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Reactivity of Bovine Blood Coagulation Factor $IX_{a\beta}$, Factor $X_{a\beta}$, and Factor XI_a toward Fluorogenic Peptides Containing the Activation Site Sequences of Bovine Factor IX and Factor X†

Mario J. Castillo, Kotoku Kurachi,‡ Norikazu Nishino, Iwao Ohkubo, and James C. Powers*

ABSTRACT: The published activation site sequences of bovine factors IX and X have been utilized to synthesize a number of peptides specifically designed respectively as substrates for bovine factors XI_a and $IX_{a\beta}$. The substrates contain a fluorophore (2-aminobenzoyl group, Abz) and a quenching group (4-nitrobenzylamide, Nba) that are separated upon enzymatic hydrolysis with a resultant increase in fluorescence that was utilized to measure hydrolysis rates. Factor XI_a cleaved all of the peptides bearing factor IX activation site sequences with Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba having the highest k_{cat}/K_M value. The kinetic behavior of factor XI_a toward the synthetic peptide substrate indicates that it has a minimal extended substrate recognition site at least five residues long spanning S_4 to S_1' and has favorable interactions over seven subsites. The hexapeptide Abz-Glu-Phe-Ser-Arg-Val-Val-Nba was the most specific factor XI_a substrate and was not hydrolyzed by factors $IX_{a\beta}$ or $X_{a\beta}$ or thrombin. Factor $IX_{a\beta}$ failed to hydrolyze any of the synthetic peptides bearing the activation site sequence of factor X. This enzyme slowly cleaved four hexa- and heptapeptide substrates with factor IX activation site sequences extending from P_4 or P_3 to P_3'. Factor $X_{a\beta}$ poorly hydrolyzed all but one of the factor XI_a substrates and failed to cleave any of the factor $IX_{a\beta}$ substrates. Thrombin failed to hydrolyze any of the peptides examined while trypsin, as expected, was highly reactive and not very specific. Phospholipids had no effect on the reactivity of either factors $IX_{a\beta}$ or $X_{a\beta}$ toward synthetic substrates. Both factor $IX_{a\beta}$ and $X_{a\beta}$ cleaved the peptide substrates at similar rates to their natural substrates under comparable conditions. However the rates were substantially lower than optimum activation rates observed in the presence of Ca^{2+}, phospholipids, and protein cofactors. In the future, it may be useful to investigate synthetic substrates that can bind to phospholipid vesicles in the same manner as the natural substrates for factors $IX_{a\beta}$ and $X_{a\beta}$.

The blood coagulation cascade comprises a complex series of biochemical reactions in which inactive zymogens of serine proteases are converted to active blood coagulation enzymes. These enzymes have very high substrate specificities when compared with those of the digestive enzymes such as trypsin. Some stages of the pathway require the formation of discrete complexes between a protease, zymogen, phospholipids, and a protein cofactor. Ca^{2+} ions mediate the interactions among these various species. Factor X, the merging point for both the intrinsic and extrinsic branches of the coagulation pathway, is activated by factor $IX_{a\beta}$.[1] An optimal rate for its activation

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332 (M.J.C., N.N., and J.C.P.), and the Department of Biochemistry, University of Washington, Seattle, Washington 98195 (K.K. and I.O.). *Received June 23, 1982; revised manuscript received November 5, 1982.* This research was supported by grants to the Georgia Institute of Technology (HL-22530) and to the University of Washington (HL 16919) from the National Institutes of Health.
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[1] The nomenclature for the various coagulation factors is that recommended by an international committee (Wright, 1959). Factor IX_α refers to factor IX with the peptide bond between Arg(146)*Ala(147) cleaved. Factor $IX_{a\alpha}$ refers to factor IX_a with the peptide bond between Arg(181)*Val(182) cleaved. Factor $IX_{a\beta}$ refers to factor IX_a, which does not have activation peptide [Ala(147)–Arg(181)].

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is achieved only in the presence of Ca^{2+}, phospholipids, and factor $VIII_a$. Factor XI_a participates in the early phase of the intrinsic pathway and activates factor IX after two hydrolytic cleavages at specific regions of the zymogen. This activation process requires only Ca^{2+} ions for optimal efficiency.

The reactivity of blood coagulation proteases has been examined with peptide 4-nitroanilides and peptide 7-amino-4-methylcoumarin substrates (Morita et al., 1977; Aurell et al., 1976, 1977). Most of these synthetic substrates have not been characterized kinetically in terms of k_{cat}/K_M. Furthermore, the bulk of the research in this area has centered on thrombin and factor $X_{a\beta}$. Only a few papers have appeared in the literature dealing with the use of synthetic substrates for assaying blood coagulation factors $IX_{a\beta}$ and XI_a (Wuepper, 1972; Kurachi et al., 1976; Kurachi & Davie, 1977; Byrne & Castellino, 1978; Byrne et al., 1980). Recently, McRae et al. (1981) utilized a series of sensitive peptide thio esters to examine the subsite specificities of various coagulation serine proteases. In general, synthetic peptide substrates of blood coagulation proteases have suffered from a lack of specificity. A second disadvantage has been the inability to examine enzyme–substrate interactions on the P′ side[2] of the scissile peptide bond. With the exception of thrombin (Liem et al., 1971; Liem & Scheraga, 1973, 1974; van Nispen et al., 1977; Meinwald et al., 1980), no studies have been reported on the cleavage of peptide amide bonds in synthetic substrates containing amino acids on both sides of the scissile bond.

Previously, hydrolysis rates of peptide bonds have been determined with ninhydrin and fluorescamine reagents in colorimetric or fluorogenic assays (McRae et al., 1980; van Nispen et al., 1977). In this paper, we have developed an assay method that employs the fluorophore (2-aminobenzoyl, Abz)–quencher (4-nitrobenzylamide, Nba) pair.[3] These aromatic groups are linked via amide bonds to the N- and C-terminal ends, respectively, of the synthetic peptides. In designing these peptide substrates, we have exploited the published activation site sequences for various blood coagulation factors (Radcliffe & Nemerson, 1975; Fujikawa et al., 1974a,b, 1977; Titani et al., 1975; Katayama et al., 1979). We have synthesized and measured the enzymatic hydrolysis rates for peptides of varying length that mimic the activation site sequences of factors X and IX, the natural substrates for factors $IX_{a\beta}$ and $X_{a\beta}$, respectively. The active site length of factor XI_a has been mapped, and the relative importance of P and P′ residues on the catalytic efficiency of this protease has been assessed. Furthermore, ten highly specific substrates for factor XI_a have been developed, six of which are the most reactive peptide amide substrates known for this protease.

Materials and Methods

Bovine thrombin, factor $IX_{a\beta}$, factor $X_{a\beta}$, and factor XI_a were prepared according to published procedures (DiScipio et al., 1978; Kurachi & Davie, 1977; Kurachi et al., 1976, 1980). High-grade reagents were obtained from the following sources: bovine pancreatic trypsin type III (EC 3.4.4.4), dioleoylphosphatidylcholine, phosphatidylserine, NPGB, and fluorescamine from Sigma Chemical Co., St. Louis, MO; Tris and boric acid from Fisher Scientific Co., Norcross, GA; Hepes and 4,4′-dithiodipyridine from Aldrich Chemical Co., Inc., Milwaukee, WI; ninhydrin from Eastman Kodak Co., Rochester, NY. All other chemicals and solvents were of high grade. The supplementary material contains a description of the syntheses for all the new peptides employed in this study (see paragraph at end of paper regarding supplementary material).

Fluorogenic Assays. All enzymes except factor XI_a were active sites titrated with NPGB according to a modified version of the procedure of Chase & Shaw (1967). Titrations were conducted in 0.10 M Hepes and 10 mM $CaCl_2$ at pH 7.5 and 25 °C. Z-Trp-Arg-SBu-*i* was utilized to determine the active site concentration of factor XI_a with kinetic constants previously reported (McRae et al., 1981). All glassware was silylized (Castillo et al., 1979). Trypsin was prepared and stored in a 1.0 mM HCl solution to minimize autodegradation.

