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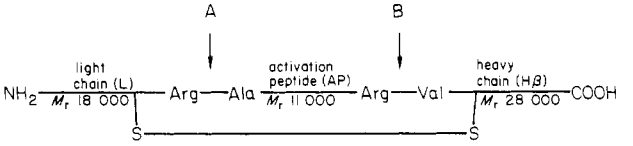
Redetermination of the Rate-Limiting Step in the Activation of Factor IX by Factor XIa and by Factor VIIa/Tissue Factor. Explanation for Different Electrophoretic Radioactivity Profiles Obtained on Activation of ^{3}H- and ^{125}I-Labeled Factor IX†

S. Paul Bajaj,* Samuel I. Rapaport, and William A. Russell

ABSTRACT: During activation of factor IX by factor XIa or by factor VIIa/tissue factor, two peptide bonds are cleaved, an Arg–Ala bond toward the NH_2 terminus and an Arg–Val bond toward the COOH terminus of the molecule. Investigators have disagreed as to the order of bond cleavages and as to which of the peptide bond cleavages constitutes the rate-limiting step. Utilizing sialyl-^{3}H human factor IX and monitoring the activation of the molecule by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis, we demonstrate that activation by each enzyme results initially in the accumulation of the inactive intermediate, factor IXα, which is the product formed by cleavage of the Arg–Ala bond. Subsequently, factor IXα is converted, by the cleavage of the Arg–Val bond, to factor IXaβ. Thus, the cleavage of this second peptide bond is slow and rate limiting when factor IX is activated by either factor XIa or factor VIIa. Furthermore, these data do not support a recent hypothesis, proposed for the bovine molecule, that factor XIa and factor VIIa, by differing in their rate-limiting steps, act synergistically in catalyzing factor IX activation. Steady-state kinetic analysis utilizing purified human factor VIIa in the presence of 5 mM Ca^{2+} and human brain tissue factor sufficient to saturate factor VIIa gave an apparent K_m of about 250 nM and k_{cat} of 13 min^{-1}. The comparable values for human factor IX utilizing XIa as the enzyme are K_m = 2 μM and k_{cat} = 10 min^{-1} [Bajaj, S. P. (1982) *J. Biol. Chem. 257*, 4127–4132]. In additional experiments, we compared the $NaDodSO_4$ gel electrophoretic radioactivity profiles obtained on activation of ^{3}H factor IX and on activation of ^{125}I factor IX. A radioactivity peak corresponding to the "smaller" heavy chain, Hβ, of factor IXaβ was not found upon activation of ^{3}H factor IX. In contrast, a radioactivity peak corresponding to the activation peptide released on formation of factor IXaβ was not found on activation of ^{125}I factor IX. This explains the striking differences in the radioactivity profiles observed with these two labels.

During the activation of single chain human and bovine blood coagulation factor IX by factor XIa and by factor VIIa/tissue factor two peptide bonds are cleaved to yield a smaller molecule and an activation peptide (Fujikawa et al., 1974; Østerud & Rapaport, 1977; Lindquist et al., 1978; DiScipio et al., 1978; Østerud et al., 1978; Zur & Nemerson, 1980). From the data of DiScipio et al. (1978), the following partial structure for human factor IX may be constructed:



human factor IX

Utilizing bovine proteins, Fujikawa et al. (1974) and Lindquist et al. (1978) reported that the first step in the

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activation of factor IX by factor XIa is the cleavage of the peptide bond A (Arg–Ala) that results in the accumulation of a two-chain inactive intermediate, factor IXα.[1] In the second step, which they concluded was rate limiting, a specific Arg–Val bond (peptide bond B) is cleaved, giving rise to factor IXaβ and an activation peptide. DiScipio et al. (1978) obtained similar data for the activation of human factor IX by factor XIa. In contrast, Østerud et al. (1978) reported that factor IXα intermediate was not observed in their studies of the activation of human factor IX by factor XIa. Therefore, these investigators concluded that the initial peptide bond cleavage is rate limiting in this reaction.

Few published data are available on the products of activation of factor IX by factor VIIa. P. A. Lindquist and E. W. Davie[2] have indicated that the factor IXa generated by activation with factor VIIa is identical with the factor IXaβ molecule generated by activation with factor XIa. Zur & Nemerson (1980) reported that they could not detect factor IXα in gels prepared from activation mixtures of bovine factor IX, factor VIIa, tissue factor, and calcium. Therefore, they concluded that the initial Arg–Ala bond (peptide bond A) cleavage is the rate-limiting step during the activation of bovine factor IX by factor VIIa. These data raise the possibility that factor XIa and factor VIIa activate factor IX by proteolytic events with different rate-limiting steps and that these enzymes, therefore, could act synergetically in the physiological activation of factor IX (Zur & Nemerson, 1980).

This laboratory has been involved in studies of the activation of variant factor IX molecules found in different patients in hemophilia B. However, analysis of such data requires a clear understanding of the mechanism of proteolysis of normal factor IX by both factor XIa and factor VIIa. Therefore, we have reinvestigated the activation of normal human factor IX by factor XIa and by factor VIIa. We report herein that the cleavage of the Arg–Ala peptide bond occurs first with both enzymes and that they do not differ in their rate-limiting steps. In addition we have identified the reasons for striking differences in the sodium dodecyl sulfate ($NaDodSO_4$)[3] gel electrophoretic profiles of activation mixtures containing ^3H factor IX and activation mixtures containing ^{125}I factor IX.

