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Kinetic Analysis of Covalent Hybrid Plasminogen Activators: Effect of CNBr-Degraded Fibrinogen on Kinetic Parameters of Glu_1-Plasminogen Activation†

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ABSTRACT: The kinetic parameters of three activator species of Glu_1-plasminogen (Glu_1-Plg) were compared in their reaction at pH 7.4 and 37 °C, in the presence and absence of CNBr-digested fibrinogen (CNBr-Fg). The urokinase- (u-PA-) derived covalent hybrid activator Pln_A-u-PA_B had an apparent Michaelis constant (K_{plg}) of 7.44 μM, a catalytic rate constant (k_{plg}) of 51.1 min^{-1}, and a second-order rate constant (k_{plg}/K_{plg}) of 6.87 μM^{-1} min^{-1}. The tissue plasminogen activator (t-PA) derived covalent hybrid activator Pln_A-t-PA_B was characterized by a K_{plg} of 3.33 μM, a k_{plg} of 1.03 min^{-1}, and a k_{plg}/K_{plg} of 0.309 μM^{-1} min^{-1}. The k_{plg}/K_{plg} values for the parent u-PA and t-PA activators were 6- and 16-fold higher than the respective hybrids, mainly due to an ~10-fold increase in the apparent K_{plg} for the hybrids. In the presence of CNBr-Fg, the increase of the k_{plg}/K_{plg} values for u-PA and its hybrid was 1.1-fold, but for t-PA and its hybrid, the increases were 7- and 12-fold, respectively. In both the absence and presence of CNBr-Fg, activator t-PA_B had an apparent K_{plg} of 19.1 and 27.6 μM and a k_{plg} of 2.9 and 5.0 min^{-1}, respectively. The increase in the k_{plg}/K_{plg} value with CNBr-Fg was 1.2-fold. The streptokinase- (SK-) derived activators Glu_1-plasmin·SK (Glu_1-Pln·SK), Val_{442}-Pln·SK, and Val_{561}-Pln·SK had apparent K_{plg} values of 0.458, 0.268, and 0.121 μM and k_{plg} values of 20.0, 126.0, and 63.3 min^{-1}, respectively. In the presence of CNBr-Fg, the first two activators showed an ~1.4-fold increase and the last showed a 1.4-fold decrease in their k_{plg}/K_{plg} values. The catalytic efficiency (k_{plg}/K_{plg}) of the various activator species fell in the decreasing order SK > u-PA > t-PA, in either the presence or absence of CNBr-Fg. CNBr-Fg enhanced significantly the activities of only two activators, t-PA and Pln_A-t-PA_B.

Plasmin (Pln)[1] is the principal plasma enzyme responsible for the dissolution of blood clots. It is formed from plasminogen (Plg) by an activation system that includes tissue plasminogen activator (t-PA), urokinase (u-PA), or the plasmin(ogen)·streptokinase (SK) species. Pln, Plg, and the activators all share the common characteristics of having a fibrin-binding domain and a catalytic domain (Bachmann,

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[1] Abbreviations: CNBr-Fg, CNBr-degraded fibrinogen; Plg, plasminogen; Pln, plasmin; Pln_A, NH_2-terminal plasmin-derived heavy A chain; SK, streptokinase; u-PA, urokinase; u-PA_B, COOH-terminal urokinase-derived catalytic B chain; t-PA, tissue plasminogen activator; t-PA_B, COOH-terminal tissue plasminogen activator derived catalytic B chain; HMW, high molecular weight; LMW, low molecular weight; P_i, inorganic phosphate; IU, international units established by World Health Organization for SK, t-PA, and u-PA; S-2251, H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide.

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1987; Robbins, 1987). The binding domain consists of homologous regions called kringles, each of which has approximately 80 amino acids in the peptide chain held together with three disulfide bonds. Glu_1-Plg and Lys_{77}-Pln both have five kringles; u-PA has one kringle, homologous to the fifth kringle of Plg; and t-PA has two kringles, the second kringle being homologous to the fifth kringle of Plg. The kringles are responsible for the binding of Plg and Pln to fibrin. The catalytic domain (active site) is homologous to other serine proteases. The activator active site catalyzes the hydrolysis of the Arg_{560}–Val scissle bond in Plg to produce Pln. Recombinant cDNA technology has produced recombinant t-PA and u-PA, which also have the fibrin-binding and catalytic domains, and their catalytic properties are similar to the naturally occurring species (Ichinose et al., 1986; Lijnen et al., 1986). Recently, two hybrid activators have been synthesized by covalently linking, through one disulfide bond, the binding domain of Plg (Pln_A) with the catalytic domain of either t-PA (t-PA_B) or u-PA (u-PA_B) to form Pln_A-t-PA_B and Pln_A-u-PA_B, respectively (Robbins & Tanaka, 1986; Robbins & Boreisha, 1987). In comparison with the parent activators, the fibrin-binding ability decreased ~1.1-fold for the t-PA hybrid but increased ~8-fold for the u-PA hybrid.

Alkjaersig et al. (1959) first suggested the importance of fibrin in the regulation of fibrinolytic activity. The fibrin enhancement of t-PA activation of Glu_1-Plg increased when the fibrin was slightly degraded by plasmin (Suenson et al., 1984). The binding of Glu_1-Plg to fibrin was induced by t-PA and u-PA, and this binding increased with progressive digestion of fibrin by Pln (Harpel et al., 1985). The specificity of this binidng is demonstrated by the inability of elastase-degraded fibrinogen to bind Glu_1-Plg. Observing that some fragments of fibrin(ogen) could enhance the activation of a mixture of Lys_{77}-Plg and Glu_1-Plg by t-PA, Nieuwenhuizen et al. (1983) had postulated that a site capable of accelerating t-PA activation of Plg existed on fibrinogen, which became exposed upon fibrin formation or when fibrinogen was hydrolyzed by Pln or CNBr. This site is within the stretch $A_{\alpha 148-197}$ of the A_α chain remnants of the fibrinogen degradation products. The only stimulatory CNBr fragment found is FCB-2 of molecular weight ~43 000 containing the $A_{\alpha 148-197}$ site, which is structurally related to plasmic fragment D. The lysine residue $A_{\alpha 157}$ is crucial for stimulation (Voskuilen et al., 1987). Chibber et al. (1985) had concluded that the stimulation by fibrinogen and fragment D of the activation of Glu_1-Plg by SK was due to the formation of an activator complex whose early fibrinogen-sensitive active sites changed to become fibrinogen-insensitive. In the case of u-PA, Lucas et al. (1983a) concluded that the interaction of fibrinogen or its fragments with Glu_1-Plg, not with u-PA, was the reason for enhancement of activation.

