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Articles

Kinetic Properties of Bovine Blood Coagulation Factors IXaα and IXaβ toward Synthetic Substrates†

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ABSTRACT: A series of kinetic properties for bovine blood coagulation factors IXaα and IXaβ have been determined toward synthetic substrates. With the thio ester substrate Cbz-Trp-Arg-SBzl (TAS), the K_m for factor IXaα, in the absence of Ca^{2+} (those obtained in the presence of 10 mM Ca^{2+} are in parentheses), at 30 °C was 1.4 ± 0.2 mM (1.5 ± 0.2 mM). The k_{cat} was found to be 7.8 ± 0.5 s^{-1} (15.0 ± 0.5 s^{-1}). For factor IXaβ, the corresponding values in the presence of 10 mM Ca^{2+} were 0.70 ± 0.5 mM and 3.8 ± 0.4 s^{-1}, respectively. Leupeptin (acetyl-Leu-Leu-argininal) was found to be a competitive inhibitor of hydrolysis of TAS for both factor IXaα and factor IXaβ in the presence of 10 mM Ca^{2+}, with a K_I of 80 ± 5 μM. For factor IXaα, in the absence of Ca^{2+}, a similar K_I for leupeptin was obtained. With the pre-steady-state substrate *p*-nitrophenyl *p*-guanidinobenzoate (NPGB), leupeptin also displayed a competitive inhibition pattern with a K_I of 75–80 μM, at 30 °C, for both factor IXaα and factor IXaβ, in the absence of Ca^{2+}. Thermodynamic properties of factors IXaα and IXaβ were determined by utilizing NPGB. The values (±5%) for the appropriate constants for factor IXaα (those for factor IXaβ are in parentheses) in the presence and absence of 10 mM Ca^{2+}, at 30 °C where applicable, were the following: E_a, 8.1 kcal mol^{-1} (9.2); $\Delta G^{\ddagger}$, 15.7 kcal mol^{-1} (15.7); $\Delta H^{\ddagger}$, 7.5 kcal mol^{-1} (8.6); $\Delta S^{\ddagger}$, −27.0 cal deg^{-1} mol^{-1} (−23.4); ΔH, −6.6 kcal mol^{-1} (−9.4); ΔG, −4.6 kcal mol^{-1} (−4.7); ΔS, −6.5 cal deg^{-1} mol^{-1} (−15.5).

The coagulation of blood can occur by either the intrinsic or the extrinsic pathways, both of which merge at the point at which factor X becomes activated. In the intrinsic system, factor X is activated by the enzyme factor IXa in a reaction accelerated by factor VIII, Ca^{2+}, and phospholipid (Lundblad & Davie, 1964; Chuang et al., 1972).

Although bovine factor IXa is not inhibited by diisopropyl fluorophosphate (iPr_2PF)[1] (Kurachi et al., 1976), its reactivity with substrates such as NPGB (Byrne et al., 1980) and with inhibitors such as antithrombin III–heparin (Rosenberg et al., 1975; Kurachi et al., 1976), as well as the identification of a peptide sequence common to other serine proteases (Enfield et al., 1974; Katayama et al., 1979), suggests that factor IXa is also a serine protease.

Factor IXa exists in plasma in a precursor form, factor IX. This glycoprotein has been purified and characterized from bovine (Fujikawa et al., 1973) and human (Soumela, 1976; DiScipio et al., 1978) plasma. Factor IX is a vitamin K dependent protein, containing 12 Gla residues (Fujikawa et al., 1973; Katayama et al., 1979) and possessing a molecular weight of approximately 55 400 in a single polypeptide chain of 416 amino acid residues. Factor IX can be activated by a variety of enzymes. In the intrinsic pathway, activation is accomplished by the serine protease, factor XIa, in the presence of Ca^{2+} (Schiffman et al., 1963; Kingdon et al., 1964). A possible link between the intrinsic and extrinsic pathways has been forwarded by the observation that factor IX can be activated by factor Xa, the latter enzyme generated through the extrinsic pathway (Kalousek et al., 1975), and by the observation that factor IX can be activated by factor VIIa/tissue factor/Ca^{2+} (Osterud & Rappoport, 1977; Zur & Nemerson, 1980). Activation of factor IX has also been observed by an enzyme present in polymorpholeukocytes (Kingdon et al., 1978). This suggests that activation can occur in a system which bypasses the above pathways. Two steps are involved in the activation of factor IX with these activators. In the first, activator-catalyzed cleavage of the Arg_{146}–Ala_{147} peptide bond occurs, resulting in a two-chain disulfide-linked inactive intermediate, factor IXα. Next, the Arg_{181}–Val_{182} peptide bond

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[1] Abbreviations: iPr_2PF, diisopropyl fluorophosphate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Gla, γ-carboxyglutamic acid; TAS, Cbz-Trp-Arg-SBzl; L-BAEE, N^{α}-benzoyl-L-arginine methyl ester; L-TAME, N^{α}-tosyl-L-arginine methyl ester; Me_2SO, dimethyl sulfoxide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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is cleaved, resulting in liberation of an activation glycopeptide of molecular weight of approximately 10000 (Fujikawa et al., 1974), and also yielding the serine protease factor IXaβ. Factor IX is also activated by a protease (RVV-X) from the venom of Russell's viper (Lindquist et al., 1978), forming factor IXaα. In this case, only the Arg_{181}–Val_{182} peptide bond is cleaved, and no peptide material is released (Lindquist et al., 1978).

