 μM phospholipid (PS/PC SUV) and Ca^{2+} (0–10 mM), and the increase in scattered intensity was monitored. Molecular weights used for the proteins were as follows: factor IX (abnormal and normal) and factor IX_{aCH}, 57 000; factor IX_{aN}, factor IX_{aAL}, and factor IX_{aMOD}, 45 000 (Discipio et al., 1977). Measurements were analyzed on a Southwestern Technical Products 6809 microcomputer interfaced with the spectrofluorometer. Scattered intensities were corrected for the scattering of unbound protein and otherwise analyzed essentially by the method of Nelsestuen & Lim (1977). In control experiments, the mixtures were shown to be at equilibrium, as the measured dissociation constant was independent of the initial phospholipid concentration. Each experiment was repeated between 2 and 9 times. Following completion of a binding isotherm, a 10-fold excess of Na_2EDTA (relative to total Ca^{2+}) was added to determine the reversibility of the measurement.

Results

Phospholipid Binding. The binding of normal factor IX to PS/PC small, unilamellar vesicles was measured as a function of Ca^{2+} concentration and vesicle PS content in order to establish the most suitable conditions for comparing the lipid binding and kinetic properties of the normal and abnormal proteins. A representative binding isotherm obtained by the light scattering technique (see Materials and Methods) is shown in Figure 2 for the binding of factor IX to PS/PC

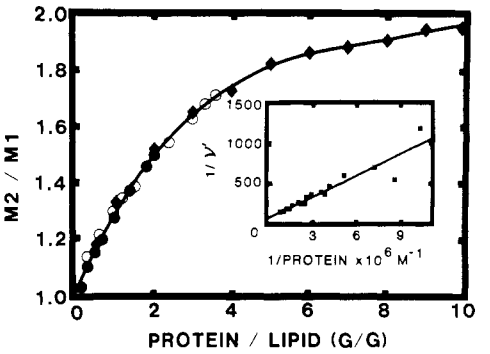

FIGURE 2: Binding of human factor IX to PS/PC (20/80) SUV in the presence of 5 mM Ca^{2+}, as measured by 90° light scattering. The mass ratio (M_2/M_1) of the complex (protein with vesicles) relative to the vesicle alone is shown as a function of the weight ratio of the protein to lipid. Data shown were collected from three different experiments. The inset shows the Hildebrand transformation of the pooled data.

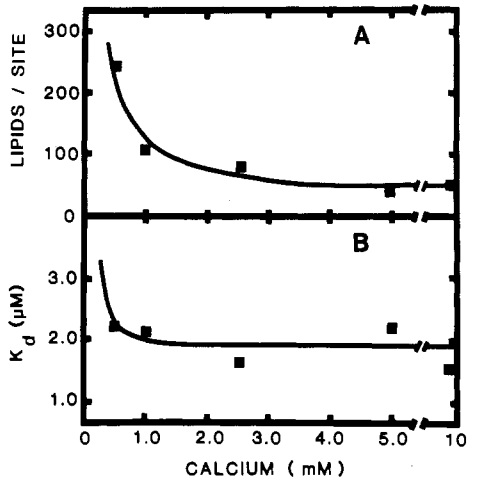

FIGURE 3: Stoichiometries (panel A) and dissociation constant values (panel B) for the binding of human factor IX to PS/PC (20/80) SUV as a function of Ca^{2+} concentration.

(20/80) SUV in 5 mM Ca^{2+}. The inset shows the Hildebrand transformation of these data from which the intrinsic dissociation constant (K_d, 2.2 ± 0.8 μM) and stoichiometry (number of lipids in the outer monolayer/site, 34 ± 9) were obtained. The binding parameters calculated from the entire isotherm (up to a protein to lipid weight ratio of 10) were the same as those calculated from the initial portion of the isotherm (up to a protein to lipid weight ratio of 2). The initial portion of the isotherm was used to derive the binding parameters whenever an excessive quantity of the rare, abnormal protein would have been required to reach complete saturation.

Data presented in Figure 3 demonstrate that changes in the Ca^{2+} concentration have a much stronger influence on the stoichiometry than on the dissociation constant for binding of factor IX to PS/PC (20/80) SUV. The greatest change in stoichiomery (Figure 3A) occurred between 0.5 and 1.0 mM ca^{2+}, which is in the range of the dissociation constant for Ca^{2+} binding to human factor IX (0.6 mM; Bajaj, 1982). The K_d values (Figure 3B) were fairly insensitive to the Ca^{2+} concentration between 0.5 and 10 mM, although no binding could be detected in the absence of Ca^{2+} or at 0.2 mM Ca^{2+}. The measured K_d value for human factor IX at 2.5 mM Ca^{2+} (2.6 ± 0.4 μM) agrees with that reported for bovine factor IX at 2 mM Ca^{2+} (2 ± 1 μM; Nelsestuen et al., 1978).