Experimental Procedures

Materials. $Na^{125}I$ and sodium [^3H]borohydride were purchased from the Amersham Corp. Ammonium persulfate, 2-mercaptoethanol, acrylamide, *N,N,N′,N′*-tetramethylethylenediamine, and glycine were all of electrophoretic purity grade and were products of Bio-Rad. Other reagents and chemicals were of the best commercially available grade.

Proteins. Human factor VII was purified as described previously (Bajaj et al., 1981a). Initial steps for isolation of human factor IX and factor X were the same as described for factor VII. Further purification of factor IX and factor X was achieved exactly as described (Bajaj et al., 1981b). Human factor XI was purified by the method of Kurachi & Davie (1977) except the last step of purification, benzamidine–agarose column chromatography, was not performed. RVV was purified by the procedure of Furie et al. (1974). Factor Xa (Bajaj et al., 1981b) and factor XIa (Bajaj, 1982) were prepared as described. All protein preparations were greater than 95% homogeneous upon examination by $NaDodSO_4$ gel electrophoresis. Protein concentrations were determined from their known extinction coefficients.

Sialyl-^3H factor IX was prepared by the technique of Van Lenten & Ashwell (1971) as reported (Bajaj, 1982). Specific activity of the preparation used was 2.2×10^8 cpm/mg of protein; the labeled protein possessed 82% of the biological activity of the nonlabeled control. Tyrosyl-^{125}I factor IX was prepared by using Bio-Rad Enzymobead reagent. The method employed was the same as described for prothrombin (Bajaj et al., 1981c). ^{125}I factor IX had 1.1×10^9 cpm/mg of protein and 94% of the biological activity of the nonlabeled control.

Tissue Factor. Human brain tissue factor was prepared as described by Owren (1949). Briefly, after the cerebral hemispheres were washed with copious amounts of distilled water, the brain was cut into 1–2-cm^2 pieces and soaked in 2.85% sodium citrate diluted 10-fold with 25 mM Na_2EDTA. The pieces were extensively washed first with this citrate–EDTA solution and then with Tris/NaCl buffer. The tissue was ground for 30 s in a Waring blender with a volume of Tris/NaCl equal to the weight of brain. The suspension was centrifuged for 10 min at 3000*g*, and the pellet was discarded.

The finer suspension clotted recalcified plasma in 14 s (prothrombin time) and was centrifuged at 12000*g* for 10 min. The supernatant clotted recalcified plasma in 18 s and was used in experiments dealing with the kinetics of activation of radiolabeled factor IX and mixtures of radiolabeled and nonlabeled factor IX. The pellet was washed 4 times with Tris/NaCl containing 25 mM Na_2EDTA and then 5 times with Tris/NaCl without EDTA. The volume of each wash was the same as the volume of starting tissue factor suspension. The pellet was then suspended in Tris/NaCl by using one-third the volume of starting tissue factor suspension. It clotted recalcified plasma in 25 s and was used in experiments with nonlabeled factor IX.

Measurement of Radioactivity Profiles of $NaDodSO_4$ Gels. $NaDodSO_4$ gel electrophoresis was performed according to the method of Laemmli (1970). The protein standards used to determine apparent molecular weights have been described elsewhere (Bajaj, 1982). Unless otherwise noted, ^3H radioactivity profiles of gels were obtained by adding 0.5 mL of water extracts of 1-mm gel slices to 4 mL of Aquasol 2 (New England Nuclear), measuring radioactivity in a Beckman LS 7500 β counter, and utilizing a computer program to plot the data. ^{125}I radioactivity profiles of 1-mm slices were obtained on a Tracor Analytic 1197 γ counter.

Rate of Activation of Factor IX. The rate of activation of ^3H factor IX by factor VIIa/Ca^{2+}/tissue factor was monitored by the activation peptide release assay of Zur & Nemerson (1980) as described (Bajaj, 1982). The buffer used was Tris/NaCl/Alb, pH 7.5. Complete activation of factor IX, as evaluated by $NaDodSO_4$ gel electrophoresis, yielded approximately 40% of the total ^3H counts in the trichloroacetic acid supernatant. This control experiment was carried out for each set of activation reactions and was used to determine the amount of factor IXaβ formed at given reaction times. In

[1] The nomenclature used for factor IX and its activation products is that of Davie and co-workers (Fujikawa et al., 1974; Lindquist et al., 1978; DiScipio et al., 1978): IX, single-chain native factor IX (M_r 57 000); IXα, two-chain inactive intermediate (IX cleaved at Arg–Ala bond) consisting of a heavier heavy chain (Hα; M_r 39 000) and light chain (L; M_r 18 000); IXaα, a factor IXa molecule (IX cleaved at Arg–Val bond) consisting of two chains L plus AP (M_r 29 000) and Hβ (M_r 28 000); IXaβ, the final two-chain factor IXa (IX cleaved at both Arg–Ala and Arg–Val bonds) consisting of L and Hβ. AP (M_r 11 000) denotes activation peptide.