Previous studies from this laboratory have shown that the presence of divalent metal cations results in increases in the k_{cat} for factors IXaα and IXaβ toward the ester substrate N^{α}-benzoyl-L-arginine ethyl ester, but these same metal cations do not affect the K_m for this substrate (Byrne & Castellino, 1978; Byrne et al., 1980). Further, the pre-steady-state rate constants, k_2 and K_s, toward NPGB were similar for both factors IXaα and IXaβ and unaffected by divalent cations, whereas the deacylation rate constant, k_3, showed significant differences for the two enzymes. The latter was influenced by the presence of Ca^{2+} (Byrne et al., 1980). The purpose of this paper is to explore further the possible similarities and differences between the two forms of factor IXa and to examine the effect of divalent cations on the kinetics of these enzymes. Since the organism is capable of providing a factor IXa with only a single peptide bond cleavage (factor IXaα), it is possible that the reasons for generation of a factor IXa with two peptide bond cleavages (factor IXaβ) might be revealed through kinetic analysis of the two enzymes.

Materials and Methods

Proteins. Factor IX was purified from fresh citrated bovine plasma by the procedure of Fujikawa et al. (1973), as modified by Amphlett et al. (1979). Details of the preparation and methods for isolation of factors IXaα and IXaβ have been published previously (Amphlett et al., 1979).

Steady-State Kinetics of Factor IXa Employing the Thio Ester Substrate TAS. The steady-state kinetic parameters of factor IXa toward the thio ester TAS (Enzyme Systems Products) were determined by using the assay procedure described by McRae et al. (1981). The enzymatic hydrolysis of TAS [$(0.5–2.0) \times 10^{-4}$ M] was measured by using 4,4′-dithiodipyridine (Aldrithiol). Stock solutions of TAS were prepared in Me_2SO. The assay buffer was 0.1 M Hepes, pH 7.5, containing 9.8% (v/v) Me_2SO. A 2.0 mM solution of Aldrithiol was prepared in the assay buffer. For assays performed in the presence of Ca^{2+}, both the assay buffer and the additional solutions contained 10 mM $CaCl_2$. All assays were conducted at 30 °C by using a Cary 219 recording spectrophotometer. In a typical assay, 885 μL of assay buffer and 75 μL of Aldrithiol were placed in a cuvette. A 20-μL aliquot of TAS was added, the solution was mixed by inversion, and a background hydrolysis rate was measured at 324 nm. Next, a 20-μL aliquot of factor IXaα or factor IXaβ was then added to the cuvette, the solution was again mixed, and the enzymatic hydrolysis rate was recorded. For rapid completion of the reaction, a 10-μL sample of trypsin (2 mg/mL) was then added to the cuvette and a final absorbance (A_∞) determined. The total substrate concentration was calculated from the A_∞ reading at 324 nm, using $\epsilon_{324} = 19\,800$ M^{-1} cm^{-1} (Grassetti & Murray, 1967). Factor IXa catalyzed hydrolysis of TAS was determined by subtracting the background rate from the enzymatic rate and converting ΔA_{324} per second units into appropriate molar concentrations. Lineweaver–Burk plots were used to obtain the K_m and k_{cat} values.

The concentrations of factor IXaα and factor IXaβ were determined by titration of each enzyme with NPGB (Byrne & Castellino, 1978).

Determination of Thermodynamic and Activation Parameters for Factor IXaα and Factor IXaβ. These parameters were obtained from pre-steady-state rate measurements, by employing the substrate NPGB. It has been demonstrated previously (Byrne et al., 1980) that the kinetic mechanism of factors IXaα and IXaβ proceeds through an acyl-enzyme intermediate (Bender et al., 1966; Bender & Brubacher, 1966), as follows:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow[+P_1]{k_3} E + P_2$$

where E represents the enzyme, S is the substrate, and ES is the adsorptive complex of the two. Upon acylation, the acyl-enzyme complex, ES′, is formed along with the released alcohol, P_1. Deacylation occurs, yielding $E + P_2$, the acid moiety of the substrate. Methods of determination of K_s, k_2, and k_3 have been published for factors IXaα and IXaβ (Byrne et al., 1980).

The effect of temperature on the pre-steady-state rate constants was conducted from 8 to 38 °C, with the temperature monitored during the assay by a Lauda RM3 circulating water bath, attached to a Cary 219 recording spectrophotometer. The values for k_2 and K_s were determined at various temperatures, as previously described (Byrne et al., 1980). According to the van't Hoff expression for the variation of K_s as a function of temperature

$$d \ln K_s/dT = -\Delta H/(RT^2)$$

the value of ΔH can be determined from a graph of $-\ln K_s$ vs. $1/T$. From the slope of this plot, the ΔH for the formation of the enzyme–substrate complex was determined. The change in free energy, ΔG, and the change in entropy, ΔS, at 30 °C, were determined from basic thermodynamic relationships, with the values for K_s at 30 °C and ΔH.