The effect of binding of fibrin(ogen) and its fragments on the activation of Plg has been studied under various experimental conditions, making a quantitative comparison of the different activators difficult. In this study, we have analyzed the activation of Glu_1-Plg, using a steady-state kinetic model (Wohl et al., 1980), by three SK species, u-PA, Pln_A-u-PA_B, t-PA, t-PA_B, and Pln_A-t-PA_B, at pH 7.4 and 37 °C, in the absence and presence of CNBr-Fg. Due to the experimental limitations in the use of fibrin to examine its effect on the fibrinolytic system, soluble CNBr-Fg was used (Zamarron et al., 1984; Cassels et al., 1987). The kinetic results have permitted us to understand better the differences between these activators in their reaction with Glu_1-Plg and the regulatory role of fibrin and its degradation products on fibrinolysis.

Experimental Procedures

Materials. The chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251) and human fibrinogen, grade L (KabiVitrum AB), were purchased from Helena Laboratories. Glu_1-Plg (22 casein units/mg of protein) and SK (100 000 IU/mg of protein) were a gift from KabiVitrum AB. Single-chain melanoma t-PA (500 000 IU/mg of protein) was a gift from Dr. D. Collen, Belgium. Lys_{77}-Pln (30 IU/mg of protein) was prepared from Glu_1-Plg with u-PA (Wohl et al., 1980) and stored at −70 °C in a 25% glycerol buffer. Human urinary HMW-u-PA (113 333 IU/mg of protein) was purified from a partially purified preparation as described by Robbins and Tanaka (1986). It was stabilized with 1% mannitol and kept at −70 °C. Val_{442}-Pln·SK (46 540 IU/mg of protein) was prepared from Val_{442}-Plg (Powell & Castellino, 1980) and SK and lyophilized. Val_{561}-Pln·SK (43 000 IU/mg of protein) was prepared from Val_{561}-Pln and SK and lyophilized (Summaria & Robbins, 1976). Glu_1-Pln·SK was prepared daily before use by incubating together equimolar concentrations of Glu_1-Plg with SK at 4 °C (Wohl et al., 1980). Hybrid Pln_A-u-PA_B (45 000 IU/mg of protein) was prepared from Lys_{77}-Pln_A and human u-PA_B (Robbins & Tanaka, 1986) and lyophilized. Hybrid Pln_A-t-PA_B (200 000 IU/mg of protein) was prepared from Lys_{77}-Pln_A and t-PA_B and lyophilized (Robbins & Boreisha, 1987). The lyophilized proteins were kept at −20 °C. Activator t-PA_B (62 000 IU/mg of protein) was prepared from two-chain t-PA and stored in 0.01 M potassium phosphate (P_i) buffer, pH 7.4, containing 50 μM imidazole and 0.01% Tween at −70 °C (Robbins & Boreisha, 1987). The concentrations of Pln and all the activators, except t-PA and t-PA_B, were determined by active-site titration with *p*-nitrophenyl *p'*-guanidinobenzoate (Chase & Shaw, 1969). The extinction coefficient of the *p*-nitrophenol at 410 nm is 12 700 in 0.1 M KP_i, pH 7.4 (Wohl, 1984). The concentrations of t-PA and t-PA_B were determined from their specific activities. Fibrinogen fragments were prepared by CNBr digestion of fibrinogen in formic acid solution (Zamarron et al., 1984) and stored at −70 °C.

Steady-State Kinetic Methods. In the kinetic assays the hydrolysis of S-2251 was used to monitor the reactions. The extinction coefficient of *p*-nitroaniline is 1×10^4 at 405 nm. The total reaction volume was 200 μL. The reaction was carried out in a masked microcuvette at 37 °C and was followed at 405 nm in the Cary 219 spectrophotometer. In the amidolytic reactions the substrate S-2251 varied from 0.1 to 1 mM. The reaction time did not exceed 3 min. The activation reactions were measured indirectly by measuring the hydrolysis of S-2251 (0.5 mM) by Pln generated by the action of the activators on Glu_1-Plg. Except for the t-PA species, all reactions were in 0.1 M KP_i, pH 7.4. The t-PA activator buffer was 0.05 M NaP_i, 0.12 M NaCl, 2.7 mM EDTA, and 0.01% Tween 80 (P_i–Tween), pH 7.4. Glu_1-Plg and S-2251 were equilibrated in buffer at 37 °C before the activator (6–10 μL) was added, and the absorbance was recorded. The assay periods for the SK and u-PA species were less than 3 min and up to 5 min for the t-PA species. The concentrations of the activators were 2.6×10^{-8}–9.3×10^{-10} M. Plg concentrations were 0.029–5.20 μM, where it varied at least 10-fold for each activator. In the reactions with CNBr-Fg, it (0.217 μM) was incubated for 2 min with the Plg and buffer before S-2251 was added. The activator was added last.

The Plg activation kinetics were performed and calculated according to the steady-state model proposed by Wohl et al. (1980), using the integrated form of the Michaelis–Menten rate equation. For all of the u-PA and t-PA species, the

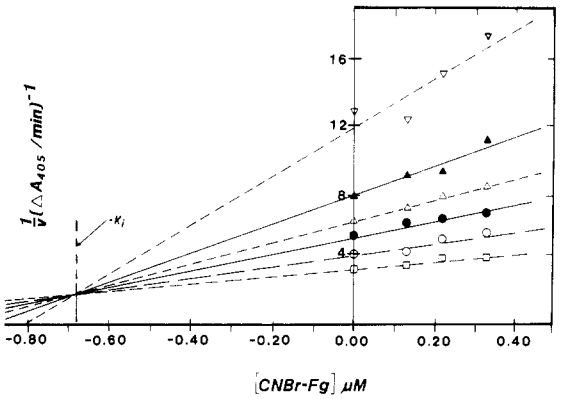

FIGURE 1: Dixon plot for CNBr-Fg inhibition of Lys_{77}-Pln. Lys_{77}-Pln concentration was 4.06×10^{-8} M. CNBr-Fg concentration varied between 0 and 0.326 μM. The molar concentrations of S-2251 were (□) 5×10^{-4}, (○) 3.5×10^{-4}, (●) 2.5×10^{-4}, (△) 2×10^{-4}, (▲) 1.5×10^{-4}, and (▽) 1×10^{-4}.

assumptions stated for the activation by u-PA were maintained, and all the assumptions made for the activation by SK were applied to all the SK species in these experiments. The raw data were analyzed with the ENZFITTER program developed by R. J. Leatherbarrow (Elsevier, Amsterdam, The Netherlands).