[2] Personal communication.

[3] Abbreviations: RVV, factor X activating protein of Russell's viper venom; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Tris/NaCl buffer, 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.5; Tris/NaCl/Alb buffer, Tris/NaCl buffer containing 1 mg/mL bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

other control experiments, factor Xa alone and a mixture of tissue factor (50% v/v of final reaction volume) and factor Xa at the concentrations used to convert factor VII to factor VIIa failed to generate factor IXaβ in the time frame (up to 1 h) of these experiments. The statistical analyses of the kinetic data are described elsewhere (Bajaj, 1982).

For the kinetic experiments it was necessary to determine the amount of our tissue factor (supernatant preparation; see above) needed to saturate a given amount of factor VIIa. This was determined as follows: Fifteen microliters of factor VII, 2.5 μg/mL, was incubated with varying volumes of tissue factor from 25 to 85 μL and sufficient Tris/NaCl/Alb buffer to give a final volume of 100 μL. Then, 5 μL of factor Xa, 6 μg/mL, and 2 μL of $CaCl_2$, 0.5 M, were added, and the mixture was incubated for 10 min to allow full activation of factor VII to factor VIIa. Ten microliters of the incubation mixture was added to 90 μL of Tris/NaCl/Alb, pH 7.5, containing 5 mM EDTA. The sample was further diluted 10–50-fold in Tris/NaCl/Alb, pH 7.5, without EDTA, and assayed in a modified one-stage factor VII clotting assay. In this assay 50 μL of factor VII deficient plasma was incubated with 50 μL of either tissue factor or cephalin for 3 min at 37 °C. Then, 25 μL of the test sample and 50 μL of 35 mM $CaCl_2$ were added, and the clotting time was noted. The factor VIIa of the initial incubation mixture was considered saturated with tissue factor if the test sample gave the same clotting time in the factor VII assay with cephalin and the factor VII assay with tissue factor. By using this technique, we found that 23 μL of our tissue factor preparation saturated 10 ng of factor VII.

The following kinetic experiments also validate our above conclusion. Thus, when we used 50 ng/mL factor VIIa, 5 mM Ca^{2+}, 10 μg/mL ^{3}H factor IX, and 4% (v/v) tissue factor preparation in our reaction mixtures, we obtained a velocity of generation of factor IXaβ of 0.12 μg mL^{-1} min^{-1}; when we used 11% (v/v) tissue factor, the rate of factor IXaβ formation was 0.25 μg mL^{-1} min^{-1}. When 12–17% (v/v) tissue factor was used, a further increase in the rate of activation of factor IX was not found, and velocities of generation of factor IXaβ were from 0.21 to 0.25 μg mL^{-1} min^{-1}. Virtually the same rate (0.23 μg mL^{-1} min^{-1}) was obtained when tritiated factor IX was diluted with an equal amount of unlabeled factor IX. (The rate predicted from kinetic constants given in Table I will be 0.30 μg mL^{-1} min^{-1}.) These data provide reasonable evidence that 20–25 μL of our tissue factor preparation saturated 10 ng of factor VIIa and that tritiated factor IX and unlabeled factor IX behaved similarly in our kinetic experiments.

Results

Activation of Unlabeled Factor IX by Factor VIIa/Tissue Factor Complex and by Factor XIa. A reaction mixture (total volume 1 mL) was prepared containing 0.5 mg/mL unlabeled factor IX, 50 ng/mL factor VIIa, 5 mM Ca^{2+}, and 65% (v/v) tissue factor (from which soluble proteins had been removed by centrifugation; see Experimental Procedures). Under these conditions, whether all of factor VIIa was bound to tissue factor was not determined; however, conclusions drawn from this experiment are not affected by the presence of free factor VIIa in solution (see control experiment below; Figure 1C). Fifty-microliter aliquots of the reaction mixture were withdrawn at different times and centrifuged for 1 min in the Eppendorf centrifuge. Forty microliters of the supernatant was then added to an equal volume of $NaDodSO_4$ gel buffer, heated for 20 min at 80 °C, and analyzed by $NaDodSO_4$ gel electrophoresis. The representative electrophoretograms (from

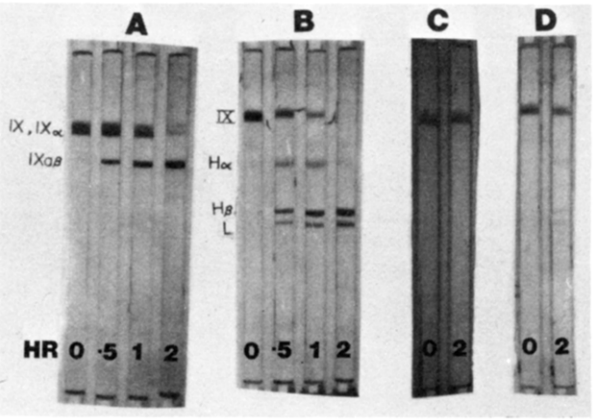

FIGURE 1: $NaDodSO_4$ gel electrophoretic analysis of the activation of unlabeled human factor IX by factor VIIa/tissue factor (see text for details). Each gel (12% acrylamide) represents 20 μL (containing 10 μg of protein) of the reaction mixture components. Gels are shown from three incubation mixtures. The numbers on the gels refer to the incubation times in hours, and the anode is at the bottom of the gels. A representative sample of the total number of samples is depicted. The A panel represents the unreduced samples, and the B panel represents the reduced samples of the activation mixture containing factor IX, factor VIIa, tissue factor, and Ca^{2+}. The C panel represents the reduced samples from a reaction mixture in which Tris/NaCl replaced tissue factor, and the D panel represents the reduced samples from a reaction mixture in which Tris/NaCl replaced factor VIIa.