The activation energy, E_a, was determined from the slope of a plot of $-\ln k_2$ vs. $1/T$, using the Arrhenius relationship

$$d \ln k_2/dT = E_a/(RT)$$

The free energy of activation, $\Delta G^{\ddagger}$, at 30 °C was calculated from the following expression derived from the transition-state theory:

$$k_2 = \frac{kT}{h} e^{-\Delta G^{\ddagger}/(RT)}$$

where k is Boltzmann's constant and h is Planck's constant. The value for the enthalpy of activation, $\Delta H^{\ddagger}$, at 30 °C was determined from the relationship $\Delta H^{\ddagger} = E_a - RT$. The value for the entropy of activation, $\Delta S^{\ddagger}$, at 30 °C, could then be calculated, with the knowledge of $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ from the equilibrium thermodynamic relationships.

Kinetic Assays in the Presence of Inhibitors. A series of protein inhibitors were tested for their ability to inhibit factor IXa catalyzed hydrolysis of both TAS and NPGB. When required, the inhibitors were added to the assay mixture, usually in the assay buffer, prior to initiation of the enzymatic reaction rate. The following inhibitors were employed: soybean trypsin inhibitor (Sigma); lima bean trypsin inhibitor (Sigma); trasylol (FBA Pharmaceuticals); leupeptin (acetyl-Leu-Leu-argininal; Protein Research Foundation); GGA (Glu-Gly-Arg-CH_2Cl; provided by Dr. Elliot Shaw); iPr_2PF.

Results

Steady-State Kinetic Study of Factors IXaα and IXaβ with TAS in the Presence and Absence of Ca^{2+}. A steady-state kinetic study of factors IXaα and IXaβ toward TAS was performed in the presence and absence of Ca^{2+}. The rate of

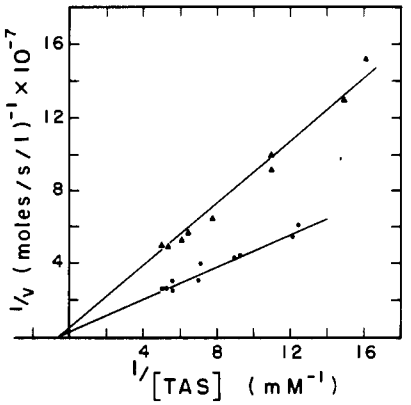

FIGURE 1: Lineweaver–Burk plots of TAS hydrolysis by factor IXaα in the absence and presence of Ca^{2+}. The assays were conducted with titrated 2.6 × 10^{-8} M factor IXaα, in an assay buffer of 100 mM Hepes (pH 7.5)/9.8% Me_2SO (v/v)/150 μM Aldrithiol, at 30 °C: (●) with 10 mM $CaCl_2$; (▲) without Ca^{2+}.

TAS hydrolysis, at 30 °C, was measured by the reaction of the released thiol with 4,4′-dithiodipyridine (Aldrithiol) contained in the assay mixture. A linear rate of substrate hydrolysis was observed, and less than 3% of the substrate was hydrolyzed in the assay. The substrate did hydrolyze slowly in the assay buffer, and this blank rate was subtracted from the rate of hydrolysis with factor IXa. The upper limit of the substrate concentration employed in these assays was determined by the solubility of the substrate in the assay buffer.

The initial rate of hydrolysis of TAS, with 10 mM Ca^{2+}, increased linearly with the amount of factor IXaα over the enzyme concentration range of 0.5 and 5.0 μg/mL of active site titrated factor IXaα. The initial rates of hydrolysis of TAS by factor IXaα were determined in the absence of Ca^{2+} and in the presence of 10 mM $CaCl_2$. The assays performed in the absence of Ca^{2+} employed Chelex-100-treated factor IXaα and Ca^{2+}-free buffers and reagents. The data are presented in Figure 1 in the form of Lineweaver–Burk plots. In the presence of 10 mM Ca^{2+}, the K_m for TAS is 1.5 ± 0.2 mM and the k_{cat} is 15.0 ± 0.5 s^{-1}. In the absence of Ca^{2+}, the K_m for TAS is 1.4 ± 0.2 mM and the k_{cat} is 7.8 ± 0.5 s^{-1}. The kinetic parameters presented are the average of three separate determinations, utilizing two different enzyme concentrations.

In the presence of Ca^{2+}, the initial rate of TAS hydrolysis was directly proportional to the factor IXaβ concentration between 0.3 and 9.0 μg/mL. Subsequent assays employed active site titrated factor IXaβ concentrations within this range. The rate of hydrolysis of TAS by factor IXaβ was determined in the presence of 10 mM $CaCl_2$. The data are presented in Figure 2, in the form of a Lineweaver–Burk plot. The K_m for TAS of 0.7 ± 0.5 mM and the k_{cat} of 3.8 ± 0.5 s^{-1} are the average values obtained from three separate determinations, utilizing two enzyme concentrations. In the absence of Ca^{2+}, the hydrolysis of TAS by factor IXaβ was not linear, showing an initial "burst" of activity, followed by a much slower linear rate. This final linear rate was found to be dependent on enzyme concentration but not on substrate concentration over the range employed. A faster linear rate was observed when Ca^{2+} was added to the assay mixture to a final concentration of 10 mM after the slow linear rate was obtained. This former rate was dependent on substrate concentration and was approximately 70% of the rate obtained with 10 mM Ca^{2+}, initially. This atypical pattern of hydrolysis of a steady-state substrate was only observed for factor IXaβ in the absence of Ca^{2+}. Factor IXaβ with Ca^{2+} and factor IXaα in the absence or presence of Ca^{2+} displayed linear initial rates of TAS hydrolysis. A summary of the results of factors IXaα and IXaβ for TAS is seen in Table I.