To a cuvette containing 2.0 mL of a fluorogenic peptide solution in the indicated buffer with 10% Me_2SO also included was added 25 μL of trypsin. The change in fluorescence at λ_{ex} = 340 nm and λ_{em} = 415 nm, 25 °C, was continuously monitored with a Perkin-Elmer 204S fluorometer equipped with a Model 56 chart recorder. The instrument was standardized daily with a 1.0 μM quinine sulfate solution in 0.1 N H_2SO_4. The hydrolysis rate was linear well above the percent hydrolysis required for obtaining accurate measurements of initial velocities.

Blood clotting proteases were diluted in 50 mM Tris-HCl, 200 mM NaCl, and 50 mM $CaCl_2$, pH 7.8, immediately prior to use, and 250-μL aliquots were used. With these enzymes, the hydrolysis rates were too slow to follow continuously. Fluorescence measurements were taken initially and after completion of a 2-h incubation period to determine initial hydrolysis rates when assaying for factor XI_a. For factors $IX_{a\beta}$ and $X_{a\beta}$, the incubation period extended from 18 to 24 h. Phospholipid vesicles were prepared in buffer containing Me_2SO and substrate in a low-energy Cole-Parmer ultrasonic cleaner. An approximate molecular weight of 750 was employed to calculate the molar concentration of phosphatidylserine.

During the course of the incubation period, usually less than 5% of the substrate was hydrolyzed by these enzymes. The linearity of the initial rate was checked with a number of the substrates by measuring the fluorescence change frequently throughout a major part of the incubation period. No background hydrolysis was obtained in controls lacking enzyme. All fluorogenic substrates were found to be stable to autohydrolysis over a period of several weeks when stored in buffer containing 10% Me_2SO.

Fluorescence intensity vs. concentration of substrates (and hydrolysis products) curves were constructed for each peptide tested (Figure 1 shows one example). The fluorescent change at each substrate concentration was measured by subtracting the tangent of the starting peptide curve from the tangent of the hydrolysis products curve at the appropriate concentration of substrate and hydrolysis products. These tangents fell usually in the linear portion of the starting peptide curve and always in the linear region of the hydrolysis products' curve. The fluorescence change was used to convert fluorescence rate measurements into absolute molar velocities. The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were calculated by the Lineweaver–Burk method with a linear regression analysis. With these assays as well as with the ninhydrin and fluorescamine assays described below, no fewer than five separate

[2] The nomenclature used for the individual amino acid residues (P_1, P_1', P_2', etc.) of a substrate and the subsites (S_1, S_1', S_2', etc.) of the enzyme is that of Schechter & Berger (1967).

[3] Abbreviations: NPGB, nitrophenyl guanidinobenzoate; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NA, 4-nitroanilide; AMC, 7-amino-4-methylcoumarinamide; Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamide; SBu-*i*, thioisobutyl ester; Me_2SO, dimethyl sulfoxide; Ac, acetyl; Z, benzyloxycarbonyl; Bz, benzoyl; TLC, thin-layer chromatography.

substrate concentrations were assayed and no fewer than three separate determinations went into each calculated hydrolysis rate.

Ninhydrin Assays. So that the accuracy of the fluorogenic assay method in determining kinetic parameters could be checked, the tryptic hydrolysis of Abz-Phe-Ser-Arg-Val-Val-Gly-Nba was measured by a modified version of the ninhydrin analysis for amino acids (Rosen, 1957). Reactions were conducted in 50 mM phosphate, 200 mM NaCl, 5.0 mM $CaCl_2$, and 9.9% v/v Me_2SO at pH 7.8 and 25 °C. After adding trypsin to the reaction mixture, 250-μL aliquots were removed periodically and quenched in test tubes containing 100 μL of 5% w/v trichloroacetic acid. Color development was effected upon addition of 100 μL each of 0.2 mM NaCN in acetate buffer (pH 5.3) and 10% w/v ninhydrin in ethyl glycol methyl ether, followed by heating for 20 min over a steam bath. The test tubes were allowed to cool after adding to them 1.0 mL of a 50% v/v 2-propanol solution. The A_{570} was promptly read and subsequently converted to absolute molar velocities as has been previously described (McRae et al., 1980).

Fluorescamine Assays. For evaluation of the effects of the 2-aminobenzoyl (Abz) and 4-nitrobenzylamide (Nba) groups on enzymatic hydrolysis, the kinetic behavior of Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2 with thrombin, factors $IX_{a\beta}$, $X_{a\beta}$, and XI_a, and trypsin was examined via fluorescamine assays. These assays were conducted with a modification of a previously reported procedure (van Nispen et al., 1977). To a plastic vial containing 4.0 mL of the peptide in a borate buffer and 10% Me_2SO was added 100 μL of trypsin. Periodically, 1.0-mL aliquots were removed and mixed rapidly into a solution of fluorescamine in acetone (1.0 mg/mL). For blood coagulation proteases, 150-μL aliquots were added to 1.0 mL of substrate solution. After an incubation period ranging from 2 to 24 h, 1.0 mL of the fluorescamine solution was added as before. The increase in fluorescence was measured at λ_{ex} = 390 nm and λ_{em} = 480 nm. Fluorescence increases due to enzyme alone were subtracted from the gross fluorescence change. Following the procedure delineated under Fluorogenic Assays, we converted net fluorescence change into absolute molar velocities, and the kinetic constants were subsequently calculated.

Results

A series of peptides bearing factor IX and factor X activation site sequences were synthesized via solution methods. They were covalently linked through amide bonds to a fluorophore (2-aminobenzoyl, Abz) and a fluorescence quenching group (4-nitrobenzylamide, Nba) at the N and C terminus, respectively. Under the conditions of the assay, the excited fluorophore of the intact peptide will transfer some its excitation energy to the fluorescence quenching group. This transfer of energy has been demonstrated to occur through a non-Förster type mechanism involving direct collisions between the fluorophore and the quencher (Carmel et al., 1977). Enzymatic hydrolysis of the Arg–Ile or Arg–Val peptide bond in the substrate results in the separation of these two groups. The quenching phenomenon previously observed in the intact peptide is now abolished. The resultant fluorescence increase was proportional to the concentration of liberated, unquenched Abz-peptide, allowing for a continuous assessment of the enzymatic hydrolysis rate. Fluorescence intensity vs. substrate concentration curves indicated that these parameters were linearly related to each other up to concentrations of approximately 0.1 mM. In this concentration range all quenching is intramolecular. At higher substrate concentrations, intermolecular quenching destroys the linearity of this relationship (see Figure 1). Previously, fluorogenic peptide substrates of this type have been employed in assays for leucine aminopeptidase (Carmel et al., 1977), angiotensin converting enzyme (Persson & Wilson, 1977; Carmel & Yaron, 1978), and *Pseudomonas aeruginosa* elastase (Nishino & Powers, 1980). The substrate developed for the latter enzyme, Abz-Ala-Gly-Leu-Ala-Nba, allowed for the development of a highly sensitive rate assay.

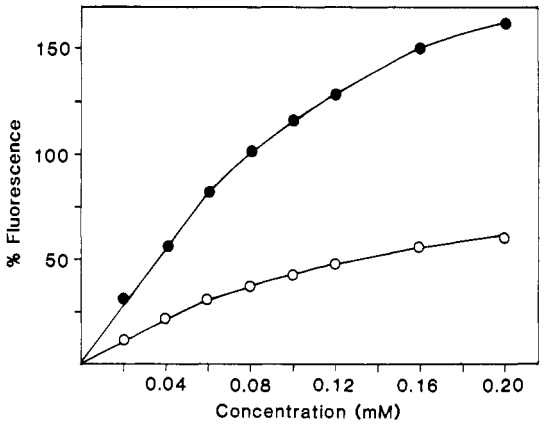

FIGURE 1: Fluorescence of Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba (open circles) and its hydrolysis products (Abz-Glu-Phe-Ser-Arg-OH and H-Val-Val-Gly-Nba) after exhaustive digestion with bovine trypsin (closed circles).