RESULTS

Inhibition of Plasmin by CNBr-Fg. The initial study of the effect of CNBr-Fg on Pln activity showed no effect at concentrations of CNBr-Fg less than 0.1 μM; however, inhibition increased with increasing and higher CNBr-Fg concentrations and leveled off to ~60–70% when it exceeded 1 μM. This effect was observed in both the P_i and the P_i–Tween buffers. At CNBr-Fg concentrations greater than 0.35 μM, turbidity interfered with the spectrophotometric assay. Thus, in all activation studies with CNBr-Fg, we kept its concentration at 0.217 μM, which inhibited 17% of the plasmin activity. In the activation studies involving CNBr-Fg, the observed velocities were corrected for inhibition of Pln by multiplying them by 1.20. The Lineweaver–Burk plots of the inhibition of Pln with three different fixed concentrations of CNBr-Fg, 0.130, 0.217, and 0.326 μM, intersect on the ordinate, indicative of competitive inhibition. The Dixon plot in Figure 1 gives an inhibition constant, K_i, of 6.82×10^{-7} M.

Amidase Activities. The amidolytic activities of Pln, Glu_1-Pln·SK, Val_{422}-Pln·SK, and Val_{561}-Pln·SK with substrate S-2251 at pH 7.4 and 37 °C are compared in Table I. All the SK reactions were carried out in 0.1 M P_i buffer, but the Lys_{77}-Pln reactions were carried out in both the P_i and P_i–Tween buffers. The latter buffer [similar to that used by Zamarron et al. (1984)] was chosen for the study of the activation by the t-PA species since their activities in the P_i buffer were much lower than in the P_i–Tween buffer. For Lys_{77}-Pln, the catalytic efficiency or second-order rate constant, k_{cat}/K_m, was 1.3-fold higher in the P_i–Tween buffer due to a lower K_m, indicating greater substrate–enzyme binding, and a higher k_{cat}, a faster turnover of substrate to product. This higher efficiency is likely caused by the presence of Tween since Pln activity is 1.2-fold less in the buffer without Tween (see below).

The apparent Michaelis constant, K_m, increased from 548 μM for Lys_{77}-Pln to 586–1540 μM for the SK activators, with the smallest enzyme molecule having the higher K_m. This pattern of increase was also reported by Wohl et al. (1980). The catalytic rate constants, k_{cat}, increased from 24.3 s^{-1} for Lys_{77}-Pln to 26.6–97.5 s^{-1} for the SK activators. Val_{422}-Pln·SK had the highest k_{cat} value, also reported by Wohl et al. (1980).

Table I: Amidase Kinetic Parameters at pH 7.4 and 37 °C with Substrate S-2251[a]

		amidase parameters		
enzyme	CNBr-Fg	K_m (μM^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Lys_{77}-Pln				
in P_i buffer	–	548 ± 31	24.3 ± 0.74	0.044
	+	749 ± 30	25.4 ± 0.64	0.034
in P_i–Tween	–	505 ± 39	29.8 ± 1.2	0.059
buffer	+	699 ± 57	26.1 ± 1.2	0.037
Glu_1-Pln·SK	–	598 ± 53	26.6 ± 1.3	0.044
	+	699 ± 67	24.1 ± 1.4	0.034
Val_{442}-Pln·SK	–	586 ± 22	97.5 ± 2.0	0.166
	+	581 ± 83	102 ± 7.6	0.176
Val_{561}-Pln·SK	–	1540 ± 218	93.7 ± 9.4	0.061
	+	1150 ± 102	79.9 ± 4.6	0.069

[a] In the assay, the molar concentrations of Pln, Glu_1-Pln·SK, Val_{442}-Pln·SK, and Val_{561}-Pln·SK were 4.06×10^{-8}, 1.45×10^{-7}, 1.33×10^{-8}, and 3.13×10^{-8}, respectively. CNBr-Fg (0.217 μM): absence (–), presence (+). The methodology is described under Experimental Procedures. Values given are mean ± standard error.

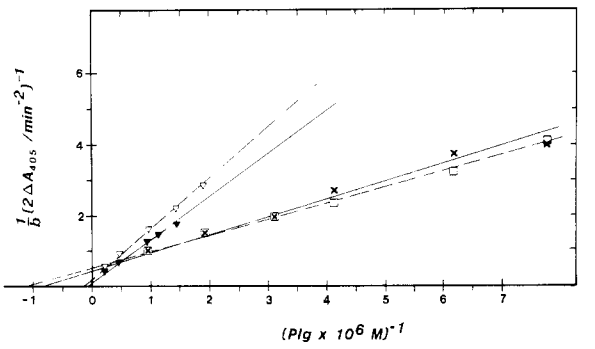

FIGURE 2: Lineweaver–Burk plots of activation of Glu_1-Plg by u-PA and Pln_A-u-PA_B. The molar concentrations of u-PA and Pln_A-u-PA_B were 8.56×10^{-9} and 1.59×10^{-8}, respectively. CNBr-Fg concentration was 0.217 μM. Glu_1-Plg concentrations varied between 0.1 and 5.2 μM; S-2251 was 0.5 mM. (□) u-PA; (×) u-PA + CNBr-Fg; (▽) u-PA hybrid; (▼) u-PA hybrid + CNBr-Fg.