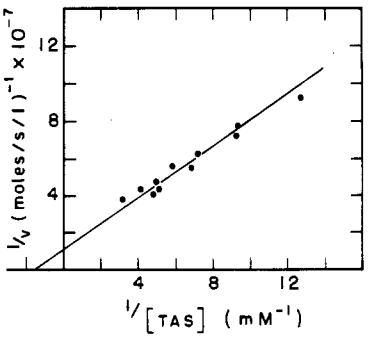

FIGURE 2: Lineweaver–Burk plot of TAS hydrolysis by factor IXaβ in the presence of Ca^{2+}. The rates were obtained with 2.4 × 10^{-8} M factor IXaβ, in an assay buffer of 100 mM Hepes/10 mM $CaCl_2$ (pH 7.5)/9.8% Me_2SO (v/v)/150 μM Aldrithiol, at 30 °C.

Table I: Steady-State Parameters for the Hydrolysis of TAS with Factor IXa at 30 °C

enzyme	Ca^{2+} (10 mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
factor IXaα	–	1.4 ± 0.2	7.8 ± 0.5	5.7 × 10^3
	+	1.5 ± 0.2	15.0 ± 0.5	10 × 10^3
factor IXaβ	–	ND[a]	ND	
	+	0.7 ± 0.05	3.8 ± 0.5	5.4 × 10^3

[a] Not determined.

Effect of Serine Protease Inhibitors on the Activity of Factors IXaα and IXaβ toward TAS. A series of serine protease inhibitors was studied for their effect on factors IXaα and IXaβ toward TAS. Three trypsin inhibitors were investigated: soybean trypsin inhibitor, lima bean trypsin inhibitor, and bovine pancreatic trypsin inhibitor (trasylol). A sample of the desired factor IXa was incubated with the inhibitor at a minimum of 100:1 molar ratio, inhibitor:factor IXa, for 1 h at room temperature. The samples were then assayed for activity toward 0.12 mM TAS in 100 mM Hepes/10 mM $CaCl_2$ (pH 7.5)/9.8% Me_2SO (v/v)/150 μM Aldrithiol, at 30 °C, with the inhibitor to factor IXa molar ratio doubled in the assay. None of the trypsin inhibitors showed any inhibitory effect on either factor IXaα or factor IXaβ. The levels of trypsin inhibitor employed greatly reduced the activity of trypsin toward TAS.

Leupeptin, a small bacterially derived trypsin inhibitor, was also tested for its possible effect on factors IXaα and IXaβ. Because of the high solubility properties of this inhibitor, an inhibitor to factor IXa ratio of 1000:1 was employed for both the preincubation and the assay. The activities of both factors IXa were inhibited 80% under these conditions.

The interaction of leupeptin with factor IXa was investigated further in order to determine the type of inhibition and K_I values. As seen in Figure 3, a competitive inhibition pattern was observed in the Lineweaver–Burk plot of factor IXaβ with TAS, in the presence of Ca^{2+}, at varying leupeptin concentrations. From a plot (Figure 4) of leupeptin concentration vs. slopes, the latter obtained from the double-reciprocal plots of Figure 3, a K_I of 75 μM was determined for leupeptin with factor IXaβ. The leupeptin interaction with factor IXaα also showed competitive inhibition (data not presented). A K_I of 80 μM was determined for leupeptin with factor IXaα, from the replot of leupeptin concentrations vs. slopes similar to those of Figures 3 and 4. The K_I determinations for each factor IXa were based on two separate experiments at three different inhibitor concentrations in the presence of 10 mM Ca^{2+}. Similar patterns of inhibition and K_I values were observed for factors IXaα and IXaβ.

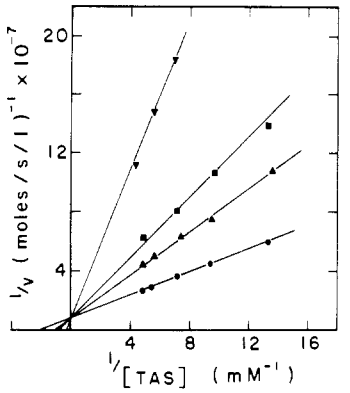

FIGURE 3: Lineweaver–Burk plots of TAS hydrolysis by factor IXaβ in the presence of leupeptin. The thioesterase activity of 2.4×10^{-8} M factor IXaα was determined, at 30 °C, in 100 mM Hepes/10 mM $CaCl_2$ (pH 7.5)/9.8% Me_2SO/150 μM Aldrithiol, with the following concentrations of leupeptin: (●) none; (▲) 70 μM; (■) 146 μM; (▼) 305 μM.

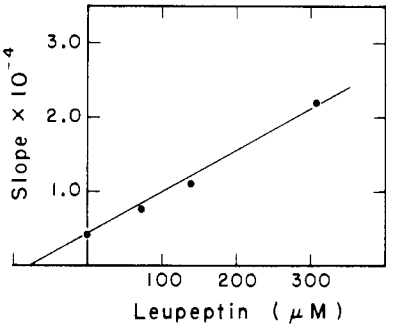

FIGURE 4: Determination of the K_I for leupeptin with factor IXaβ. A plot of the leupeptin concentration vs. the slopes obtained from Figure 3.