Figure 1 depicts the fluorescence change that occurs upon exhaustive tryptic cleavage of Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba. This pattern was characteristic for all of the fluorogenic peptides examined. The hydrolysis product had 2–3 times greater fluorescence than an equimolar amount of the uncleaved peptide. In general, the observed increase in fluorescence was inversely proportional to the peptide length. Unhydrolyzed substrates exhibited some intrinsic fluorescence of their own as indicated in the figure. Incomplete energy transfer may arise from conformational considerations that preclude effective collisions between the Abz and the Nba group or from the nature of the quencher group itself. The Nba group may be absorbing only a portion of the Abz excitation energy. The unabsorbed energy would correspond then to the observed intrinsic fluorescence of the intact peptide. Similar results were obtained with the *P. aeruginosa* elastase substrate, Abz-Ala-Gly-Leu-Ala-Nba (Nishino & Powers, 1980). Our fluorogenic assay yielded linear double-reciprocal plots with correlation coefficients greater than 0.95 over the substrate concentration ranges examined.

Tables I–IV list the kinetic constants for the enzymatic hydrolysis of all synthetic peptides. The accuracy of the fluorogenic assay in determining standard kinetic parameters was verified via an alternate assay employing ninhydrin. The kinetic constants derived by this method for the behavior of Abz-Phe-Ser-Arg-Val-Val-Gly-Nba with trypsin (K_M = 148 ± 201 μM, k_{cat} = 52 ± 22 s^{-1}, k_{cat}/K_M = 1.0 × 10^6 ± (9.2 × 10^5) M^{-1} s^{-1}) from several independent determinations are within reasonable agreement of those reported for the peptide in Table IV. The high reactivity and low K_M of trypsin toward this substrate demanded the use of low concentrations of both enzyme and fluorogenic peptide in ascertaining the kinetic parameters. As a result, the background absorbance inherent in the method was considerably greater than the net absorbance change recorded for any particular time interval (t_n–t_0) during the course of the assay. This unavoidable condition is responsible for the indicated variances in the kinetic constants derived with the ninhydrin assay.

Table I: Kinetic Constants for Hydrolysis of Synthetic Fluorogenic Peptide Substrates by Bovine Factors $IX_{a\beta}$ and XI_a[a]

substrate	factor $IX_{a\beta}$				factor XI_a			
P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	[S] range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	[S] range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
-Ser-Gln-Val-Val-Arg-Ile-Val-Gly-Gly-[b]	15-220[c]	181	0.000175	0.967				
-Arg-Val-Val-Gly-Gly-[d]								
-Lys-Lys-Leu-Thr-Arg-Ala-Glu-Thr-Ile-[e]								
-Asp-Glu-Phe-Ser-Arg-Val-Val-Gly-Gly-[f]						15[g]		
Abz-Val-Val-Arg-Ile-Val-Nba	1.6-53			N.R.[h]	16-47			N.R.
Abz-Val-Arg-Ile-Val-Gly-Nba	44			N.R.	44			N.R.
Abz-Val-Val-Arg-Ile-Val-Gly-Nba	46			N.R.	46			N.R.
Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba	18-180			N.R.	36-180[i]			1 100
Abz-Ser-Arg-Val-Val-Gly-Nba	47			N.R.	16-160			N.R.
Abz-Phe-Ser-Arg-Val-Val-Gly-Nba	18-180	20	0.011	550	18-89	230	1.1	4 800
Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba	18-89			N.R.	18-89	36	0.74	21 000
Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2[j]	110			N.R.	9-110	13	0.41	32 000
Abz-Glu-Phe-Ser-Arg-Val-Val-Nba	18-110			N.R.	18-110	220	1.0	4 500
Abz-Glu-Phe-Ser-Arg-Val-Nba	110			N.R.	18-110	370	0.68	1 800
Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba	18-89	24	0.013	540	18-89	130	1.5	12 000
Abz-Leu-Ser-Arg-Val-Val-Gly-Nba	18-110			N.R.	18-110	56	0.68	12 000
Abz-Leu-Thr-Arg-Val-Val-Gly-Nba	18-71[k]				18-89	43	1.1	26 000
Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba	36-110	75	0.0062	83	18-110	99	1.9	19 000

[a] pH 7.8, 50 mM Tris-HCl buffer, 200 mM NaCl, 5.0 mM $CaCl_2$, 0.5 mg/mL bovine serum albumin, and 8.8% v/v Me_2SO at 25 °C. The factor $IX_{a\beta}$ concentration ranged from 21 to 36 nM and the factor XI_a concentration from 2.7 to 3.6 nM. [b] Bovine factor X activation site sequence. Cleavage catalyzed by factor $IX_{a\beta}$ (Fujikawa et al., 1974a; Titani et al., 1975). [c] pH 7.9, 50 mM Tris-HCl buffer, 175 mM NaCl, 10 mM $CaCl_2$, 0.5 mg/mL ovalbumin, and 1.1 μM factor $IX_{a\beta}$ at 37 °C. The k_{cat}/K_M value was calculated from a reported V_{max} of 0.0105 mol of factor X_a generated min^{-1} (mol of factor $IX_{a\beta})^{-1}$ (van Dieijen et al., 1981). [d] Bovine factor XII activation site sequence. Cleavage catalyzed by factor XI_a (Fujikawa et al., 1977, 1980). [e] Cleavage by factor XI_a at this site in bovine factor IX generates an inactive two-chain disulfide-linked species, factor IX_α (Fujikawa et al., 1974b; Katayama et al., 1979). [f] Bovine factor IX activation site sequence. Cleavage catalyzed by factor XI_a (Fujikawa et al., 1974a; Katayama et al., 1979). [g] This value is a composite K_M for the complete activation of bovine factor IX by bovine factor XI_a and plasma (Steinberg et al., 1980). [h] N.R., no reaction after incubation for 24 h. [i] Double-reciprocal plot goes through the origin. The K_M value is higher than can be accurately measured. [j] Same conditions as in footnote *a* except that borate buffer was used instead of Tris-HCl. The serum albumin was omitted from the reaction mixture. A fluorescamine assay described under Materials and Methods was employed to determine the kinetic constants. [k] Double-reciprocal plot reveals much scatter in the data points.

Table II: Kinetic Constants for Hydrolysis of Synthetic Fluorogenic Peptide Substrates by Bovine Factor $X_{a\beta}$[a]

substrate	[S] range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'				
-Ala-Ile-Glu-Gly-Arg-Thr-Ser-Glu-Asp-[b]	7.6-55[c]	84	0.011	130
-Tyr-Ile-Glu-Gly-Arg-Ile-Val-Glu-Gly-[d]				
-Ala-Ala-Gly-Ser-Arg-Gly-His-Ser-Glu-[e]				
-Arg-Ile-Val-Gly-Gly-[f]				
Abz-Val-Val-Arg-Ile-Val-Nba	16			N.R.[g]
Abz-Val-Arg-Ile-Val-Gly-Nba	44			N.R.
Abz-Val-Val-Arg-Ile-Val-Gly-Nba	46			N.R.
Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba	18-180			N.R.
Abz-Ser-Arg-Val-Val-Gly-Nba	89			N.R.
Abz-Phe-Ser-Arg-Val-Val-Gly-Nba	18-110	94	0.014	150
Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba	18-89	456	0.011	24
Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2[h]	18-110	130	0.0049	38
Abz-Glu-Phe-Ser-Arg-Val-Val-Nba	18-110			N.R.
Abz-Glu-Phe-Ser-Arg-Val-Nba	18-110	150	0.013	87
Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba	18-89	45	0.014	310
Abz-Leu-Ser-Arg-Val-Val-Gly-Nba	18-110	82	0.016	200
Abz-Leu-Thr-Arg-Val-Val-Gly-Nba	36-89	36	0.0053	150
Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba	18-71	64	0.0062	97