40 gels) prepared in this fashion are depicted in Figure 1A,B. At zero time, factor IX appeared as a single chain molecule. In nonreduced gels, a new faster moving band (factor IXaβ, M_r ~45 000) was observed during the activation (Figure 1A). In reduced gels (Figure 1B), three new bands were observed. The two faster moving bands (labeled Hβ and L, Figure 1B) correspond to the heavy and light chain of factor IXaβ. The third protein band (labeled Hα, Figure 1B) appeared very early, remained fairly constant up to 90 min, and then disappeared as the reaction went to completion. Since the activation peptide stains poorly (DiScipio et al., 1978), it was not observed in these gels. In control experiments, in which Tris/NaCl buffer replaced tissue factor, the activation of factor IX was not observed (Figure 1C). In another control experiment, incubation of factor IX with tissue factor and Ca^{2+} in the absence of added factor VIIa resulted in less than 5% activation of factor IX up to 2 h (Figure 1D). This activation could be due to a small amount of factor VII (or other proteases) present as a contaminant in our factor IX or in our tissue factor preparation.

The data presented in Figure 1 suggest that the mechanism of activation of human factor IX by factor VIIa/tissue factor complex is essentially identical with that observed by DiScipio et al. (1978) by utilizing factor XIa as the enzyme. We also obtained identical results (data not given) when we activated human factor IX by factor XIa employing the conditions described by DiScipio et al. (1978). Thus, it appears that the activation mechanism and the rate-limiting step in the activation of human factor IX by factor XIa and by factor VIIa/tissue factor complex are the same.

In the following sections we performed experiments utilizing radioactive human factor IX and soluble tissue factor (see Experimental Procedures). The purpose of these experiments is to test the above conclusions derived from the data by using nonlabeled factor IX and to understand the reason(s) for different radioactivity profiles obtained during the activation of ^{3}H factor IX and ^{125}I factor IX.

Activation of ^{3}H Factor IX by Factor VIIa/Tissue Factor Complex. An activation mixture was made containing ^{3}H

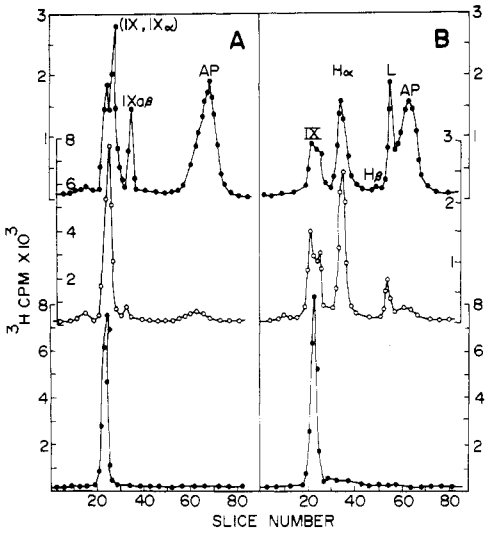

FIGURE 2: $NaDodSO_4$ radioactivity profiles of the activation of ^3H factor IX by factor VIIa/tissue factor complex. In the plots on the left (A) disulfide bonds are intact; in the plots on the right (B) disulfide bonds are reduced. The reaction mixture contained the following: ^3H factor IX, 30 μg/mL; factor VIIa, 50 ng/mL; tissue factor, 12% by volume; Ca^{2+}, 5 mM, at 37 °C. The bottom profiles are at zero time, the middle profiles are at 10 min, and the top profiles are at 120 min. At these times 50-μL aliquots were withdrawn and inactivated by adding an equal volume of $NaDodSO_4$ protein buffer in the presence or absence of 2-mercaptoethanol and by immediate heating at 80 °C for 20 min. $NaDodSO_4$ gel electrophoresis was performed by using 12% acrylamide concentration. One-millimeter gel slices were incubated for 24 h in 0.5 mL of distilled water, and a 50-μL aliquot was then counted as described under Experimental Procedures. Abbreviations are explained in footnote 1. For validation of identification of components, see data of Figure 3.