An irreversible factor Xa inhibitor, Glu-Gly-Arg-CH_2Cl (GGA), was tested for its ability to inhibit factor IXa. When either factor IXaα or factor IXaβ was incubated for 30 min with up to 7×20^{-4} M GGA and assayed for TAS activity, no inhibition was observed. This concentration of GGA is greater than 100 times the amount required for total inhibition of factor Xa in 30 s.

The effect of iPr_2PF on factor IXaβ was also studied with TAS with 10 mM Ca^{2+}. This compound was investigated as an irreversible inhibitor of factor IXa in the presence of Ca^{2+} with phospholipid. For these experiments, the factor IXaβ samples were prepared in 100 mM Hepes/100 mM NaCl, pH 7.4, which maintained a stable pH at high iPr_2PF concentrations. Samples were prepared of factor IXaβ, factor IXaβ/8 mM $CaCl_2$, and factor IXaβ/8 mM $CaCl_2$/80 μM PC/PS (egg phosphatidylcholine/bovine brain phosphatidylserine, 4/1 w/w). Each sample was adjusted to 20 mM in iPr_2PF, with 1 M iPr_2PF in 2-propanol, and incubated at room temperature. The samples were assayed for TAS activity at 30 and 60 min, and compared to a control in which an equal volume of 2-propanol was added to the sample. No inhibition of factor IXaβ activity toward TAS was observed. A sample of factor IXaα with 8 mM $CaCl_2$ was prepared and assayed in the same manner. Factor IXaα showed no appreciable difference in the initial enzymatic rate after treatment with 20 mM iPr_2PF as compared to the control. To test for reversible inhibition by iPr_2PF, factor IXaβ was assayed for TAS activity with 0.12 mM TAS in 100 mM Hepes/10 mM $CaCl_2$ (pH 7.5)/9.8% Me_2SO (v/v)/150 μM Aldrithiol, with 10 or 25 mM DPF at 30 °C. Again no significant change in rate was observed when compared to assays in which iPr_2PF was absent.

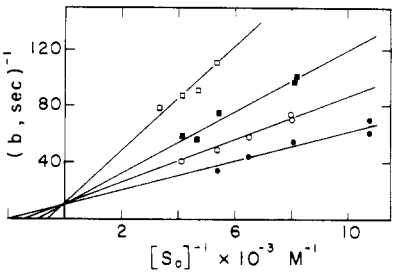

FIGURE 5: Double-reciprocal plot of the first-order rate constant obtained for factor IXaα hydrolysis of NPGB, $[S_0]$, in the presence of leupeptin. The esterase activity of factor IXaα (1.3×10^{-6} M) was determined in 100 mM Hepes/100 mM NaCl, pH 8.3, at 30 °C in the presence of the following concentrations of leupeptin: (●) none; (○) 35 μM; (■) 70 μM; (□) 140 μM.

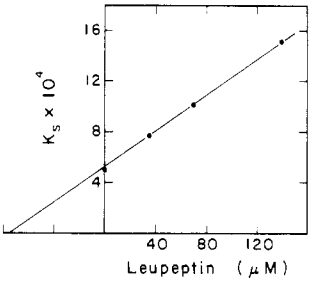

FIGURE 6: Determination of K_I for leupeptin with factor IXaα. A plot of the leupeptin concentration vs. K_s derived from the data in Figure 5.

Effect of Serine Protease Inhibitors on the Activity of Factors IXaα and IXaβ with NPGB. Those same inhibitors were analyzed for their effects on the pre-steady-state rate parameters of factor IXaα and factor IXaβ toward the substrate NPGB. The only effective inhibitor was leupeptin.

The inhibition by leupeptin was investigated further for both forms of factor IXa to determine the type of inhibition and K_I values. Initial studies showed little difference in inhibition in the absence or presence of 10 mM Ca^{2+}. Therefore, all subsequent experiments were performed in the absence of Ca^{2+}. Figure 5 shows the competitive inhibition pattern seen in the double-reciprocal plot for factor IXaα with NPGB, at varying leupeptin concentrations. From the plot of leupeptin concentration vs. K_s for NPGB, shown in Figure 6, a K_I of 75 μM was determined with factor IXaα. A similar pattern was observed in the $1/b$ vs. $1/[NPGB]$ plots for factor IXaβ (data not shown) at varying leupeptin concentrations. The K_I of 75 μM was determined for factor IXaβ from the plot of leupeptin concentration vs. the K_S for NPGB, similar to Figure 6. The K_s values were based on two separate determinations at each leupeptin concentration.

Determination of Thermodynamic and Activation Parameters of Factors IXaα and IXaβ with NPGB. The thermodynamic and activation parameters for factors IXaα and IXaβ were determined with NPGB. The values for k_2 and K_S were obtained at various temperatures, from 8 to 38 °C, by using the procedures and methods of calculation previously described (Byrne et al., 1980). Since no measurable difference was observed in the presence or absence of Ca^{2+}, at either 30 or 38 °C, for either factor IXaα or factor IXaβ, all experiments were performed in the presence of 10 mM $CaCl_2$.