[a] pH 7.8, 50 mM Tris-HCl buffer, 200 mM NaCl, 5.0 mM $CaCl_2$, 0.5 mg/mL bovine serum albumin, and 8.8% v/v Me_2SO at 25 °C. The concentration of factor $X_{a\beta}$ ranged from 32 to 39 nM. [b] Activation site sequence for the cleavage of prothrombin by factor X_a to yield prethrombin 2 (Jackson & Nemerson, 1980; Magnusson et al., 1975). [c] pH 7.5, 20 mM Tris-HCl buffer, 100 mM NaCl, 20 mM $CaCl_2$, 0.5 mg/mL ovalbumin, and 91 nM bovine factor $X_{a\beta}$ at 37 °C. The k_{cat} value was calculated from a reported V_{max} of 0.68 mol of thrombin generated min^{-1} (mol of factor $X_{a\beta})^{-1}$ (Rosing et al., 1980). [d] Prothrombin activation site sequence. Cleavage by factor X_a yields the A and B chains (Jackson & Nemerson, 1980; Magnusson et al., 1975). [e] Factor X_a autolysis site sequence. Cleavage by factor X_a yields factor $X_{a\beta}$ (Jesty et al., 1975). [f] Bovine factor VII activation site sequence. Cleavage catalyzed by factor X_a (Radcliffe & Nemerson, 1975). [g] N.R., no reaction after incubation for 24 h. [h] Same conditions as in footnote *a* except that borate buffer was used instead of Tris-HCl. The serum albumin was omitted from the reaction mixture. The kinetic constants were determined via a fluorescamine assay described under Materials and Methods.

A nonfluorogenic peptide, Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2, was synthesized and tested with factor XI_a in a fluorescamine assay in order to assess the effects of the 2-aminobenzoyl and 4-nitrobenzylamide groups on the enzymatic hydrolysis rate. The observed kinetic parameters (see Table I) indicate that the peptide devoid of these aromatic moieties was a slightly better substrate for factor XI_a.

Digestion of Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba with factor XI_a for 24 h under the assay conditions employed for the fluorogenic assays and subsequent TLC analysis [1-bu-

Table III: Kinetic Constants for Hydrolysis of Selected Synthetic Peptide Substrates by Bovine Factors $IX_{a\beta}$ and $X_{a\beta}$ in the Presence of Phospholipids[a]

substrate	factor $IX_{a\beta}$ [S] range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	factor $X_{a\beta}$ [S] range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
-Ser-Gln-Val-Val-Arg-Ile-Val-Gly-Gly-[b]		0.058[c]	0.000041	710				
-Ala-Ile-Glu-Gly-Arg-Thr-Ser-Glu-Asp-[d]					0.02–0.79[e]	0.032	0.018	550 000
Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba	36–89[f]			N.R.[g]	36–89			N.R.
Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba	18–110	21	0.016	760	18–110	28	0.013	460
Abz-Leu-Thr-Arg-Val-Val-Gly-Nba	18–110[h]				18–110	30	0.0052	180
Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba	36–110	78	0.0082	110	18–110	88	0.0089	100

[a] pH 7.8, 50 mM Tris-HCl buffer, 200 mM NaCl, 5.0 mM $CaCl_2$ 0.5 mg/mL bovine serum albumin, 11 μM dioleoylphosphatidylcholine, 12 μM phosphatidylserine, 14 μM cholesterol, 8.9% v/v Me_2SO, and 26 nM factor $IX_{a\beta}$ or 36 nM factor $X_{a\beta}$ at 25 °C. [b] See Table I, footnote *b*. [c] pH 7.9, 50 mM Tris-HCl, 175 mM NaCl, 10 mM $CaCl_2$, 0.4 mg/mL ovalbumin, 7.5 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 2.5 μM 1,2-dioleoyl-*sn*-glycero-3-phosphoserine, and 10.9 nM factor $IX_{a\beta}$ at 37 °C. The k_{cat} value was calculated from a reported V_{max} of 0.00247 mol of factor X_a generated min^{-1} (mol of factor $IX_{a\beta})^{-1}$ (van Dieijen et al., 1981). [d] See Table II, footnote *b*. [e] pH 7.5, 20 mM Tris-HCl buffer, 100 mM NaCl, 8.0 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 1.3 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1.3 μM 1,2-dioleoyl-*sn*-glycero-3-phosphoserine, and 0.387 nM factor $X_{a\beta}$ at 37 °C (Rosing et al., 1980). [f] Same conditions as in footnote *a* except that the concentrations of the phospholipids and Me_2SO were as follows: 250 μM dioleoylphosphatidylcholine, 250 μM phosphatidylserine, and 3.6% v/v Me_2SO. Cholesterol was omitted. [g] N.R., no reaction after incubation for 24 h. [h] Double-reciprocal plots reveal much scatter in the data points.

Table IV: Kinetic Constants for Hydrolysis of Synthetic Fluorogenic Peptide Substrates by Bovine Pancreatic Trypsin[a]

substrate	[S] range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Abz-Val-Val-Arg-Ile-Val-Nba	1.8–18	55	6.1	110 000
Abz-Val-Arg-Ile-Val-Gly-Nba	0.66–48	57	25	440 000
Abz-Val-Val-Arg-Ile-Val-Gly-Nba	0.79–43	77	53	690 000
Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba	9.9–5.9	56	23	410 000
Abz-Ser-Arg-Val-Val-Gly-Nba	8.8–7.0	79	6.1	77 000
Abz-Phe-Ser-Arg-Val-Val-Gly-Nba	9.0–72	82	66	800 000
Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba	18–110	78	37	470 000
Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2[b]	18–110	260	40	150 000
Abz-Glu-Phe-Ser-Arg-Val-Val-Nba[c]	20–100	140	35	250 000
Abz-Glu-Phe-Ser-Arg-Val-Nba[c]	20–120	65	39	600 000
Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba	16–97	46	75	1 600 000
Abz-Leu-Ser-Arg-Val-Val-Gly-Nba	18–70	35	20	570 000
Abz-Leu-Thr-Arg-Val-Val-Gly-Nba	8.8–70	36	27	750 000
Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba	8.3–67	70	60	860 000

[a] pH 7.8, 50 mM Tris-HCl buffer, 200 mM NaCl, 5.0 mM $CaCl_2$, and 9.9% v/v Me_2SO at 25 °C; trypsin ranged from 9.0 to 10.0 nM. [b] Same conditions as in footnote *a* except that borate buffer was used instead of Tris-HCl. The kinetic constants were determined via a fluorescamine assay as described under Materials and Methods. [c] Same conditions as in footnote *a* except that a 0.5 mg/mL concentration of bovine serum albumin was included.

tanol–acetic acid–H_2O (4:1:2)] of the reaction mixture indicated that the sole hydrolysis products were Abz-Lys-Phe-Ser-Arg-OH and H-Val-Val-Gly-Nba. Two spots were observed on the TLC plate (silica gel G-60 with fluorescent indicator F254, Merck). The lower Sakaguchi-positive spot (R_f 0.29) was fluorescent and corresponded to Abz-Lys-Phe-Ser-Arg-OH. The upper nonfluorescent spot (R_f 0.62) comigrated with a sample of H-Val-Val-Gly-Nba independently introduced onto the same TLC plate.

Discussion

Factors $IX_{a\beta}$ and XI_a are two blood coagulation proteases whose reactivities toward synthetic peptide substrates have not been thoroughly assessed. Factor XI_a participates in the early phase of the intrinsic blood coagulation pathway. It activates factor IX in a two-step process requiring the excision of a M_r 9000 activation peptide (Fujikawa et al., 1974b). In the first step of this activation process, a limited hydrolysis of an Arg(146)*Ala(147) peptide bond (asterisk indicates bond cleaved) converts factor IX into an inactive two-chain disulfide-linked species, factor IX_α (Fujikawa et al., 1974a). In the second and rate-determining step, cleavage of an Arg(181)*Val(182) bond transforms factor IX_α into a fully active protease, factor $IX_{a\beta}$ (Katayama et al., 1979). In the design of all fluorogenic peptide substrates for factor XI_a, the P′ sequence *Val-Val-Gly corresponding to the slow step in the factor IX activation process was specifically chosen over that of the fast step, *Ala-Glu-Thr. The former sequence is common also to the activation site of factor XII, a second blood coagulant precursor that factor XI_a can cleave (Fujikawa et al., 1977, 1980). Moreover, nearly all blood coagulation zymogens have either *Val-Val-Gly or *Ile-Val-Gly as their P′ activation site sequences [see McRae et al. (1981) for a table of activation site sequences]. For these reasons, we surmised that *Val-Val-Gly-containing peptides would make better factor XI_a substrates than those containing *Ala-Glu-Thr.