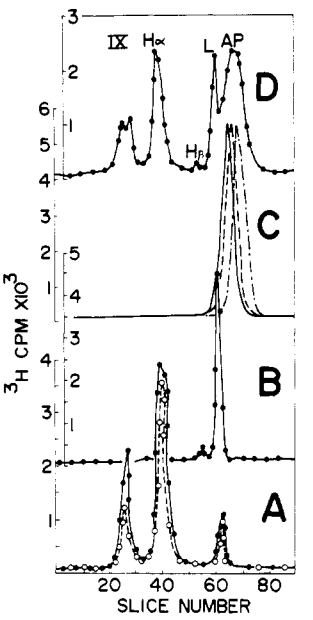

FIGURE 3: Identification of the radioactivity peaks of the products of activation of ^3H factor IX. The remaining 450-μL aliquots of protein eluates of the selected gel slices of 120-min activated sample (top curve, Figure 2A) were pooled, filtered (to remove gel particles) and lyophilized. The lyophilized samples were then subjected to $NaDodSO_4$ gel electrophoresis (12% acrylamide concentration) in the presence of 2-mercaptoethanol. The gels were sliced and counted for radioactivity as described under Experimental Procedures. ^3H radioactivity profiles of pooled and reduced protein eluate are shown as follows: (A) slices 22–24 (○) and 26–28 (●); (B) slices 33–34 (●); (C) slices 59–62 (—), 63–66 (- - -), and 67–71 (· · ·). (D) illustrates a complete 120-min activation mixture subjected to reduced $NaDodSO_4$ gel electrophoresis under conditions similar to the top curve of Figure 2B.

factor IX (0.6 μM), factor VIIa (1 nM), tissue factor (12% v/v), and Ca^{2+} (5 mM), and samples were taken at various time intervals for analysis by $NaDodSO_4$ gel electrophoresis. Figure 2 depicts the ^3H radioactivity profiles of samples removed at zero time and 10 and 120 min. Their examination leads to two important observations. The first is that the 10-min samples contain substantial amounts of factor IXα but only insignificant amounts of activation peptide. Thus, the initial cleavage has occurred at the Arg–Ala site, with resultant formation of an inactive intermediate molecule containing the "heavier heavy chain", Hα, and the light chain, L. The second observation is that the appearance of large amounts of activation peptide on the 120-min gel profiles is not associated with the appearance of a peak of ^3H radioactivity corresponding to Hβ. Apparently the labeling of the Hα segment of factor IX by ^3H is confined to the activation peptide portion of the chain, and as a consequence of the second cleavage at the Arg–Val site, and release of the activation peptide, the resultant Hβ possesses no significant ^3H radioactivity.

The analysis of the peaks of radioactivity depicted in Figure 2 was validated by the experiment illustrated in Figure 3. For each radioactivity peak of the 120-min nonreduced gel (top curve, Figure 2A), the remaining 450 μL of elutes of selected slices was pooled. (For further explanation, see legends to Figures 2 and 3.) The protein of each pooled eluate was reduced with 2-mercaptoethanol, and the samples were subjected to $NaDodSO_4$ gel electrophoresis. As shown in Figure 3A, each of the peaks of the doublet between slices 22 and 28 of the top curve of Figure 2A contains both single-chain native factor IX and the two-chain factor IXα intermediate, composed of Hα and L chains. Of particular importance is the demonstration in Figure 3B that reduction of the protein in slices 33–34 of the top curve of Figure 2A (factor IXaβ) yields a major peak of radioactivity corresponding to the light chain, L, and only an insignificant peak of radioactivity corresponding to the heavy chain, Hβ. The peaks of Figure 3C obtained from different pooled slices of the broad activation peptide peak of the top curve of Figure 2A suggest that the breadth of that peak is attributable to heterogeneity of the activation peptide, perhaps secondary to variation in its carbohydrate moieties. The profile of Figure 3D, obtained from a 120-min, reduced total activation mixture, is shown for orientation.

Comparison of the Activation Products Formed by Activation of ^3H Factor IX with Factor VIIa/Tissue Factor, by Factor XIa, and by RVV. The bottom curve of Figure 4 is a radioactivity profile obtained on reduced $NaDodSO_4$ gel electrophoresis of the activation products of ^3H factor IX incubated with tissue factor, Ca^{2+}, and factor VIIa. A similar profile was obtained for the activation products of ^3H factor IX incubated with factor XIa and Ca^{2+} (see bottom curve of Figure 5). A mixture of equal amounts of the two reaction mixtures gave the radioactivity profile shown in the top curve of Figure 4. This illustrates that the activation products of ^3H factor IX produced by the factor VIIa/tissue factor complex and by factor XIa are electrophoretically indistinguishable.

Figure 5 shows the different radioactivity profiles obtained on reduced $NaDodSO_4$ gel electrophoresis of ^3H factor IX activated with RVV (middle curve) and activated with factor XIa (bottom curve). As reported earlier by DiScipio et al. (1978), the primary product of RVV activation of factor IX was factor IXaα, an activated molecule in which only the Arg–Val bond is cleaved. This molecule yields, on reduced $NaDodSO_4$ gel electrophoresis, Hβ, which as described above