The effects of vesicle PS content on the binding of normal factor IX to PS/PC SUV at 5 mM Ca^{2+} are summarized in Figure 4. While the stoichiometry was insensitive to PS

Table I: Kinetic and Lipid Binding Properties of Factor IX and IX_a from Various Sources

source	rate of factor X activation: mmol of factor X min^{-1} (mol of factor IX_a)$^{-1}$	rate of factor X activation: relative	K_d (μM): factor IX	K_d (μM): factor IX_a	percent factor IX_a bound under assay conditions: absolute	percent factor IX_a bound under assay conditions: relative
normal	8.64	100	0.10 ± 0.05	0.10[a]	92	100
Chapel Hill	3.07	36	0.04 ± 0.02	0.08 ± 0.02	94	102
Alabama	3.71	43	0.05 ± 0.02	0.05[a]	96	104
Gla-modified	0.17	2	9.8 ± 0.6	10.8 ± 0.5	10	11

[a] Protein caused irreversible changes in the SUV during light scattering experiment, and assumed value is the same as that measured for the zymogen.

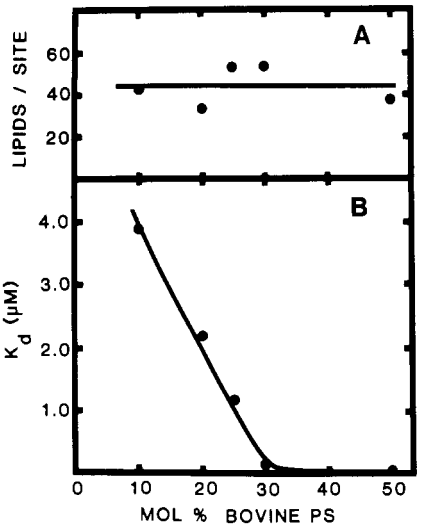

FIGURE 4: Stoichiometries (panel A) and dissociation constant values (panel B) for the binding of human factor IX to PS/PC SUV in the presence of 5 mM Ca^{2+} as a function of vesicle PS content.

content (Figure 4A), the K_d values depended strongly on the vesicle composition (Figure 4B) and dropped by almost 1 order of magnitude when the PS content increased from 25 to 30 mol % or from 30 to 50 mol %. A log–log plot of K_d vs. mole percent of PS gave a slope of 6.3, suggesting that about six PS molecules are associated with a bound factor IX molecule (Hermans & Scheraga, 1961). No binding to pure PC vesicles could be detected. A steep dependence of binding affinity on the membrane PS content has been observed for at least two other vitamin K dependent proteins, prothrombin and factor X (Nelsestuen & Broderius, 1977; Dombrose et al., 1979). A comparison of the dissociation constants for binding of equimolar PS/PC vesicles to factor IX (measured here by light scattering, 14 ± 9 nM) vs. binding to factor IX_a [measured by a centrifugation sedimentation technique, 12 nM (Mertens et al., 1984)] indicates that the zymogen and activated forms of human IX bind with similar affinities to these membranes.

The results shown in Figure 3 and 4 for the native factor IX were used to establish the most favorable conditions for measuring the membrane-binding and kinetic abilities of the abnormal factor IX and factor IX_a molecules. If the decrease in the coagulant activity of the abnormal molecules were due to impaired phospholipid binding, then at any given membrane composition or Ca^{2+} concentration the amount of the abnormal protein bound would be less than that of normal factor IX_a. The membrane composition [PS/PC (30/70) SUV] selected for further studies had a low K_d value (0.10 ± 0.05 μM, Figure 4B) with normal human factor IX (and presumably factor IX_a; see above). A membrane with a low K_d was selected so that its titration with the variant factor IX species would not require the consumption of unreasonable quantities of protein even if the abnormal protein's dissociation constant were 10-fold larger than that of normal proteins. A Ca^{2+} concentration of 5 mM was selected for further studies both because the stoichiometry and dissociation constant of binding were insensitive to Ca^{2+} concentration at this level (Figure 3B) and because the rate of activation of factor X in the presence of phospholipid is optimal around 5 mM Ca^{2+} (Griffith et al., 1982).

The measured K_d values for the binding of normal and abnormal factors IX and IX_a to PS/PC (30/70) SUV in the presence of 5 mM Ca^{2+} are summarized in Table I. The K_d values for normal factor IX, factor IX_{CH}, factor IX_{AL}, and factor IX_{aCH} were identical within experimental error. A 100-fold increase in the K_d value was observed for factor IX_{MOD} and factor IX_{aMOD} over factor IX_N. Since an average of 3 of the 12 total Gla residues of factor IX are chemically altered by the treatment to produce factor IX_{MOD}, the higher K_d value for the modified protein demonstrates that all Gla residues need not be modified to result in a decrease in membrane binding.