But unlike the latter, the k_{cat} for Val_{561}-Pln·SK was 3.5-fold higher than that for Glu_1-Pln·SK. Our activators were lyophilized, whereas previous workers in this laboratory (Wohl et al., 1980) prepared them on ice before each experiment. Similar to the Wohl et al. (1980) data, the k_{cat}/K_m value for Glu_1-Pln·SK is the same as that obtained for Lys_{77}-Pln. However, the absolute values for these enzymes are different than those found in the previous study, as are all the amidase kinetic parameters. The different buffers are the most likely cause of the different kinetic data. In these experiments, we have used 0.1 M KP_i; previously, this laboratory used 0.05 M Tris–0.1 M NaCl buffer. We have observed that Pln in 0.1 M KP_i is 1.6-fold more active than that found both in 0.05 M KP_i and in 0.05 M KP_i–0.12 M NaCl, a buffer with the same ionic strength as 0.1 M KP_i. A comparison of the k_{cat}/K_m values indicated that Val_{442}-Pln·SK is 3.8-fold and Val_{561}-Pln·SK is 1.4-fold more efficient than both Lys_{77}-Pln and Glu_1-Pln·SK in their amidase activities.

The effect of CNBr-Fg on the amidase activities appears to be similar for Lys_{77}-Pln and Glu_1-Pln·SK. Their k_{cat}/K_m values decreased 1.3-fold in comparison with those in the absence of CNBr-Fg. On the other hand, Val_{442}-Pln·SK and Val_{561}-Pln·SK both showed a 1.1-fold increase in the presence of CNBr-Fg. Thus, CNBr-Fg caused Lys_{77}-Pln and Glu_1-Pln·SK to be less efficient but Val_{442}-Pln·SK and Val_{561}-Pln·SK to be more efficient enzymes in their hydrolysis of a synthetic substrate. The magnitude of the CNBr-Fg effect on all the amidase reactions, however, is insignificant.

Table II: Steady-State Kinetic Parameters of Activation of Human Glu_1-Plg by Various Activator Species at pH 7.4 and 37 °C[a]

activator	CNBr-Fg	activation parameters			CNBr-Fg enhancement[b]
		K_{plg} (μM)	k_{plg} (min^{-1})	k_{plg}/K_{plg} (μM^{-1} min^{-1})	(x-fold)
u-PA species					
u-PA	−	0.872 ± 0.065	33.1 ± 1.47	38.0	
	+	1.41 ± 0.075	58.8 ± 2.07	41.7	1.1
Pln_A-u-PA_B	−	7.44 ± 0.865	51.1 ± 4.12	6.87	
	+	12.3 ± 0.008	97.2 ± 0.05	7.90	1.2
t-PA species					
t-PA	−	0.331 ± 0.027	1.58 ± 0.04	4.77	
	+	0.122 ± 0.014	3.84 ± 0.13	31.5	6.6
Pln_A-t-PA_B	−	3.33 ± 0.442	1.03 ± 0.080	0.309	
	+	0.244 ± 0.027	0.919 ± 0.023	3.77	12.2
t-PA_B	−	19.1 ± 0.014	2.90 ± 0.002	0.152	
	+	27.6 ± 7.68	5.03 ± 1.33	0.181	1.2
SK species					
Glu_1-Pln·SK	−	0.458 ± 0.07	20.0 ± 1.80	43.6	
	+	0.354 ± 0.02	21.7 ± 0.79	61.3	1.4
Val_{442}-Pln·SK	−	0.268 ± 0.038	126 ± 10.7	470	
	+	0.220 ± 0.043	138 ± 17.4	627	1.3
Val_{561}-Pln·SK	−	0.121 ± 0.015	63.3 ± 3.72	523	
	+	0.345 ± 0.016	131 ± 3.90	380	0

[a] The molar concentrations of the activators are given in parentheses: Glu_1Plg·SK (7.7×10^{-9}); Val_{442}-Plg·SK (2.55×10^{-9}); Val_{561}-Pln·SK (9.39×10^{-10}); u-PA (8.56×10^{-9}); Pln_A-u-PA_B (1.59×10^{-8}); t-PA (7.14×10^{-9}); t-PA_B (2.31×10^{-8}); Pln_A-t-PA_B (2.63×10^{-8}). CNBr-Fg (0.217 μM): absence (−), presence (+). All other conditions are described under Experimental Procedures. Values given are mean ± standard error. [b] CNBr-Fg enhancement of k_{plg}/K_{plg}.

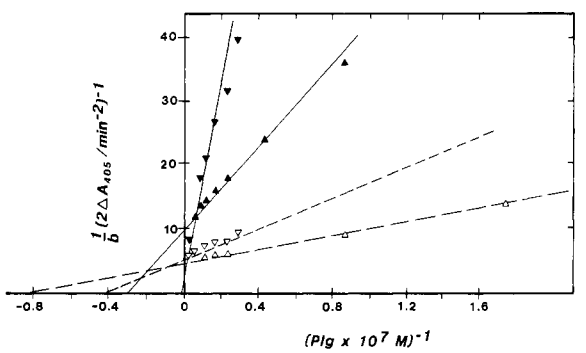

FIGURE 3: Lineweaver–Burk plots of activation of Glu_1-Plg by t-PA and Pln_A-t-PA_B. The molar concentrations of t-PA and Pln_A-t-PA_B were 7.14×10^{-9} and 2.63×10^{-8}, respectively. CNBr-Fg concentration was 0.217 μM. Glu_1-Plg concentrations varied between 0.029 and 3.6 μM; S-2251 was 0.5 mM. (▲) t-PA; (△) t-PA + CNBr-Fg; (▼) t-PA hybrid; (▽) t-PA hybrid + CNBr-Fg.