Factor $IX_{a\beta}$ activates factor X through excision of an activation peptide on the N terminus of the zymogen's heavy chain (Fujikawa et al., 1974a). The resulting intermediate, factor $X_{a\alpha}$, is a two-chain disulfide-linked species having full catalytic activity. In vitro, factor $X_{a\alpha}$ autodegradates, releasing a small peptide from the C terminus of the heavy chain (Jesty et al., 1974). The resulting factor $X_{a\beta}$ is catalytically indistinguishable from factor $X_{a\alpha}$ and is the form employed in nearly all laboratory studies. Factor $IX_{a\beta}$ must form a complex composed of the protease, factor X, factor $VIII_a$, phospholipids, and Ca^{2+} in order to attain maximal catalytic turnover of its natural substrate (Van Dieijen et al., 1981). The factor X activation site sequence, Glu-Val-Val-Arg*Ile-Val-Gly was chosen in designing and synthesizing all fluorogenic peptide

substrates for factor $IX_{a\beta}$. The P residues correspond to the C-terminal sequence of the activation peptide while the P′ residues are those of the new N terminus of the heavy chain.

The goals of this research were to develop highly specific synthetic peptide substrates for factors $IX_{a\beta}$ and XI_a, to examine the relative importance of P and P′ residues on their amidolytic activities, to assess the effects of phospholipids on synthetic substrate hydrolysis, and to compare the synthetically derived peptides with the natural substrates in terms of the kinetic constants k_{cat} and K_M. Given the low reactivity of factors $IX_{a\beta}$ and XI_a toward peptide amides (Kurachi & Davie, 1977; McRae et al., 1981), our success with the *P. aeruginosa* elastase substrate, and a knowledge of the activation site sequences of the blood coagulation zymogens, we developed and successfully tested a series of fluorogenic peptide substrates for these two proteases. The peptides varied in length about the hydrolytically susceptible Arg*Ile or Arg*Val bonds. On the basis of the high specificity of the blood coagulation proteases, we expected that the longer synthetic peptides would be hydrolyzed more efficiently and specifically than the shorter ones.

Bovine Factor $IX_{a\beta}$. Surprisingly, factor $IX_{a\beta}$ failed to hydrolyze even the longest of the four fluorogenic peptides bearing factor X activation site sequences from P_4 to P_3' (Table I). McRae et al. (1981) have reported that factor $IX_{a\beta}$ also failed to hydrolyze a tripeptide thiobenzyl ester substrate with the sequence Val-Val-Arg corresponding to the P_3–P_1 residues of the factor X activation site. Factor $IX_{a\beta}$ has also been reported to be completely unreactive toward Bz-Ser-Gln-Val-Val-Arg-NA (Suomela et al., 1977). The table also shows that under these conditions, factor $IX_{a\beta}$ was unreactive toward its natural substrate. Instead, this enzyme poorly cleaved four synthetic substrates bearing factor IX activation site sequences. Within this set, Abz-Phe-Ser-Arg-Val-Val-Gly-Nba was most prone to hydrolytic cleavage. Apparently, factor $IX_{a\beta}$ is the most specific and least reactive of the blood-clotting enzymes tested.

The enzyme prefers aromatic amino acids at S_3. While Abz-Leu-Ser-Arg-Val-Val-Gly-Nba was unhydrolyzed, the analogous peptide containing a P_3 Phe was the most reactive substrate with factor $IX_{a\beta}$. McRae et al. (1981) have recorded similar observations with regard to the high specificity and unreactive nature of this protease and to the hydrophobicity of its S_3 subsite. Extension of Abz-Phe-Ser-Arg-Val-Val-Gly-Nba to include a P_4 Lys resulted in enhanced reactivity for substrates with a P_3 Leu but had no effect on those with a P_3 Phe. The S_4 subsite, however, could not accomodate a Glu. The abrasive interaction of a P_4 Glu with the enzyme is significant to the extent that no Glu-containing peptides were cleaved. The S_2 pocket of factor $IX_{a\beta}$ appears to prefer the slightly more hydrophobic and bulky side chain of Thr as opposed to that of Ser. Perhaps the greater steric resemblance between the side chains of Thr and Val, the amino acid bond at S_2 in the natural substrate, explains this observation.

In spite of their low reactivity, Abz-Phe-Ser-Arg-Val-Val-Gly-Nba and the corresponding P_4 Lys peptide are the best known synthetic peptide amide substrates of factor $IX_{a\beta}$. They are cleaved 5- and 7.5-fold better than Z-Trp-Arg-NA and the corresponding AMC derivative, respectively (McRae et al., 1981). Moreover, in the absence of phospholipids and under virtually identical assay conditions, factor $IX_{a\beta}$ cleaved these fluorogenic peptides 560-fold more efficiently than they were cleaved by factor X (van Dieijen et al., 1981).

Bovine Factor XI_a. Factor XI_a exhibited considerable reactivity toward peptides bearing factor IX activation site sequences. The protease was also substantially specific in that with one exception (Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba) it only hydrolyzed fluorogenic substrates with these sequences. Within the series of peptides that mimicked the second activation site of factor IX, the best substrates were always the longest peptides. Progressively lower K_M values are directly correlated to an increase in the peptide chain length. The concomitant increase in k_{cat}/K_M is, therefore, always attributable principally to more effective binding between enzyme and substrate since turnover numbers remained approximately constant.

The data clearly demonstrate that P residues are more important than P′ residues in substrate recognition: in comparing the two pentapeptide substrates (cf. Abz-Ser-Arg-Val-Val-Gly-Nba and Abz-Glu-Phe-Ser-Arg-Val-Nba), one observes that while the peptide extending in the P′ direction remained unhydrolyzed, the analogous P extended sequence was rapidly cleaved. Both hexapeptides in the series that mimic the second activation site sequence (Abz-Phe-Ser-Arg-Val-Val-Gly-Nba and Abz-Glu-Phe-Ser-Arg-Val-Val-Nba) were equally good substrates of factor XI_a. One may infer from this result that a P_4 Glu is equally as important as a P_3' Gly in substrate recognition. In sharp contrast, a P_3 Phe contributes significantly more than a P_2' Val in this regard. Whereas a P_3 Phe extension of Abz-Ser-Arg-Val-Val-Gly-Nba resulted in a hydrolyzable substrate (Abz-Phe-Ser-Arg-Val-Val-Gly-Nba), the effect on k_{cat}/K_M was not as pronounced in a P_2' Val extension of Abz-Glu-Phe-Ser-Arg-Val-Nba.