In the cases of factor IX_{aN} and factor IX_{aAL}, the reported K_d values (Table I) are assumed to be those identified for the zymogen. These assumptions were necessary due to irreversible changes in the light scattering properties of PS/PC SUV observed upon exposure to these proteins. Changes included a steady rather than a discrete increase of the intensity of scattered light following addition of protein and an inability to return to the predicted scattering of the SUV alone plus protein after addition of EDTA. In analogy to the reported fusion of SUV by prothrombin fragment 1 (Lentz et al., 1985), we presume that the irreversible changes in light scattering reflect fusion of SUV into larger vesicles. These changes in light scattering were also observed when factor IX_a was added to PS/PC (20/80) small and large, unilamellar vesicles, to phosphatidylglycerol/PC (20/80) large, unilamellar vesicles, and even to pure PC SUV and were accelerated by the presence of Ca^{2+}. It appears also that this fusogenic ability of factor IX_a required the proteolytic release[2] of the activation peptide (not possible with factor IX_{CH}) and the presence of intact Gla residues (not found in IX_{MOD}). Because of these irreversible changes in light scattering, the dissociation constants for binding of factor IX_{aN} and factor IX_{aAL} to PS/PC membranes could not be measured directly by the light scattering technique. Presumably, the activated protein and zymogen have identical dissociation constants, since equivalent binding parameters were obtained for the activated and unactivated forms of factor IX_{CH} and factor IX_{MOD} (Table I) and even for normal factor IX and factor IX_a when measured by different techniques [compare our results with those of Mertens et al. (1984); see above].

[2] Physical separation of the activation peptide from the parent zymogen is not implied by our use of the term "proteolytic release". We know of no evidence that physical separation accompanies proteolytic release.

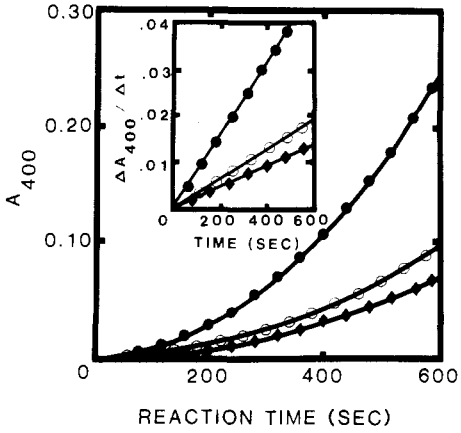

FIGURE 5: Factor X activation by factor IX_a in the presence of Ca^{2+} and PS/PC (30/70) SUV. Factor IX_a (normal, closed circles; Chapel Hill, triangles; Alabama, open circles) was added (final concentration 40 nM) to a solution containing 0.5 μM factor X, 50 μM phospholipid, 5 mM Ca^{2+}, and 0.3 M Tos-Gly-Pro-Arg-NA. The change in absorbance at 400 nM was recorded as described under Experimental Procedures. The rate of change in absorbance ($\Delta A/\Delta t$) was calculated over 10-s time intervals and is plotted (OD units/min) in the inset as a function of reaction time.

Kinetic Studies. The initial rate of factor X activation by factor IX_a in the presence of PS/PC (30/70) SUV and 5 mM Ca^{2+} was measured as described under Materials and Methods with a chromogenic assay for factor X_a. The time course of factor X activation is shown in Figure 5 for factor IX_{aN}, factor IX_{aCH}, and factor IX_{aAL}. Initial and relative rates for these proteins as well as for factor IX_{aMOD} are listed in Table I.

The dissociation constants (Table I) and the average stoichiometry (44 lipids per binding site) have been used to calculate the fraction of bound factor IX_a (both normal and abnormal) in the factor X activation assay system. The fraction bound and the relative fraction bound (compared to normal factor IX_a) are listed in Table I. Under the chosen assay conditions, more than 90% of the normal and genetic-variant factor IX_a molecules were surface-bound, although only 10% of the Gla-modified factor IX_a was bound. Clearly, inadequate binding of the genetic-variant factor IX_a molecules does not account for their decreased abilities to activate factor X.

Discussion

Structure–Function Relationships in Factor IX_a/Membrane Complexes. In the present study, three abnormal factor IX molecules, which are structurally altered at known positions in the light-chain region, have been used as tools both for evaluating the role of lipid binding in protein function and for examining the functional interactions between the heavy and light chains of factor IX_a. Both factor IX_{AL} and factor IX_{CH} are genetic variants, purified from the plasma of hemophilia B patients. Both proteins have known amino acid substitutions (Figure 1) that decrease the abilities of the activated zymogens to catalyze the proteolysis of factor X (Table I). The third abnormal factor IX molecule (factor IX_{MOD}) can be prepared chemically by modifying an average of three of the γ-carboxyglutamic acid residues per molecule to γ-methyleneglutamic acid (Figure 1). This modification effectively eliminates all enzymatic activity (Table I).