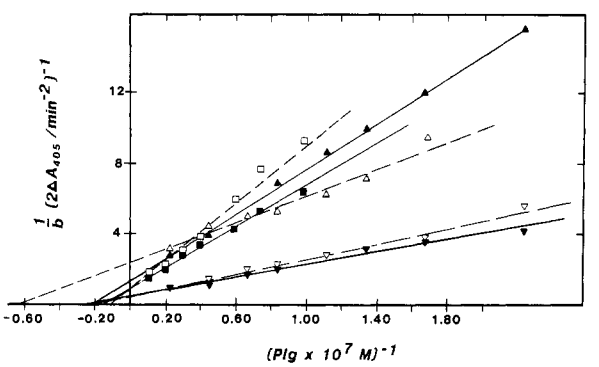

FIGURE 4: Lineweaver–Burk plots of activation of Glu_1-Plg by SK species. The molar concentrations were 7.7×10^{-9} for Glu_1-Pln·SK (□, ■), 2.55×10^{-9} for Val_{442}-Pln·SK (▽, ▼), and 9.4×10^{-10} for Val_{561}-Pln·SK (△, ▲). The open and filled figures are in the absence (□, ▽, △) and presence (■, ▼, ▲) of 0.217 μM CNBr-Fg, respectively. Glu_1-Plg concentration varied between 0.045 and 1 μM; S-2251 was 0.5 mM.

Activation Parameters. Urokinase (u-PA) Species. The u-PA and hybrid Pln_A-u-PA_B activators obeyed Michaelis–Menten kinetics in both the presence and absence of CNBr-Fg (Figure 2). Table II summarizes the kinetic parameters derived from Lineweaver–Burk plots. The second-order rate constants, k_{plg}/K_{plg}, indicated that u-PA is a 5.5-fold more efficient activator than its hybrid Pln_A-u-PA_B. The reason for the decrease in catalytic efficiency of the hybrid is an 8.5-fold increase in K_{plg} over that of u-PA. The large fibrin-binding domain, Pln_A, in the hybrid appears to interfere with the binding of the activator to Glu_1-Plg, even though the catalytic rate constant, k_{plg}, is increased 1.5-fold over that of u-PA.

The effect of CNBr-Fg is similar for u-PA and its hybrid. The enhancement was small in the k_{plg}/K_{plg} values for both activators, 1.1- and 1.2-fold for u-PA and its hybrid, respectively. Their K_{plg} increased ~1.6-fold, and their k_{plg} increased ~1.8-fold. The opposite effects of CNBr-Fg on the binding and catalytic rate constants cancel each other out, so that essentially CNBr-Fg has no significant effect on the activation on Glu_1-Plg by u-PA or its hybrid.

Tissue Plasminogen Activator (t-PA) Species. In both the presence and absence of CNBr-Fg, the activation of Glu_1-Plg by t-PA, the Pln_A-t-PA_B hybrid, and t-PA_B obeyed Michaelis–Menten kinetics (Figure 3; Table II). The second-order rate constant, k_{plg}/K_{plg}, indicated that t-PA is 15- and 31-fold more efficient than its hybrid and t-PA_B, respectively. The main difference may be attributed to a 10-fold increase in the apparent K_{plg} and a 1.5-fold decrease in k_{plg} of the hybrid. For t-PA_B, the difference is due to a 58-fold increase in the apparent K_{plg} and a 1.8-fold increase in the k_{plg}. CNBr-Fg increased the catalytic efficiency of t-PA and Pln_A-t-PA_B by 6.6- and 12.2-fold, respectively. Both activators showed increased binding with Glu_1-Plg as indicated by the decreased K_{plg}. The increase in binding is 2.7-fold for t-PA and 13.6-fold for the hybrid; k_{plg} increase 2.4-fold for t-PA but decreased 1.1-fold for the hybrid. In the absence of CNBr-Fg, the large Pln_A fibrin-binding domain (kringles) in the hybrid seems to interfere with the activation; but in the presence of CNBr-Fg, interaction of CNBr-Fg with the fibrin-binding domain probably changed the conformation of the hybrid favorably so that the reaction was enhanced. On the other hand, CNBr-Fg increased the catalytic efficiency of t-PA_B only 1.2-fold, as a result of a 1.4-fold decrease in K_{plg} and a 1.73-fold increase in k_{plg}.

Streptokinase (SK) Species. The SK species activators obeyed Michaelis–Menten kinetics in both the presence and

Table III: Comparison of Kinetic Parameters of Published Data on the Activation of Glu_1-Plg by Different Activator Species at pH 7.4 and 37 °C

activator	fibrin[a]	kinetic parameters			assay method[b]	Cl^-[c]
		K_{plg} (μM^{-1})	k_{plg} (min^{-1})	k_{klg}/K_{plg} (μM^{-1} min^{-1})		
Glu_1-Plg·SK (Glu_1-Pln·SK)						
Wohl et al. (1980)	−	0.12	8.21	68.4	initial rates	+
Chibber & Castellino (1986)	−	0.62	78	125	initial rates	−
	+	0.16	66	412	initial rates	−
	−	1.7	12	7	initial rates	+
	+	0.67	19.2	28.6	initial rates	+
this work	−	0.458	20	43.6	initial rates	−
	+	0.354	21.7	61.3	initial rates	−
u-PA, HMW						
Wohl et al. (1980)	−	1.72	47	27.4	initial rates	+
Watahiki et al. (1987)	−	1.45	55.8	38.5	initial rates	+
	+	1.45	75–138	57.1–95.2	initial rates	+
this work	−	0.872	33.1	38.0	initial rates	−
	+	1.41	58.8	41.7	initial rates	−
Lucas et al. (1983b)	−	2.5	3.4	1.36	two-step, 45 s	+
	+			6	two-step, 45 s	+
Lijnen et al. (1984)	−	53	90	1.7	two-step, 10 min	+
	+	1.1	21	19	two-step, 10 min	+
Lijnen et al. (1986)	−	67	276	4.12	two-step, na[d]	+
u-PA, LMW						
Wohl et al. (1980)	−	2.64	63	23.9	initial rates	+
Sumi et al. (1983)	−	2.21	44.1	20.2	initial rates	+
Urano et al. (1987)	−	2.2	96–120	43.6–54.5	initial rates	−
	−	25	96–120	3.84–4.8	initial rates	+
t-PA, one chain						
this work	−	0.331	1.58	4.77	initial rates	+
	+	0.122	3.84	31.5	initial rates	+
Zamarron et al. (1984)	−	83	4.2	0.0506	two-step, 10 min	+
	+	0.18	16.8	93.3	two-step, 10 min	+
t-PA, two chain						
Hoylaerts et al. (1982)	−	65	3.6	0.055	two-step, 10 min	+
	+	28	18	0.643	two-step, 10 min	+
	+	0.16	6	37.5	two-step, 20 min	+