The value for ΔH was determined from the slope of the plot of $-\ln K_S$ vs. $1/T$ (Figure 7). The values for ΔG and ΔS were calculated from the K_S obtained at 30 °C and the ΔH. The E_a value was determined from the slope of the plot of $-\ln k_2$ vs. $1/T$ (Figure 8), and the $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ values were calculated from E_a and basic relationships. The k_2 and K_S

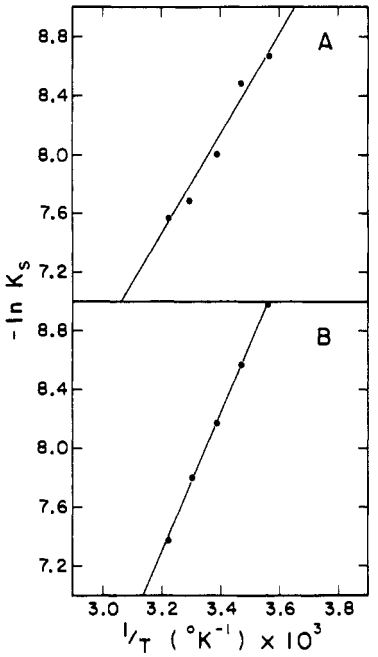

FIGURE 7: Determination of ΔH values for factors IXaα and IXaβ with NPGB. The values for ΔH were determined from the slope of this plot by using the van't Hoff expression as described under Materials and Methods. (A) Data obtained for factor IXaα with 10 mM Ca^{2+}, from which a slope of 3.3 × 10^3 K was calculated. (B) Data obtained for factor IXaβ with 10 mM Ca^{2+}, from which a slope of 4.7 × 10^3 K was calculated.

Table II: Activation and Thermodynamic Parameters for Bovine Factor IXaα and Factor IXaβ with NPGB[a]

parameter[b]	factor IXaα	factor IXaβ
E_a	8.1	9.2
$\Delta G^{\ddagger}$	15.7	15.7
$\Delta H^{\ddagger}$	7.5	8.6
$\Delta S^{\ddagger}$	−27.0	−23.4
ΔH	−6.6	−9.4
ΔG	−4.6	−4.7
ΔS	−6.5	−15.5

[a] All values are at 30 °C with the exception of E_a and ΔH.
[b] Values are expressed as kilocalories per mole with the exception of $\Delta S^{\ddagger}$ and ΔS which are in calories per degree per mole.

values were determined at five different temperatures, from triplicate points at six NPGB concentrations. The calculated thermodynamic and activation parameters for factors IXaα and IXaβ are listed in Table II.

Discussion

A comparative study of the enzymic properties of bovine factor IXaα and factor IXaβ toward synthetic substrates has been undertaken. Although factor IXa is reasonably assumed to be a serine protease, its ability to hydrolyze typical ester and amide substrates is very limited. The only simple ester substrates thus far reported for factor IXa are L-TAME (Lindquist et al., 1978), L-BAEE (Byrne & Castellino, 1978), and NPGB (Byrne et al., 1980). Recently, a series of peptide thio esters have been tested as potential factor IXaβ substrates, and the most reactive substrate found was Z-Trp-Arg-SBzl (McRae et al., 1981). Few detailed studies of the kinetic properties of factor IXa toward synthetic substrates have appeared. Available data for L-BAEE, at 30 °C (Byrne & Castellino, 1978; Bryne et al., 1980), show that the K_m values for factors IXaα and IXaβ for this substrate are approximately 20 mM and are not altered by the presence of various divalent metal cations. On the other hand, the k_{cat} values for this

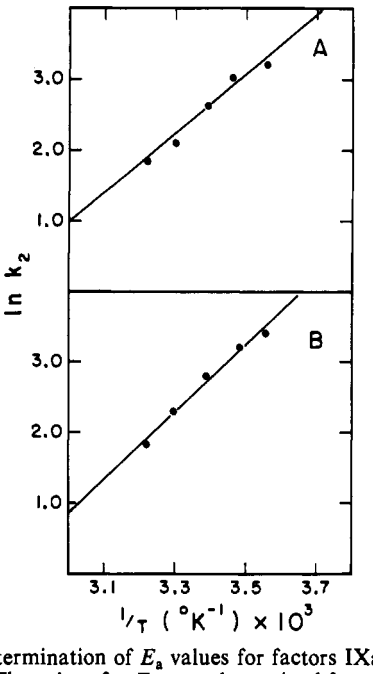

FIGURE 8: Determination of E_a values for factors IXaα and IXaβ with NPGB. The values for E_a were determined from the slopes of these plots and use of the Arrhenius relationship as described under Materials and Methods. (A) Data obtained for factor IXaα with 10 mM Ca^{2+}, which provided a slope of 4.1 × 10^3 K. (B) Data obtained for factor IXaβ with 10 mM Ca^{2+}, from which a slope of 4.7 × 10^3 K was calculated.

substrate in the absence of divalent cations were found to be 3.9 s^{-1} for factor IXaα and 6.4 s^{-1} for factor IXaβ. In the presence of 10 mM Ca^{2+}, the k_{cat} values were increased to 9.2 and 9.0 s^{-1} for factors IXaα and IXaβ, respectively. Thus, from analysis of hydrolysis of this particular substrate, it appears as though factor IXaβ is a more efficient enzyme for ester hydrolysis than factor IXaα in the absence of Ca^{2+}, an effect which is alleviated in the presence of Ca^{2+}.

An evaluation of the pre-steady-state rate parameters, at 30 °C, for factor IXaα and factor IXaβ catalyzed hydrolysis of NPGB has been published (Byrne et al., 1980). It was found that the K_s and k_2 values for these two enzymes toward this substrate are virtually identical and not influenced by Ca^{2+}. On the other hand, the deacylation rate constant, k_3, was larger for factor IXaα than for factor IXaβ, a difference which was reduced in the presence of Ca^{2+}.