In an attempt to enhance the reactivity of these peptides and under the consideration of the greater importance of P residues, amino acids corresponding to the P region of the first activation site of factor IX were covalently linked to the P′ sequence Val-Val-Gly to generate four "hybrid" peptides (last four in Table I). The results indicate that Leu-Thr was favored over Phe-Ser at P_3–P_2 by a 5.4 margin (cf. k_{cat}/K_M values for Abz-Phe-Ser-Arg-Val-Val-Gly-Nba and Abz-Leu-Thr-Arg-Val-Val-Gly-Nba). The hybrid peptide Abz-Leu-Thr-Arg-Val-Val-Gly-Nba turned out to be the best fluorogenic peptide substrate of factor XI_a. The interactions of Leu and Thr with the enzyme subsites S_3 and S_2, respectively, may be dependent on the binding of P′ residues. Since factor IX activation requires a slow cleavage of a Leu-Thr-Arg*Ala peptide bond, the greater ease with which factor XI_a hydrolyzed Abz-Leu-Thr-Arg-Val-Val-Gly-Nba relative to the corresponding second activation site sequence analogue may be attributed to inherently more favorable enzyme–substrate interactions conferred by a P′ Val-Val-Gly sequence as opposed to an Ala-Gly-Thr one. The cummulative data indicate that factor XI_a has a minimal recognition site spanning no fewer than five residues (from P_4 to P_1') but has favorable interactions at least over seven subsites (S_4–S_3'). The best fluorogenic peptide substrate of factor XI_a was 5 times more prone to enzymatic hydrolysis than Z-Trp-Arg-AMC, the best amide substrate reported in the literature for this protease (McRae et al., 1981). Six of the reported substrates were better while two were approximately as reactive as this dipeptide derivative. No comparison between synthetic substrates and factor XI_a can be made at this point since a detailed kinetic analysis of factor IX activation is lacking. However, a preliminary report indicated a K_M of 15 μM for factor IX in plasma (Steinberg et al., 1980). Our best factor XI_a fluorogenic substrates had a similar K_M value. On the basis of the results with factors $IX_{a\beta}$ and $X_{a\beta}$, the fluorogenic synthetic factor XI_a peptide substrates are expected to be at least as prone as factor IX to factor XI_a hydrolytic cleavage.

The effects of the terminal Abz and Nba groups on enzymatic hydrolysis were slightly detrimental. The control peptide lacking these end groups more closely resembled factor IX and was slightly more reactive. This peptide has a K_M value that is approximately the same as that of the natural substrate.

Bovine Factor $X_{a\beta}$. Factor $X_{a\beta}$ hydrolyzed the fluorogenic peptides specifically designed as factor XI_a substrates very poorly if at all (Table II). In the most favorable cases (e.g., Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba and Abz-Leu-Ser-Arg-Val-Val-Gly-Nba), factor $X_{a\beta}$ was still 40–60 times less reactive than factor XI_a. Peptides with factor X activation site sequences remained unhydrolyzed by factor $X_{a\beta}$ even though their P′ sequences closely resembled those cleaved in prothrombin and factor VII (Magnusson et al., 1975; Radcliffe & Nemerson, 1975). Factor $X_{a\beta}$ showed no preference for either a Ser or Thr at P_2 or a Leu or Phe at P_3. This observation is somewhat surprising since a P_2 Ser occurs at the factor X_a autolysis site sequence (Jesty et al., 1975). As with factor $IX_{a\beta}$, the low efficiency constant values toward the fluorogenic substrates are attributed to poor turnover rather than poor binding of these peptides to the enzyme.

Bovine Trypsin. Trypsin was highly reactive toward all fluorogenic peptide substrates with k_{cat}/K_M values in the range of 10^4–10^6 (Table IV). Unlike the blood coagulation proteases, the effects on the efficiency constant were mostly the result of variations in k_{cat}. Generally, peptide chain extension in either the P or P′ direction had little bearing on a substrates susceptibility to hydrolysis. For the series of substrates with factor IX activation site sequences, a number of observations can be made: a P_3 Phe or Leu chain extension of Abz-Ser-Arg-Val-Val-Gly-Nba markedly increases the turnover number with the Abz-Phe-Ser-Arg-Val-Val-Gly-Nba peptide being the more reactive one. The two best trypsin substrates contain a P_4 Lys (Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba and Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba). In these cases, the preferred point of cleavage was not investigated so that enzymatic hydrolysis at both Lys and Arg are possible. Nonetheless, the reported kinetic constants reflect cleavage at either one or the other of these two possible foci since the initial scission leads to the abolition of the quenching phenomenon and to a concomitant increase in fluorescence.

The high reactivity of trypsin toward our fluorogenic peptides is not unusual in light of the sequence homology that exists at the activation site sequences of serine protease zymogens. The P′ activation site sequences of most of these zymogens, including the pancreatic ones, is either *Ile-Val-Gly or *Val-Val-Gly, as with our peptides (Jackson & Nemerson, 1980). Furthermore, trypsin, like factor XII_a, is known to activate factor XI to a fully active protease presumably after cleavage of an Arg*Ile-Val-Gly peptide bond (Mannhalter et al., 1980; Kurachi & Davie, 1977). Nearly all of the fluorogenic peptides reported here are among the best amide substrates of trypsin known. The best trypsin substrate, Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba, had a k_{cat}/K_M value 11-fold greater than that for Z-Trp-Arg-AMC (McRae et al., 1981). Carmel et al. (1973), however, reported di-, tri-, and tetralysyl fluorogenic substrates for trypsin with efficiency constant values of 1.2×10^6, 2.52×10^6, and 4.95×10^6 M^{-1} s^{-1}, respectively.

Effect of Phospholipids and Ca^{2+} Ions on Substrate Hydrolysis. The natural substrates for factors $IX_{a\beta}$ and $X_{a\beta}$ contain γ-carboxyglutamic acid or Gla residues. These Gla residues mediate the binding of the proteases, their zymogens, and protein cofactors to a phospholipid surface through Ca^{2+} bridging between the carboxyl groups of Gla and the polar heads of the phospholipids. These interactions result in the formation of discrete complexes where activation of zymogens occurs with high efficiency (Jackson & Nemerson, 1980).

Substrates that lack the Gla residues are much less reactive. Dahlbäck & Stenflo (1980) observed that a carboxyprothrombin that lacks the Gla residues was converted to thrombin by factor X_a 50 times more slowly than prothrombin. In addition, factors $IX_{a\beta}$ and $X_{a\beta}$ react at maximal rates with their natural substrates only in the presence of phospholipid vesicles, Ca^{2+}, and their protein cofactors (Jackson & Nemerson, 1980).

In the presence of Ca^{2+}, three out of four of the fluorogenic substrates reported for factor $IX_{a\beta}$ were considerably more prone than factor X to enzymatic hydrolysis (Table I). Addition of phospholipids to the reaction medium rendered the natural substrate approximately as reactive as the synthetic peptide. Phospholipids had no significant effect on the rates of hydrolysis of the synthetic substrates. When Ca^{2+}, phospholipids, and factor $VIII_a$ were all present, a k_{cat}/K_M value of 37 000 M^{-1} s^{-1} for the hydrolysis of factor X by factor $IX_{a\beta}$ can be calculated from the data of van Dieijen et al. (1981). This value represents a 52-fold rate enhancement over that observed in the absence of only factor $VIII_a$ and a 38 000-fold enhancement if both phospholipids and factor $VIII_a$ are omitted from the medium. Our best synthetic substrate has a k_{cat}/K_M value of 760 M^1 s^{-1}, which is 49-fold lower than the natural substrate under optimum conditions. We have not investigated the effect of factor $VIII_a$ on the hydrolysis of the synthetic fluorogenic substrates. However, we have shown that the bovine factor $IX_{a\beta}$ hydrolysis of Z-Trp-Arg-$SCH_2C_6H_5$, which decreased slightly (2-fold) in the presence of phospholipids, was not further affected by the addition of human factor $VIII_a$.

Similarly, except for Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba and Abz-Glu-Phe-Ser-Arg-Val-Val-Nba, which are the most specific and reactive factor XI_a fluorogenic substrates, all the other peptides cleaved by factor $X_{a\beta}$ in the presence of Ca^{2+} only were approximately as sensitive as prothrombin toward enzymatic cleavage. However, addition of phospholipids results in a 4200-fold rate enhancement for the activation of prothrombin by factor $X_{a\beta}$ (Rosing et al., 1980). Again the phospholipids had no effect on fluorogenic substrate hydrolysis and prothrombin is much more reactive with factor $X_{a\beta}$ than any of the synthetic peptides. In the presence of all the components of the prothrombinase complex, factor $X_{a\beta}$ activates prothrombin with a calculated k_{cat}/K_M value of 150 000 000 M^{-1} s^{-1} (Rosing et al., 1980). The rate enhancement here is 270-fold over that observed in the absence of only factor V_a and 1 200 000-fold over that observed in the absence of both phospholipids and factor V_a.