[a] The activator was studied in both the presence (+) and absence (−) of fibrin, fibrinogen, and their fragments. [b] The assay of Pln is denoted as (1) "initial rates" when substrate S-2251 was present in the activation reaction or (2) "two-step, maximum time of incubation of activation reaction" when an aliquot of activated solution at various times was used for estimation of Pln concentration. [c] This denotes the presence (+) or absence (−) of Cl^- in the reaction buffer. [d] Not available.

absence of CNBr-Fg (Figure 4). The kinetic constants for the activation of Glu_1-Plg by the SK species (Table II) are not altogether similar to those reported by Wohl et al. (1980). The sources for the differences are likely due to the difference in the buffers and the preparation of our Val-Pln·SK activators (see above). The K_{plg} values decreased as the SK species decreased in size from Glu_1-Pln·SK to the intermediate, Val_{442}Pln·SK, to the smallest complex, Val_{561}Pln·SK. With the decrease in the size of the fibrin-binding domain (Pln_A), there was an increase in the k_{plg} values; however, the intermediate-sized species had the highest k_{plg}. Thus, the bulk of the Pln_A chain, from the NH_2 terminus to Val_{442}, is not significantly involved in the activation mechanism, as concluded by Wohl et al. (1980). Val_{561}-Pln·SK was the most efficient enzyme in the activation reaction, 12- and 1.1-fold more efficient than Glu_1-Pln·SK and Val_{442}-Pln·SK, respectively.

The activation reactions catalyzed by the SK species were affected by the presence of CNBr-Fg. Glu_1-Pln·SK and Val_{442}-Pln·SK showed a small increase in both binding ($1/K_{plg}$) and k_{plg}, which translated into an increase in catalytic efficiency of 1.4- and 1.3-fold, respectively. However, for the most efficient activator, Val_{561}-Pln·SK, binding was decreased 2.9-fold but k_{plg} increased 2-fold, which resulted in a 1.4-fold decrease in catalytic efficiency.

Discussion

In this study we have compared the activation of Glu_1-Pg by three different activator species, u-PA, t-PA, and SK, to determine the catalytic efficiency differences between them. Many studies with these activators have been performed under various conditions (Table III). All of them have required an indirect determination for generated Pln. The most common procedure for measuring Pln generation is by spectrophotometrically determining its hydrolysis of a chromogenic substrate, e.g., S-2251. Pln, however, can undergo many other reactions, which will interfere with the determination of its actual concentration. In a pH 7.4 Tris buffer at 37 °C, Lys_{77}-Pln has a half-life of 12 min (Lucas et al., 1983b). Pln can hydrolyze plasminogen to other lower molecular weight plasminogens. Lys_{77}-Plg is formed readily from Glu_1-Plg in the presence of Lys_{77}-Pln (Lucas et al., 1983b); it is generally activated several times faster than Glu_1-Plg (Markus et al., 1978b, 1979; Wohl et al., 1980; Lucas et al., 1983b). Autolysis would give a low estimation of Pln, and conversion of Glu_1-Plg to Lys_{77}-Plg will result in a greater amount of Pln formed in the activation reaction. To minimize such problems, we have employed the initial-rate method, using a steady-state kinetic model, to study the activation of Gu_1-Plg by different activators (Wohl et al., 1980). The Pln formed in the activation reaction was measured instantaneously by its hydrolysis of S-2251. The concentration of S-2251 (0.5 mM) was 10^2–10^5 times that of Glu_1-Plg. The excess S-2251 decreases the autolysis of Lys_{77}-Pln and the hydrolysis of Glu_1-Plg to Lys_{77}-Plg by Lys_{77}-Pln, by competing for its active site.

In comparing kinetic data on the activation of Glu_1-Plg, we will report only on those experiments with Glu_1-Plg at pH 7.4 and 37 °C. The effect of different buffers can give different kinetic results. The activation reactions are inhibited by anions

in the decreasing order $SCN^- > Cl^- > SO_4^{2-} > P_i >$ citrate (Radcliffe & Heize, 1980). Many activation studies have used fibrin(ogen) to modulate the reaction. The inhibition of Pln by a fibrin(ogen) component can complicate the Pln assay in the two-step method. This inhibition effectively lowers the concentration of Pln that can autodigest or hydrolyze Glu_1-Plg (Lucas et al., 1983b). In the two-step assay method, Glu_1-Plg in the reaction mixture is manyfold more concentrated than that in the initial-rate method. When the reaction mixture is diluted 20–100-fold for the assay of Pln, the concentrations of fibrin(ogen) component are at the noninhibitory level, so that a Pln concentration that is more reflective of the activation reaction is obtained, whereas, in the absence of a fibrin(ogen) component, autodigestion would result in apparently low Pln concentration. The combination of Pln autodigestion and fibrin(ogen) protection in the two-step method should give catalytic efficiencies that indicate apparent higher enhancement by the fibrin(ogen) component. Another complication in studying the effect of the fibrin(ogen) component is the continuous change in fibrin(ogen) structure caused by Pln digestion with concomitant changes in binding sites (Harpel et al., 1985; Norrman et al., 1985; Suenson et al., 1986; Higgins et al., 1987). In the short periods involved in the initial-rate method, minimal structural changes would be expected.

The effect of fibrinogen on the activation reaction with Glu_1-Plg·SK was independent of the Cl^- concentration (Chibber & Castellino, 1986). In both the presence and absence of Cl^-, the enhancement of the activation reactin by fibrinogen (1 μM) was ~4-fold. These workers concluded that this fibrinogen-sensitive (enhanceable) activator species, with a half-life of 7.45 min at 4 °C, converted readily to a fibrinogen-insensitive activator species that then yielded Pln·SK (Chibber et al., 1985). Our kinetic data on the SK-activator species showed that CNBr-Fg had very little effect on the kinetic parameters. All of our SK-activator species were "aged" and fibrinogen-insensitive; i.e., Glu_1-Pln·SK was stored at 4 °C for 30–120 min, and Val_{442}-Pln·SK had been lyophilized. The catalytic efficiency of Glu_1-Pln·SK obtained in the P_i buffer (43.6 μM^{-1} min^{-1}) lies between that of null and saturating concentrations of Cl^- (125 and 7 μM^{-1} min^{-1}, respectively) for Glu_1-Plg·SK.