The thio ester substrate TAS was found by McRae et al. (1981) to be a suitable substrate for factor IXaβ. In that publication, individual kinetic constants were not reported due to difficulties encountered in the assay. However, the k_{cat}/k_m for TAS with factor IXaβ, at 30 °C, was found to be 3.4 × 10^5 M^{-1} s^{-1}. The high sensitivity of the thio ester assay allows the use of relatively low levels of enzyme. In our study, we routinely employ titrated factor IXa concentrations of approximately 1 μg/mL. A limitation of TAS as a substrate is its low solubility in aqueous solutions. We, nonetheless, optimized enzyme and substrate concentrations to obtain k_{cat} and K_m values. The double-reciprocal plots obtained did not intersect at the origin or deviate from linearity at the highest substrate concentration employed, as reported by McRae et al. (1981). However, concentrations greater than 15% of the calculated K_m values could not be used in the assay due to the low solubility properties of TAS. The data obtained for TAS hydrolysis by factors IXaα and IXaβ, in the presence of Ca^{2+}, showed linear initial rates that were proportional to the factor IXa concentration. From initial experiments, it was apparent that TAS was a more effective substrate for factor IXaα than

for factor IXaβ. This was borne out by the values for the steady-state rate parameters. The K_m for factor IXaβ was found to be approximately half of that for factor IXaα (Table I), but the k_{cat} value for factor IXaβ was also lower than that for factor IXaα. The comparative k_{cat}/K_m values for each enzyme, in the presence of Ca^{2+}, show that, overall, factor IXaα is a more efficient enzyme for hydrolysis of this substrate than factor IXaβ. In the absence of Ca^{2+}, the K_m for the factor IXaα catalyzed hydrolysis of TAS remained essentially the same, as was the case with L-BAEE as a substrate, whereas the k_{cat} was decreased, again similar to the behavior of L-BAEE. These same parameters could not be obtained for factor IXaβ in the absence of Ca^{2+}, due to problems in assuming steady-state behavior. Here, the initial rate of TAS hydrolysis appeared as a "burst", followed by a slow linear rate that was not dependent upon substrate concentration. This behavior appears to be more typical of a pre-steady-state substrate, in which $k_3 \ll k_2$. When Ca^{2+} was added to the Ca^{2+}-free assay of TAS with factor IXaβ after the steady-state rate was obtained, the rate increased and was dependent on substrate concentration. One interpretation of these results is that Ca^{2+} may have increased the k_3 for factor IXaβ with TAS. Although the effect of Ca^{2+} on factor IXaβ appeared more dramatic than that on factor IXaα, the interpretation could be the same. The primary effect of Ca^{2+} on factor IXa catalyzed hydrolysis of TAS could be an increase in the deacylation rate. The k_{cat}/K_m value obtained in this study for factor IXaβ toward TAS is approximately 60-fold lower than that reported by McRae et al. (1981). Our value is based upon determination of the individual parameters of k_{cat} and K_m under conditions where Michaelis–Menten kinetics were followed. The results of McRae et al. (1981) did not conform to typical Michaelis–Menten kinetics.

All steady-state rate data reported for synthetic substrates suggest that factors IXaα and IXaβ behave similarly in their kinetic properties. Although some subtle differences exist between the enzymes, dependent upon the substrate employed, one generalization can be forwarded. It does appear that divalent cation interaction with factor IXa stimulates its catalytic properties through increases in the k_{cat} of the reactions. Further analysis of the catalytic properties of these two enzymes awaits studies with other substrates.

Factor IXa appears to be as selective in regard to protease inhibitors as toward synthetic substrates. Of the inhibitors employed in this study, only one was effective toward this enzyme, leupeptin. Competitive inhibition patterns were observed for leupeptin with factors IXaα and IXaβ, using both NPGB and TAS. Similar K_I values were obtained for leupeptin with both substrates. This is particularly interesting since NPGB is a pre-steady-state substrate and TAS is a steady-state substrate. The same relationship:

$$v/V_{max} = ([S]/K_m)(1 + [I]/K_I) + [S]$$

can be derived from steady-state assumptions or from rapid equilibrium assumptions, in which K_s replaces K_m in the above equation. Therefore, K_I values can be determined from either observed changes in K_s or K_m values with the addition of inhibitor. The similarity in the K_I values obtained from the two different substrates supports the assumptions in which the pre-steady-state calculations are based.

Leupeptin inhibition has been studied for other proteases. Its inhibition of trypsin has been reported (Aoyagi et al., 1969). Competitive inhibition patterns with K_I values of 0.13 and 0.34 μM were determined for leupeptin and trypsin with L-BAEE and L-TAME, respectively (Umezawa, 1976). This inhibitor was also found to inhibit plasmin with a K_I of 8.5 μM (Wohl et al., 1977), urokinase with a K_I of 190 μM (Wohl et al., 1977), and two complement enzymes, $C1_s$ with a K_I of 400 μM (Andrews & Baillie, 1979) and CVFBb (cobra venom activated B) with a K_I of 40 μM (Caporale, 1981). Leupeptin also was shown to delay coagulation in human and rabbit blood (Umezawa, 1976). The average K_I value determined for either form of factor IXa and leupeptin was 75–80 mM. Of the known K_I values for leupeptin, its binding to factor IXa is more similar to that for urokinase and CVFBb than for plasmin or trypsin. Leupeptin does inhibit factor Xa and, most likely, other coagulation enzymes, but K_I values have not been determined. The structure of leupeptin (acetyl-Leu-Leu-argininal) is similar to the amino acid sequence in factor X (Val-Val-Arg) that is the site of factor IXa cleavage, thus providing a rationale for its effectiveness toward factor IXa inhibition.