The fluorogenic peptides are quite small relative to factor X and prothrombin and they lack Gla residues and a specific tertiary structure, which are an integral part of the natural substrates for factor $IX_{a\beta}$ and $X_{a\beta}$, respectively. As with the natural substrates, the low k_{cat}/K_M values of these peptides with factors $IX_{a\beta}$ and $X_{a\beta}$ under virtually identical assay conditions as reported in the tables are principally the result of poor turnover rather than poor binding. The results indicate that the Ca^{2+}, phospholipids, and the protein cofactor probably act to lower the entropy of the activation complexes by properly orienting the reacting species with respect to each other.

Substrate Specificity. None of the fluorogenic peptides bore any similarity to the fibrinogen cleavage sites (α or β chain; Timpl et al., 1977; Blombäck et al., 1965). Not surprisingly, none was hydrolyzed by bovine thrombin. As expected, all

were hydrolyzed by bovine trypsin. The results of this study clearly indicate that factors $IX_{a\beta}$ and XI_a are highly specific enzymes. Factor $IX_{a\beta}$ exhibited the least reactivity and greatest substrate specificity and has a preference for substrates with P_4 Lys, P_3 Phe, and P_2 Thr. Factor $IX_{a\beta}$ only cleaved hexapeptide or heptapeptide substrates that extended at least from P_3 to P_3'. These substrates were also cleaved more slowly by factor $X_{a\beta}$ and quite rapidly by factor XI_a.

Factor XI_a was considerably more reactive than either factor $IX_{a\beta}$ or $X_{a\beta}$. With one poor exception, it only cleaved peptides bearing factor IX activation site sequences. For the factor IX activation site sequence peptides, a P_4 Glu residue was instrumental in conferring substrate specificity toward factor XI_a hydrolysis. Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba, the longest factor XI_a fluorogenic peptide substrate mimicking the second activation site sequence of factor IX, was highly specific for this protease. It remained unhydrolyzed by factor $IX_{a\beta}$ and thrombin and was poorly hydrolyzed by factor $X_{a\beta}$ (880-fold slower than by factor XI_a). Abz-Glu-Phe-Ser-Arg-Val-Val-Nba was the most specific factor XI_a substrate and was not hydrolyzed by factor $IX_{a\beta}$ or $X_{a\beta}$ or thrombin, but it was 4.7-fold less reactive toward this protease than the P_3' Gly extended peptide. Although factor XI_a was most reactive toward Abz-Leu-Thr-Arg-Val-Val-Gly-Nba, this synthetic substrate was not as specific for factor XI_a as was Abz-Glu-Phe-Ser-Arg-Val-Val-Nba or Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba. Factor $IX_{a\beta}$ slowly hydrolyzed it, and its k_{cat}/K_M value with factor $X_{a\beta}$ was 170-fold lower than that with factor XI_a.

Conclusions. Both factors $IX_{a\beta}$ and XI_a are highly selective and unreactive blood coagulation proteases with factor XI_a being more reactive. The minimal extended substrate recognition site encompass six amino acid residues for factor $IX_{a\beta}$ (from P_3 to P_3') and five for factor XI_a (from P_4 to P_1'). In addition, favorable interactions occur at other subsites, and this study clearly demonstrates that an increase in the peptide chain length results in greater substrate specificity. As a result, two highly specific synthetic fluorogenic peptide substrates have been developed for factor XI_a. The increased specificity is primarily attributable to more effective binding between the enzyme and the synthetic peptide.

In the case of factor XI_a, P residues are more important than P′ residues. Phospholipids had no effect on the reactivity of either factor $IX_{a\beta}$ or $X_{a\beta}$ toward synthetic peptide substrates, which indicates that with their natural substrates, the phospholipids and the protein cofactor facilitate the formation of a properly oriented enzyme substrate complex. In the future, it should be profitable to investigate synthetic substrates that can bind to phospholipid vesicles in a manner similar to the natural substrate for factor $IX_{a\beta}$.

Acknowledgments

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Supplementary Material Available

Description of the syntheses for all new peptides employed in this study (21 pages). Ordering information is given on any current masthead page.