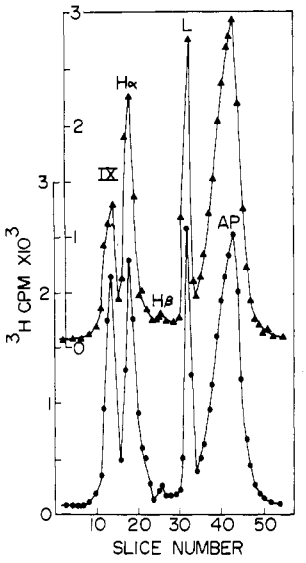

FIGURE 4: Comparative electrophoretic analysis of the activation products formed during the activation of ^{3}H factor IX by factor VIIa/tissue factor and by factor XIa. Reaction mixtures contained 10 μg/mL ^{3}H factor IX, 5 mM Ca^{2+}, and one of the following: factor XIa (4 μg/mL) or factor VIIa (50 ng/mL) and tissue factor (12% by volume). The reaction mixture was inactivated (at 20 min for factor XIa and at 120 min for factor VIIa/tissue factor) by the addition of an equal volume of $NaDodSO_4$ protein buffer containing 10% (v/v) 2-mercaptoethanol and subjected to SDS gel electrophoresis (18% acrylamide concentration). The gels were sliced and counted for radioactivity as described under Experimental Procedures. The bottom curve represents 50 μL of the reaction mixture activated by factor VIIa/tissue factor and the top curve represents a mixture of 25 μL of the reaction mixture activated by factor VIIa/tissue factor and 25 μL of the reaction mixture activated by factor XIa. The profile obtained from 50 μL of reaction mixture activated by factor XIa is shown in the bottom curve of Figure 5.

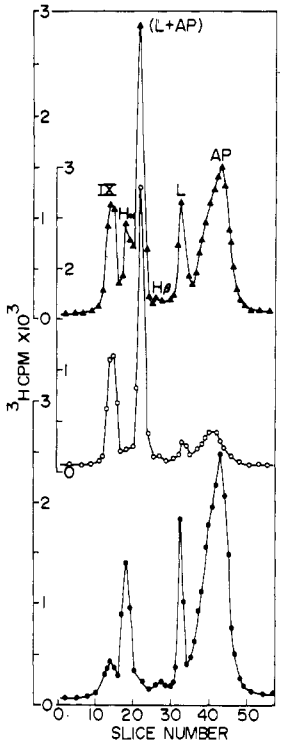

FIGURE 5: Comparative electrophoretic analysis of the activation products formed during the activation of ^{3}H factor IX by RVV and by factor XIa. Reaction mixtures contained 10 μg/mL ^{3}H factor IX, 5 mM Ca^{2+}, and either 0.4 μg/mL RVV or 4 μg/mL factor XIa. Reaction mixtures were inactivated at 20 min. Other conditions are as described in the legend to Figure 4. The bottom curve was from 50 μL of the reaction mixture activated by factor XIa, the middle curve was obtained from 50 μL of the reaction mixture activated by RVV, and the top curve was obtained from a mixture of 25 μL of each reaction mixture.

does not contain significant ^{3}H radioactivity, and a unique chain made up of the light chain plus the activation peptide (L plus AP). Small amounts of radioactivity are also seen on the profile corresponding to the separate light chain and activation peptide, which indicates that a small amount of factor IXaα has been converted to factor IXaβ.

When equal amounts of ^{3}H factor IX activated by factor XIa and by RVV were mixed and analyzed by reduced $NaDodSO_4$ gel electrophoresis, the radioactivity profile shown in the top curve of Figure 5 was obtained. It shows that the heavy chain, Hα, can be distinguished from the peptide chain composed of the L plus AP segment of factor IX. It is important to point out that this separation requires the use of 18% acrylamide in the gels.

It is interesting to compare the radioactivity profile of a mixture of ^{3}H factor IX activated by factor XIa and by factor VIIa/tissue factor complex (top curve, Figure 4) with the radioactivity profile of a mixture of ^{3}H factor IX activated by factor XIa and by RVV (top curve, Figure 5). The two profiles differ in that the profile of the former mixture does not contain a radioactivity peak corresponding to the L plus AP segment of factor IX. Thus, it would appear that factor IXaα is not generated in any substantial amount during activation of factor IX by either factor XIa or factor VIIa/tissue factor.

Comparison of the Radioactivity Profiles of the Activation Products of ^{3}H Factor IX and of ^{125}I Factor IX. Factor IX, either ^{3}H labeled or ^{125}I labeled, was activated by factor XIa, and the activation mixtures were analyzed by reduced $NaDodSO_4$ gel electrophoresis. The radioactivity profiles obtained are shown in Figure 6. Two striking differences are evident. A ^{3}H radioactivity peak is missing corresponding to the heavy

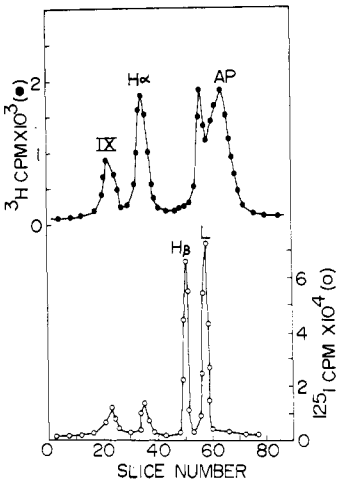

FIGURE 6: Comparison of the radioactivity profiles of the activation of ^{3}H factor IX and of ^{125}I factor IX. The reaction mixture contained 10 μg/mL either ^{3}H factor IX or ^{125}I factor IX, 5 mM Ca^{2+} and 4 μg/mL factor XIa. After 20 min at 37 °C the reaction mixture was inactivated by adding an equal volume of $NaDodSO_4$ buffer containing 10% 2-mercaptoethanol and subjected to $NaDodSO_4$ gel electrophoresis (12% acrylamide concentration). The gels were sliced and counted for radioactivity as described under Experimental Procedures. (●) Profile obtained with 50 μL of reaction mixture containing ^{3}H factor IX; (O) profile obtained with 50 μL of reaction mixture containing ^{125}I factor IX.

chain, Hβ, and a ^{125}I radioactivity peak is missing corresponding to the activation peptide, AP.