Abnormal or decreased phospholipid binding has been suggested as an explanation of the decrease in activity of these abnormal proteins relative to normal factor IX_a (Griffith et al., 1985a; Straight et al., 1985). However, our results (Table I) show that only for factor IX_{aMOD} does the decrease in activity correlate (at least in part) with a comparable loss of phospholipid binding affinity. Since the rate of factor X activation directly relates to the amount of surface-bound factor IX_a (Mertens & Bertina, 1984), comparison of the relative rates and amounts bound for factor IX_{aCH} (36% vs. 102%) and for factor IX_{aAL} (43% vs. 104%) indicates that the loss of activity of the genetic variants is not due to a loss of phospholipid binding affinity. Therefore, in addition to its lipid binding function, the light chain of human factor IX_a may influence substrate affinity, the catalytic rate constant of factor IX_a, or both.

The ability of the light chain to influence substrate affinity has already been suggested for bovine factor IX_a. In 1974, Fujikawa et al. speculated as to this possibility. Later, Link & Castellino (1983) established that the apparent K_m of factor X for factor IX_a in the presence of Ca^{2+} and PS/PC vesicles depended on the structure of factor IX_a. Specifically, the K_m of factor X for factor $IX_{a\alpha}$ (single cleavage at the Arg_{181}–Val_{182} bond) was 4 times greater than that for factor $IX_{a\beta}$ (cleavages at Arg_{146}–Ala_{147} and Arg_{181}–Val_{182} and release of activation peptide). Since the structure of bovine factor $IX_{a\alpha}$ closely resembles that of human factor IX_{aCH}, one may predict a similar increase in the K_m value for factor X would be observed for this abnormal molecule, reflecting an altered substrate affinity.

Altogether, our observations suggest that, for the proper functioning of the active site serine and histidine residues in the heavy chain of factor IX_a, the light and heavy chains must interact. This functional interaction may involve direct interaction between the light and heavy chains to produce an appropriate conformation of the active site in the heavy chain. Alternatively, the interaction may require and be mediated through a proper orientation on the phospholipid surface. In either case, it is clear from our data (Table I) that failure to proteolytically release the activation peptide (as for factor IX_{aCH}) as well as certain amino acid substitutions in the light chain (as for factor IX_{aAL}) will interfere with this interaction.

Fusion of Membrane Vesicles by Factor IX_a. Factor IX_{aN} and factor IX_{aAL} interact with PS/PC SUV in a manner qualitatively different from that of factor IX_{aCH} and factor IX_{aMOD}. Specifically, factor IX_{aN} and factor IX_{aAL} appear to aggregate and to fuse the SUV into larger vesicles that more intensely scatter light. Although the formation of large vesicles was not confirmed either by conventional lipid or contents mixing assays or by evaluating vesicle morphology, the inability of EDTA to completely reverse the increased light scattering strongly suggests that aggregation alone did not occur. The ability to induce vesicle fusion following proteolytic release of the activation peptide might reflect either an altered mechanism of protein–membrane interaction involving some bilayer destabilization or enhanced bilayer–bilayer contact mediated by factor IX_a dimerization.

With regard to the possibility of altered protein–membrane interactions, we have noted that irreversible light scattering changes also accompany the interaction of prothrombin fragment 1, the lipid binding portion of prothrombin, and uncharged SUV. In the case of prothrombin fragment 1, electron microscopy has established that the uncharged SUV are fused into larger vesicles. On this basis, we have suggested that fragment 1 is capable of Ca^{2+}-independent interactions with phospholipids (Lentz et al., 1985). By analogy with prothrombin fragment 1, the fusogenic capabilities of factors IX_{aN} and IX_{aCH} suggest the possibility that similar interactions contribute to the associations beteen other vitamin K dependent proteins and membranes. In support of this possibility,

Nelsestuen et al. (1978) have interpreted fluorescence energy transfer data in terms of calcium-independent binding of bovine factor IX to charged membranes.

The apparent fusogenic capacity found in activated factor IX and absent in the unactivated zymogen need not have a functional role in blood coagulation. Several peptides and proteins that are quite unlikely to play a role in cellular membrane fusion are known, nonetheless, to induce fusion of synthetic vesicles (Blumenthal, 1985). However, we note that this fusogenic ability has been useful in distinguishing between the different lipid binding properties of normal and variant forms of factor IX and factor IX_a.

Registry No. Ca, 7440-70-2; blood coagulation factor IX, 9001-28-9; blood coagulation factor IXa, 37316-87-3; blood coagulation factor X, 9001-29-0.

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