The catalytic efficiency of u-PA was enhanced 1.3–11-fold by the addition of a fibrin(ogen) component (Table III). The reactions that were assayed by the two-step method showed severalfold higher enhancement than that by the initial-rate method. Watahiki et al. (1987) has reported that 0.2 μM fibrin(ogen) components enhanced catalytic efficiency 1.3–2.4-fold where the order of increasing effect was E < D < fibrinogen < fibrin. Our kinetic data indicate no significant enhancement of activation by u-PA in the presence of 0.217 μM CNBr-Fg, consistent with the lower range observed by these workers.

The fibrinolytic enhancement byfibrin of the t-PA activator of Glu_1-Plg in an identical buffer varied from 12- to 1844-fold in the two-step method, as indicated by the second-order rate constants (Table III). The fibrin components used in these experiments were saturating concentrations of CNBr-Fg (Zamarron et al., 1984), fibrinogen (10 min; Hoylaerts et al., 1982), and fibrin (20 min; Hoylaerts et al., 1982). Since the catalytic efficiencies of one-chain and two-chain t-PA in the presence of fibrin are essentially the same (Rånby, 1982; Rijken et al., 1982), the wide range of enhancement effects on t-PA activation must be attributed to these fibrin(ogen) components. The fibrin(ogen) species inhibit Pln in the following order of decreasing effectiveness: CNBr-Fg > fibrinogen > fibrin (see below). Consistent with the complications caused by Pln self-digestion and Pln protection by fibrino(ogen) components, apparently higher enhancement of the second-order rate constant was obtained with the better inhibitor and with a longer incubation period for the activation reaction. The two-step assay method failed to give the true second-order rate constants. At present, our kinetic data for the enhancement of t-PA activation by fibrin(ogen) component appeared to be the only data obtained with the initial-rate method. We observe that the catalytic efficiency is enhanced only 6.6-fold by 0.217 μM CNBr·Fg in P_i–Tween buffer.

The two covalent hybrids have decreased catalytic efficiencies when compared to their parent molecules due primarily to a 10-fold increase in their apparent K_{plg}. However, with u-PA, the catalytic rate constants, k_{plg}, increased 2-fold. These activators were synthesized by oxidizing a mixture of the sulfhydryl form of the NH_2-terminal plasmin-derived heavy (A) chain (Pln_A) with either the sulfhydryl form of the COOH-terminal B-chain of u-PA to give Pln_A-u-PA_B or the sulfhydryl form of the COOH-terminal B-chain of t-PA to give Pln_A-t-PA_B. The Pln_A domain has two SH groups ($Cys_{547/557}$) capable of forming a single disulfide bond with the one SH group of the B-chain to form either Pln_A-Cys_{547}-Cys-B-chain or Pln_A-Cys_{557}-Cys-B-chain. The activator activity of these two possible hybrids may be either identical or different. Whether the kinetic parameters are a reflection of one species or a mixture of two species is unknown. This uncertainty complicates the interpretation of our results. However, for simplification of interpretation we assume that only one kind of active species was involved for both hybrids.

In our kinetic analysis of the activation reactions, our model requires the inclusion of the kinetic parameters of the amidase reaction in our calculations. The increase in the catalytic efficiency of Pln in P_i–Tween buffers, as compared with that in P_i buffer, precludes the concern that differences between these two buffers would make impossible a comparison of kinetic parameters obtained in them. Since the t-PA reaction in the P_i buffer was too slow to be measured conveniently, its reaction was studied in the P_i–Tween buffer. Thus, the catalytic efficiency of t-PA must be smaller in the former. In comparing the second-order rate constants of t-PA with that of the other activators (in P_i buffer), we found that the t-PA constants would err on the high side. Even with this consideration, a comparison of the second-order rate constants of the three species of activators shows that the catalytic efficiency falls in the decreasing order SK > u-PA > t-PA. In all three species, the activator with the intact Pln_A fibrin-binding domain is the least efficient enzyme, particularly native Glu_1-Pln·SK, which contains the fibrin-binding domain undisturbed by reduction and oxidation. Thus, none of the five kringles appear to be essential for the activation reaction. However, they must affect the allosteric control of the activation reaction.

Glu_1-Plg has one strong ω-aminocarboxylic acid binding site on kringle 1 and four to five weaker binding sites, possibly distributed one in each of the five kringles in Pln_A (Markus et al., 1978a, 1979). Saturation of the strong binding site with ω-aminocaproic acid or tranexamic acid had an insignificant effect on the activatability of Glu_1-Plg by u-PA; however, saturation of the weaker sites led to conformational changes that increase u-PA activatability (Markus et al., 1978b, 1979). The ability of Plg to bind to fibrin(ogen) and their hydrolytic products has been quantitatively analyzed (Bok et al., 1985; Lucas et al., 1983a). While the strong lysine-binding site on

kringle 1 of Plg can bind to fibrin(ogen), it is the binding of fibrin(ogen) to the lower affinity lysine-binding sites that results in the enhancement of u-PA activation (Lucas et al., 1983a). Our results show that CNBr-Fg enhanced nearly all the activator reactions, except Val_{561}Pln·SK. We suggest that the enhancement observed in the presence of CNBr-Fg is due to a favorable change in conformation in the substrate Glu_1-Plg produced by its interaction with the $A_{\alpha 157}$ lysine residue of Fg (Voskuilen et al., 1987). For each of the three activator species, the enhancement was greater for the poorer activator. The less efficient activators appear to be more sensitive to the conformational change caused by CNBr-Fg. However, the rate-determining step in the activation reaction is not the conformational change on Glu_1-Plg caused by CNBr-Fg. The binding of CNBr-Fg does not result in significant change in the activation reactions of the SK and u-PA species, indicating that these activators do not need fibrin(ogen) to be effective enzymes. The complexity of the factors involved in the catalytic reactions is indicated by the changes in different directions in the values of the apparent K_{plg} and k_{plg} in the presence of CNBr-Fg. The multiplicity of the many factors involved in the catalytic reaction precludes the selection of any one factor as the dominant force in the in vitro reaction. However, only for the t-PA activators with both fibrin-binding and catalytic domains does the presence of fibrin(ogen) cause significant rate enhancement in second-order rate constants, 6.6-fold for t-PA and 12.2-fold for Pln_A-t-PA_B. Lacking in the NH_2-terminal domain, t-PA_B was essentially not affected by CNBr-Fg. This is comparable to the activation of Lys_{77}-Plg, where there was fibrin(ogen) enhancement of activation by t-PA but not by t-PA_B (Dodd et al., 1986; Rijken et al., 1986). The second t-PA kringle contains a binding site for fibrin (Ichinose et al., 1986; van Zonneveld et al., 1986). Thus, the enhancement observed with t-PA and Pln_A-t-PA_B may be due to an interaction of CNBr-Fg with the fibrin-binding domains of the t-PA species and of Glu_1-Plg. In this process, one molecule of CNBr-Fg would interact with one molecule of the t-PA species, and/or one molecule of Glu_1-Plg, to form a trimolecular intermediate that eventually hydrolyzes the bound Plg to Pln (Hoylaerts et al., 1982).