Kinetics of Activation of Human Factor IX by Factor VIIa/Tissue Factor Complex. Reaction mixtures were made containing six concentrations of factor IX (mixtures of ^{3}H

Table I: Steady State Apparent Kinetic Parameters of the Activation of Factor IX by Factor VIIa/Tissue Factor[a]

tissue factor concn (volume fraction)	phospholipid content in reaction mixture (mM)	apparent K_m (nM)	apparent V_{max} (nmol L^{-1} min^{-1})
0.17	1.24	254	13
0.17	2.18[b]	352	15
0.51	3.72	540	33

[a] The concentration of the enzyme was 1 nM, Ca^{2+} was 5 mM, and the substrate concentration ranged from 0.175 (~10 μg/mL) to 2 μM (~10 μg/mL). In each set of experiments, a total of six substrate concentrations were utilized. [b] 0.34 volume fraction was rabbit brain phospholipid containing 2.76 mM organic phosphate.

factor IX and unlabeled factor IX) between 0.17 and 2 μM, factor VIIa at 1 nM, Ca^{2+} at 5 mM, and tissue factor at 17% by volume. This 17% volume fraction of tissue factor exceeded the 12% volume calculated to saturate 1 nM factor VII (see Experimental Procedures). Initial rates of generation of factor IXa were measured by the activation peptide release assay. In one additional set of experiments the tissue factor concentration of the reaction mixture was increased to 51% by volume. In another additional set of experiments the tissue factor concentration of the reaction mixture was kept at 17% by volume but the phospholipid concentration was increased by adding concentrated rabbit brain cephalin. Michaelis–Menten kinetic constants were calculated from Lineweaver–Burk plots obtained from the concentration and initial velocity values by utilizing the linear regression analysis method (Bajaj, 1982). The data are summarized in Table I.

Discussion

Although the cleavage sites for activation of factor IX by factor XIa (Fujikawa et al., 1974; Lindquist et al., 1978; DiScipio et al., 1978) and by factor VIIa/tissue factor[2] are established, opinion has differed as to the order of bond cleavages and as to which cleavage constitutes the rate-limiting step (Fujikawa et al., 1974; Lindquist et al., 1978; DiScipio et al., 1978; Østerud et al., 1978; Zur & Nemerson, 1980; Østerud & Rapaport, 1980).

In the data presented herein, profiles of reduced $NaDodSO_4$ gels of samples taken during the activation of human factor IX by either factor XIa (Figures 4–6) or by factor VIIa/tissue factor (Figures 2–4) consistently revealed a substantial peak of radioactivity corresponding to the Hα chain of factor IXα. In contrast, a chain corresponding to the L plus AP segments of factor IXaα, readily demonstrable in activation mixtures of factor IX by RVV, was not found in activation mixtures made with either factor XIa or factor VIIa/tissue factor (compare Figures 4 and 5). Thus, for activation of factor IX by both factor XIa and factor VIIa/tissue factor, we conclude (a) that the initial step is cleavage of the Arg–Ala bond and (b) that the subsequent step, cleavage of the Arg–Val bond, is rate limiting.

Our data for activation by factor XIa confirm the earlier observations of Fujikawa et al. (1974) with bovine factor IX and of DiScipio et al. (1978) with human factor IX. They do not support the conclusions of Østerud et al. (1978) for human factor IX. Our data for activation by factor VIIa/tissue factor confirm the preliminary observations of Østerud & Rapaport (1980). They do not agree with the conclusions of Zur & Nemerson (1980), who reported, utilizing bovine proteins, that factor XIa and factor VIIa differed in their rate-limiting steps. On the basis of this, these investigators (Zur & Nemerson, 1980) proposed that factor XIa and factor VIIa/tissue factor act in concert in catalyzing bovine factor IX activation. Although this hypothesis is appealing, our data render it untenable for human factor IX. Thus, it appears that there could be a real difference between human factor IX activation and bovine factor IX activation mechanisms.

The activation products of ^3H factor IX and of ^{125}I factor IX yielded notable different radioactivity profiles on reduced $NaDodSO_4$ gel electrophoresis (Figure 6). This resulted from the absence of a radioactivity peak corresponding to the Hβ chain on radioactivity profiles made with ^3H factor IX and the absence of a radioactivity peak corresponding to the activation peptide on radioactivity profiles made with ^{125}I factor IX. Apparently, very little tritium is incorporated into the carboxyl-terminal latent Hβ segment of native factor IX, despite the reported presence of two residues of neuraminic acid (DiScipio et al., 1978) in this segment of the molecule. It is possible that the C-7, C-8, and C-9 side chains of neuraminic acid in the Hβ segment of the molecule are partially substituted by ester groups, which by reducing the rate of periodate consumption (Schauer, 1978) would impair the incorporation of ^3H at the borohydride reduction step. Alternatively, the neuraminic acid residues in the latent portion of the factor IX molecule may be sterically hindered or unavailable for the reactions involved in periodate oxidation and borohydride reduction. The absence of a ^{125}I radioactivity peak corresponding to the activation peptide is consistent with the known low tyrosine content of the activation peptide (DiScipio et al., 1978).