Previous conclusions regarding the similarity of the two forms of factor IXa with NPGB were based on rate measurements at a single temperature, 30 °C (Byrne et al., 1980). Since it is possible that the reaction thermodynamic properties may be different, and since determination of such parameters is instructive in terms of the catalytic nature of the enzymes, a temperature study was carried out on the factor IXaα and factor IXaβ catalyzed hydrolysis on NPGB. By examination of the temperature dependence of K_s, thermodynamic parameters (ΔG, ΔH, and ΔS) were obtained for the reaction

$$\text{factor IXa} + \text{NPGB} \rightleftarrows \text{factor IXa·NPGB}$$

in the presence of Ca^{2+}. As seen in Table II, similar ΔG values were obtained for factor IXaα and factor IXaβ, indicating an overall favorable energy for binding. The negative ΔS values obtained, at least in part, reflect the ordering of the system upon binding with possible loss of translational and rotational degrees of freedom, entropy of dilution (concentration), and possible effects on H_2O structure. The fact that the ΔS for factor IXaβ·NPGB binding is smaller than the corresponding ΔS for the factor IXaα·NPGB interaction indicates that the fine details of the structure of the biomolecular complex are different in the two enzymes. These dissimilarities, however, do not apparently lead to significant differences in the rate of acylation of the two enzymes by this substrate since the k_2 values for the next step are very similar at each temperature.

The temperature dependence of k_2 allows thermodynamic activation parameters to be calculated for the energy differences between the reactants (E + S) and the transition state of the rate-determining step, i.e., $ES \rightarrow ES^{\ddagger} \rightarrow ES^{1} + P_i$. In this case, the large negative value for the entropy of activation ($\Delta S^{\ddagger}$) is noteworthy. The magnitude of $\Delta S^{\ddagger}$ involves similar considerations as does thermodynamic ΔS and is in the range of values expected for a bimolecular reaction, in this case, reactants $\rightarrow ES^{\ddagger}$. A small difference between factor IXaα and factor IXaβ is seen in this parameter.

Since thermodynamic data for other serine proteases with this substrate do not exist, it is not possible to compare the catalytic properties of factor IXa with those of other enzymes of this class. As this information becomes available, a more thorough understanding of catalysis by blood coagulation proteases will be forthcoming.

In conclusion, as discussed here, subtle kinetic and thermodynamic differences exist when the hydrolysis of synthetic substrates by both forms of factor IXa are compared. It is possible that these differences could be magnified when studies are performed comparing the kinetics of factor IXaα and factor IXaβ toward its physiologic substrate, factor X, in the presence and absence of various cofactors for this reaction. This latter topic will be the subject of a future contribution from this laboratory.

Registry No. TAS, 84280-03-5; NPGB, 21658-26-4; Ca, 7440-70-2; factor IXaα, 66526-17-8; factor IXaβ, 66526-18-9.

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Kinetic Relationships between the Various Activities of the Formyl–Methenyl–Methylenetetrahydrofolate Synthetase†

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ABSTRACT: The formyl–methenyl–methylenetetrahydrofolate synthetase from chicken liver catalyzes the formation of the 10-formyl- and 5,10-methenyltetrahydrofolate cofactors via three enzymatic activities. In this report we define the kinetic relationships between the activities of this trifunctional protein. An investigation of the time course for 10-formyl cofactor synthesis by computer modeling indicates that commencing with tetrahydropteroyltriglutamate, the activities of the synthetase/cyclohydrolase couple act as separate enzymic species. In contrast, 10-formyl cofactor formation from the 5,10-methylene cofactor utilizing the dehydrogenase/cyclohydrolase couple is described by a single or interactive site model that partitions the 5,10-methenyl intermediate primarily (85%) to the 10-formyl product. An unusual characteristic of the latter coupled activities is the negligible cyclohydrolase activity toward exogenous 5,10-methenyl cofactor, which serves as substrate in the individual activity assay. This is based on (1) competitive inhibition by 5,11-methenyltetrahydrohomofolate against the 5,10-methenyl derivative in the cyclohydrolase-catalyzed hydrolysis but the absence of such inhibition in the dehydrogenase/cyclohydrolase couple and (2) a pulse–chase experiment showing the failure of chase 5,10-methenyl cofactor to dilute the 10-formyl product derived from the coupled activities. The result of this coupling is to minimize the concentration of the 5,10-methenyl species, consistent with its noninvolvement in de novo purine biosynthesis.

The trifunctional protein (10-formyltetrahydrofolate synthetase/5,10-methenyltetrahydrofolate cyclohydrolase/5,10-methylenetetrahydrofolate dehydrogenase) from chicken liver catalyzes the formation of several reduced folate cofactors[1] via the reactions

$$\text{formate} + \text{ATP} + \text{H}_4\text{folate} \xrightarrow{\text{synthetase}} \text{10-formyl-H}_4\text{folate} + \text{ADP} + \text{P}_i$$

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