One similarity among the molecules of the fibrin(ogen) family is their inhibition of Pln. Fears et al. (1985) have reported that lower concentrations of fibrin(ogen) did not affect Pln's S-2251 activity, but 0.68 μM fibrinogen inhibited Pln 20% and 0.68 μM fibrin inhibited Pln 15%. Ranby (1982) observed that 0.1 μM fibrin did not inhibit Pln, but 0.3 μM fibrin inhibited Pln 13%. Wiman et al. (1979) reported fibrinogen competitively inhibited Pln with a $K_i = 1.9 \times 10^{-6}$ M at pH 7.3 and 25 °C. Our data on inhibition of Pln by CNBr-Fg gave a profile that is similar to that of fibrin(ogen). We suggest that fibrin(ogen) and CNBr-Fg inhibits Pln by an identical mechanism. Intact fibrin and fibrinogen are not necessary for the inhibition. The region that can interact with Pln to give inhibition is more accessible in CNBr-Fg than in fibrin(ogen). The smaller K_i for CNBr-Fg, compared to that for Fg, may be a reflection of this difference. Under physiological conditions, after fibrin-bound Pln has dissolved a blood clot, the fibrin fragments can inhibit Pln. These fragments provide a control of Pln activity when it is no longer beneficial.

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Irreversible Degradation of Histidine-96 of Prothrombin Fragment 1 during Protein Acetylation: Another Unusually Reactive Site in the Kringle†

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ABSTRACT: Acetylation of prothrombin fragment 1 in acetate–borate buffer at pH 8.5 resulted in the appearance of increased light absorbance at about 250 nm. Protease digestions resulted in isolation of a single peptide (residues 94–99) with intense absorbance at about 250 nm (estimated extinction coefficient of 5000 M^{-1} cm^{-1}). Amino acid analysis showed the expected composition except for the absence of His-96. Instead, an unidentified amino acid which had a ninhydrin product with absorption properties similar to those of proline eluted near aspartate. When sequenced, this peptide (YP?KPE containing ε-amino-acetyllysine) lacked histidine at the third position but gave a high yield of a PTH derivative that eluted near PTH-Gly from the HPLC column. Fast atom bombardment mass spectrometry of the derivatized 94–99 peptide showed a mass that was 74 units higher than expected. The histidine degradation product was identified as a di-N-acetylated side chain with an opened imidazole ring and loss of C_2 of the ring. While a similar degradation pattern has previously been reported during acylation of histidine, the high chemical reactivity exhibited by His-96 was unusual. For example, under conditions sufficient for quantitative derivatization of His-96, His-105 of fragment 1 was not derivatized to a detectable level. Furthermore, His-96 in fragment 1 was at least an order of magnitude more susceptible to degradation than His-96 in the isolated 94–99 peptide. His-96 is therefore one of several neighboring amino acids of the kringle portion of fragment 1 that displays highly unusual chemistry (see also Asn-101 [Welsch, D. J., & Nelsestuen, G. L. (1988) *Biochemistry 27* 4946–4952] and Lys-97 [Pollock, J. S., Zapata, G. A., Weber, D. J., Berkowitz, P., Deerfield, D. W., II, Olson, D. L., Koehler, K. A., Pedersen, L. G., & Hiskey, R. G. (1988) in *Current Advances in Vitamin K Research* (Suttie, J. W., Ed.) pp 325–334, Elsevier Science, New York]). Unusual ^1H NMR signals from histidine residues in the analogous position of other kringle sequences have been reported as well [Hochswender, S. M., Laursen, R. A., De Marco, A., & Llinas, M. (1983) *Arch. Biochem. Biophys. 223*, 58–67; Llinas, M., De Marco, A., Hochschwender, S. M., & Laursen, R. A. (1983) *Eur. J. Biochem. 135*, 379–391; Trexler, M., Banyai, L., Patthy, L., Pluck, N. D., & Williams, R. J. P. (1983) *FEBS Lett. 154*, 311–318]. This region of kringle structures may constitute an unusual component determined by folding of the kringle.

Prothrombin is a vitamin K dependent protein which is required in the penultimate step of the coagulation cascade (Stenflo & Suttie, 1977; Nemerson & Furie, 1980; Nelsestuen, 1984). It requires a substantial amount of posttranslational modification for function [i.e., cleavage of the pre- and propeptides (Degen et al., 1983; MacGillivray & Davie, 1984), glycosylation (Magnusson et al., 1975), and the vitamin K dependent conversion of specific Glu residues to γ-carboxyglutamic acid (Gla)[1] residues (Nelsestuen et al., 1974; Stenflo et al., 1974)]. Prothrombin fragment 1 (the amino-terminal 156 amino acids of prothrombin) contains all 10 of the Gla residues of prothrombin as well as a triple-looped sequence of amino acids known as a kringle (Magnusson et al., 1975). The binding of a variety of metal ions to fragment 1 causes the peptide to undergo a conformational change that can be observed by a decrease in intrinsic protein fluorescence (Nelsestuen, 1976; Prendergast & Mann, 1977). This metal ion induced conformational change is required for subsequent protein binding to phospholipid surfaces (Nelsestuen et al., 1976). The precise nature of the metal ion and membrane

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; fragment 1, amino acids 1–156 of the amino terminus of bovine prothrombin; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

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