The kinetic constants observed in the present study for the activation of human factor IX by factor VIIa/tissue factor are summarized in Table I. The apparent K_m value of 254 nM, which is considerably higher than the apparent K_m value of 87 nM for bovine factor IX reported by Zur & Nemerson (1980), must be interpreted with the knowledge which these investigators found that the value for K_m increases when the phospholipid content of activation mixtures is increased. Thus, when they increased the phospholipid content of their activation mixtures, they obtained values for apparent K_m from 177 to 243 nM. We were unable to lower the concentration of phospholipid, present as a component of the tissue factor preparation, below a concentration of 1.24 mM and still provide sufficient tissue factor in our activation mixtures to saturate factor VIIa. When we increased the phospholipid content of our activation mixtures, either by adding additional tissue factor or by adding phospholipid, the value for apparent K_m was further increased. Our k_{cat} values of 13–33 min^{-1} at different phospholipid concentrations agree reasonably well with the values of 9–25 min^{-1} reported for activation of bovine factor IX (Zur & Nemerson, 1980). In an earlier kinetic study from this laboratory (Bajaj, 1982), activation of human factor IX by factor XIa gave a K_m value of 2 μM and a k_{cat} value of 10 min^{-1}. Thus, in our initial studies it appears that human factor IX is activated about 10-fold faster by factor VIIa than by factor XIa. Studies are currently in progress in our laboratory to obtain kinetic constants under a variety of experimental conditions for the activation of factor IX by factor VIIa and by factor XIa.

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Registry No. Blood coagulation factor IX, 9001-28-9; blood coagulation factor XIa, 37203-61-5; blood coagulation factor VIIa, 65312-43-8; blood coagulation factor IXα, 73412-46-1; blood coag-

ulation factor IXaβ, 66526-18-9; blood coagulation factor IXa, 37316-87-3.

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Kinetics of Activation of Human Prothrombin. Use of a Fluorescein-Labeled Derivative To Obtain Kinetic Constants as a Function of Factor V Concentration and Activation State†

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ABSTRACT: Human prothrombin labeled with fluorescein to a level of 4 mol of dye/mol of protein was confirmed to be a normal substrate for prothrombinase by several criteria and used as a fluorogenic substrate for studies of the kinetics of prothrombin activation in a purified model system. In investigations of the response to modulation of prothrombinase activity the concentrations of the enzymatic component, activated factor X, and the cofactor, factor V, were varied in turn and the corresponding rates of fluorescence increase determined. When factor Xa was the variable, a hyperbolic relation of activation rate to [factor Xa] was seen at a limiting level of factor V(a), which with appropriate curve fitting gave an apparent K_d of 0.059 ± 0.0001 nM. As the factor V concentration was raised, the apparent saturation became progressively less, and at 10 nM factor V the relationship to [factor Xa] was linear. Results of a complementary experiment with factor V as the variable also showed saturation. When the level of prothrombin was varied at constant [factor Xa] and different, fixed levels of factor V, the relation of rate to prothrombin concentration was hyperbolic in all cases. At a high, saturating concentration of factor V—about one-tenth the plasma level—the apparent K_m was 0.28 ± 0.02 μM prothrombin, and the apparent V_{max} was 19.9 ± 0.8 mol s^{-1} (mol of factor Xa)$^{-1}$. Of the parameters V_{max} was the more sensitive to [factor V], dropping 7-fold with a 125-fold decrease, whereas the K_m varied in random fashion by only 60%. This variation of V_{max} reflects the amount of factor Xa bound to factor V when the latter is limiting in concentration. Even at very low concentrations of factor V it made no difference to the rates of activation that could eventually be reached whether the factor V was activated with thrombin beforehand or left to activate in situ. However, reactions in which factor V was strongly rate limiting while the concentration of prothrombin was high were characterized by lags without proteolysis of several minutes before activation began, and that maximum rate was achieved. In contrast, lags were essentially absent at low prothrombin levels. Experiments to compare the progress curves obtained when factor V had been preactivated with thrombin under various conditions showed that the lags resulted from a protective effect of prothrombin on the activation of factor V, most pronounced when the factor V level was low. One consequence of this "substrate inhibition" is that the system is damped until a threshold level of activation has been reached and/or a minimum availability of prothrombinase has been exceeded.

Thrombin is a crucial enzyme in hemostasis, with half a dozen roles to play. There is, therefore, a strong impetus to understand the kinetics and control of its formation as precisely as possible, and studies with this as one of the goals have been conducted by several investigators (Nesheim et al., 1979a; Kosow & Orthner, 1979; Rosing et al., 1980; Dahlback & Stenflo, 1980). In all cases, the strategy has been to use probes for the appearance of product (i.e., thrombin) active sites such as a chromogenic peptide substrate or a fluorogenic, reversible, active site inhibitor (Nesheim et al., 1979b). The rates measured in such experiments as a function of prothrombin concentration, and the kinetic constants derived, therefore

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