Registry No. Ser-Gln-Val-Val-Arg-Ile-Val-Gly-Gly, 84192-64-3; Arg-Val-Val-Gly-Gly, 84192-65-4; Lys-Lys-Leu-Thr-Arg-Ala-Glu-Thr-Ile, 84192-66-5; Asp-Glu-Phe-Ser-Arg-Val-Val-Gly-Gly, 84192-67-6; Abz-Val-Val-Arg-Ile-Val-Nba, 84192-68-7; Abz-Val-Arg-Ile-Val-Gly-Nba, 84192-69-8; Abz-Val-Val-Arg-Ile-Val-Gly-Nba, 84192-70-1; Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba, 84192-71-2; Abz-Ser-Arg-Val-Val-Gly-Nba, 84192-72-3; Abz-Phe-Ser-Arg-Val-Val-Gly-Nba, 84192-73-4; Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba, 84192-74-5; Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2, 84192-75-6; Abz-Glu-Phe-Ser-Arg-Val-Val-Nba, 84192-76-7; Abz-Glu-Phe-Ser-Arg-Val-Nba, 84192-77-8; Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba, 84192-78-9; Abz-Leu-Ser-Arg-Val-Val-Gly-Nba, 84192-79-0; Abz-Leu-Thr-Arg-Val-Val-Gly-Nba, 84192-80-3; Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba, 84192-81-4; Ala-Ile-Glu-Gly-Arg-Thr-Ser-Glu-Asp, 84192-82-5; Tyr-Ile-Glu-Gly-Arg-Ile-Val-Glu-Gly, 84192-83-6; Ala-Ala-Gly-Ser-Arg-Gly-His-Ser-Glu, 84192-84-7; Arg-Ile-Val-Gly-Gly, 84192-85-8; Boc-Abz-OH, 68790-38-5; H-Abz-OH, 118-92-3; Boc-ON, 80994-44-1; Z-Abz-OH, 63254-88-6; Z-Cl, 501-53-1; Boc-Val-Val-OMe, 33857-88-4; Boc-Val-OH, 13734-41-3; H-Val-OMe·HCl, 6306-52-1; Z-Abz-Leu-OBzl, 84192-86-9; H-Leu-OBzl·TosOH, 1738-77-8; Boc-Ile-Val-OBzl, 75922-53-1; Boc-Ile-OH, 13139-16-7; H-Val-OBzl·TosOH, 16652-76-9; Boc-Val-Gly-OBzl, 64918-37-2; H-Gly-OBzl·TosOH, 1738-76-7; Boc-Lys(Z)-Leu-OBzl, 70375-08-5; Boc-Lys(Z)-OH, 2389-45-9; Boc-Lys(Z)-Phe-OBzl, 81019-10-5; H-Phe-OBzl·TosOH, 1738-78-9; Ac-Glu(OtBu)-Phe-OBzl, 84192-87-0; Ac-Glu(OtBu)-OH, 84192-88-1; Boc-Gln-Val-Val-OMe, 84192-89-2; Boc-Gln-ONp, 15387-45-8; H-Val-Val-OMe·HCl, 69936-04-5; Boc-Abz-Lys(Z)-Leu-OBzl, 84192-90-5; H-Lys(Z)-Leu-OBzl·HCl, 70375-12-1; Boc-Val-Val-Gly-OBzl, 84192-91-6; H-Val-Gly-OBzl·HCl, 74221-51-5; Boc-Arg(NO_2)-Ile-Val-OBzl, 84192-92-7; Boc-Arg(NO_2)-OH, 2188-18-3; H-Ile-Val-OBzl·HCl, 75922-54-2; Boc-Abz-Gln-Val-Val-OMe, 84192-93-8; H-Gln-Val-Val-OMe·HCl, 84192-94-9; Boc-Val-Arg(NO_2)-Ile-Val-OBzl, 84192-95-0; H-Arg(NO_2)-Ile-Val-OBzl·HCl, 84192-96-1; Boc-Arg(NO_2)-Val-Val-Gly-OBzl, 84192-97-2; H-Val-Val-Gly-OBzl·HCl, 84192-98-3; Boc-Ser-Arg(NO_2)-Val-Val-Gly-OBzl, 84192-99-4; Boc-Ser-OH, 3262-72-4; H-Arg(NO_2)-Val-Val-Gly-OBzl·HCl, 84193-00-0; Z-Phe-N_2H_2-Boc, 36374-63-7; Z-Phe-OH, 1161-13-3; Boc-N_2H_3, 870-46-2; Boc-Phe-N_2H_2-Z, 7801-85-6; Boc-Phe-OH, 13734-34-4; Z-N_2H_3, 5331-43-1; Z-Abz-Phe-N_2H_2-Boc, 84193-01-1; Z-Abz-Leu-N_2H_3, 84193-02-2; Ac-Glu(OtBu)-Phe-N_2H_3, 84193-03-3; Fmoc-Glu(OtBu)-Phe-N_2H_2-Z, 84193-04-4; Fmoc-Glu(OtBu)-OH, 71989-18-9; H-Phe-N_2H_2-Z·HCl, 7801-86-7; Boc-Abz-Glu(OtBu)-Phe-N_2H_2-Z, 84193-05-5; Boc-Abz-Lys(Z)-Leu-N_2H_3, 84193-06-6; Boc-Abz-Lys(Z)-Phe-N_2H_3, 84193-07-7; H-Lys(Z)-Phe-OBzl·HCl, 84193-08-8; Boc-Abz-Gln-Val-Val-N_2H_3, 84193-09-9; Boc-Abz-Gln-Val-Val-OBzl, 84193-10-2; Boc-Val-Gly-OH, 45233-75-8; Boc-Gly-Nba, 84193-11-3; Boc-Gly-OH, 4530-20-5; H-Nba·HCl, 18600-42-5; Boc-Val-Nba, 84193-12-4; Boc-Val-Gly-Nba, 84193-13-5; Boc-Val-Val-Nba, 84193-14-6; H-Val-Nba·HCl, 84193-15-7; Boc-Arg(NO_2)-Val-Nba, 84193-16-8; Boc-Ile-Val-Gly-Nba, 84193-17-9; H-Val-Gly-Nba·HCl, 84193-18-0; Boc-Val-Val-Gly-Nba, 84193-19-1; Boc-Arg(NO_2)-Val-Val-Nba, 84193-20-4; H-Val-Val-Nba·HCl, 84193-21-5; Boc-Ser(Bzl)-Arg(NO_2)-Val-Nba, 84193-22-6; Boc-Ser(Bzl)-OH, 23680-31-1; H-Arg(NO_2)-Val-Nba·HCl, 84193-23-7; Boc-Arg(NO_2)-Ile-Val-Gly-Nba, 84193-24-8; H-Ile-Val-Gly-Nba·HCl, 84193-25-9; Boc-Arg(NO_2)-Val-Val-Gly-Nba, 84193-26-0; H-Val-Val-Gly-Nba·HCl, 84193-27-1; Boc-Ser(Bzl)-Arg(NO_2)-Val-Val-Nba, 84193-28-2; H-Arg(NO_2)-Val-Val-Nba·HCl, 84193-29-3; Boc-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-30-6; H-Arg(NO_2)-Val-Val-Gly-Nba·HCl, 84193-31-7; Boc-Thr(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-32-8; Boc-Thr(Bzl)-OH, 15260-10-3; Boc-Abz-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-33-9; H-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba·HCl, 84193-34-0; Boc-Abz-Gln-Val-Val-Arg(NO_2)-Ile-Val-Gly-Nba, 84193-35-1; H-Arg(NO_2)-Ile-Val-Gly-Nba·HCl, 84193-36-2; Z-Abz-Leu-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-37-3; Z-Abz-Phe-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-38-4; Z-Abz-Phe-N_2H_3·TFA, 84215-00-9; Boc-Abz-Glu(OtBu)-Phe-Ser(Bzl)-Arg(NO_2)-Val-Nba, 84193-39-5; Boc-Abz-Glu(OtBu)-Phe-N_2H_3·HOAc, 84193-41-9; H-Ser(Bzl)-Arg(NO_2)-Val-Nba·HCl, 84193-42-0; Boc-Abz-Glu(OtBu)-Phe-Ser(Bzl)-Arg(NO_2)-Val-Val-Nba, 84193-43-1; H-Ser(Bzl)-Arg(NO_2)-Val-Val-Nba·HCl, 84193-44-2; Boc-Abz-Glu(OtBu)-Phe-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84215-01-0; Ac-Glu(OtBu)-Phe-Ser-Arg(NO_2)-Val-Val-Gly-OBzl, 84193-45-3; Ac-Glu(OtBu)-N_2H_3, 84193-46-4; H-Ser-Arg(NO_2)-Val-Val-Gly-OBzl·HCl, 84193-47-5; Ac-Glu(OtBu)-Phe-Ser-Arg(NO_2)-Val-Val-Gly-NH_2, 84193-48-6; Boc-Abz-Lys(Z)-Phe-Ser(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-49-7; Z-Abz-Leu-Thr(Bzl)-Arg(NO_2)-Val-Val-Gly-NBa, 84193-50-0; H-Thr(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba·HCl, 84193-51-1; Boc-Abz-Lys-(Z)-Leu-Thr(Bzl)-Arg(NO_2)-Val-Val-Gly-Nba, 84193-52-2; H-Abz-Val-Val-Arg-Ile-Val-Nba·2HCl, 84193-53-3; H-Val-Arg(NO_2)-Ile-OBzl·TFA,

84193-55-5; Boc-Val-Val-Arg(NO_2)-Ile-Val-OBzl, 84193-56-6; Boc-Abz-Val-Val-Arg(NO_2)-Ile-Val-OBzl, 84193-57-7; Boc-Abz-Val-Val-Arg-Ile-Val-Nba, 84193-58-8; H-Abz-Val-Arg-Ile-Val-Gly-Nba·3HCl, 84193-59-9; H-Val-Arg(NO_2)-Ile-Val-OBzl·TFA, 84193-61-3; Boc-Abz-Val-Arg(NO_2)-Ile-Val-OBzl, 84215-02-1; Boc-Abz-Val-Arg-Ile-Val-OH, 84193-62-4; H-Gly-Nba·HCl, 84193-63-5; Boc-Abz-Val-Arg-Ile-Val-Gly-Nba, 84193-64-6; H-Abz-Val-Val-Arg-Ile-Val-Gly-Nba·2HCl, 84193-65-7; Boc-Abz-Val-Val-Arg-Ile-Val-OH, 84193-66-8; Boc-Abz-Val-Val-Arg-Ile-Val-Gly-Nba, 84193-67-9; H-Abz-Gln-Val-Val-Arg-Ile-Val-Gly-Nba·2HF, 84193-68-0; H-Abz-Ser-Arg-Val-Val-Nba·*x*HF, 84193-69-1; H-Abz-Leu-Ser-Arg-Val-Val-Gly-Nba·*x*HF, 84193-70-4; H-Abz-Phe-Ser-Arg-Val-Val-Gly-Nba·*x*HF, 84193-71-5; H-Abz-Glu-Phe-Ser-Arg-Val-Nba·*x*HF, 84193-72-6; H-Abz-Glu-Phe-Ser-Arg-Val-Val-Nba·*x*HF, 84193-73-7; H-Abz-Glu-Phe-Ser-Arg-Val-Val-Gly-Nba·*x*HF, 84193-74-8; Ac-Glu-Phe-Ser-Arg-Val-Val-Gly-NH_2·*x*HF, 84193-75-9; H-Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba·*x*HF, 84193-76-0; H-Abz-Leu-Thr-Arg-Val-Val-Gly-Nba·*x*HF, 84193-77-1; H-Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba·*x*HF, 84193-78-2; factor $IX_{a\beta}$, 66526-18-9; factor $X_{a\beta}$, 73613-00-0; factor XI_a, 37203-61-5; trypsin, 9002-07-7.

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