Effects of Human Fibrinogen and Its Cleavage Products on Activation of Human Plasminogen by Streptokinase†

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ABSTRACT: The influence of human fibrinogen (Fg) and its terminal plasminolytic digestion products, fragment D and fragment E, on the kinetics of activation of human plasminogen (Pg) by catalytic levels of streptokinase (SK) has been investigated. Both Fg and fragment D enhanced the rates of activation of human Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg. Fragment E was refractive in this regard. In the case of Glu_1-Pg, the K_m for activation by SK, 0.4 μM, was not affected by the presence of Fg or fragment D. The k_{cat} for this same reaction, 0.12 s^{-1}, was elevated to 0.3 s^{-1} at saturating levels of these effector molecules. On the other hand, the K_m for activation of Lys_{77}-Pg, 0.5 μM, was decreased to 0.09 μM, whereas the k_{cat}, 0.33 s^{-1}, was not altered in the presence of saturating concentrations of Fg or fragment D. In the case of Val_{442}-Pg, the K_m for this same activation, 2.0 μM, was lowered to 0.4 μM and 0.25 μM in the presence of Fg and fragment D, respectively. The k_{cat} for this process, 1.0 s^{-1}, was unchanged in the presence of these agents. The concentrations of Fg (K_{Fg}) and fragment D (K_{FD}) that led to half-maximal stimulation of the activation rates were determined. For Fg with Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg, the K_{Fg} values were 0.08 μM, 0.14 μM, and 0.17 μM, respectively. The K_{FD} values for these same plasminogens were 0.25 μM, 2.0 μM, and 1.7 μM, respectively. Mechanistic analysis of these data demonstrated that activation of Lys_{77}-Pg and Val_{442}-Pg, in the presence of Fg and fragment D, was ordered and sequential, with Fg or fragment D first binding to the activator complex and then to Pg. Further, we show herein that, in the SK·Glu_1-Pg activator complex, an early Fg-sensitive active site forms, which decays to a Fg-insensitive active site. During the latter process, the only molecular forms of these proteins that exist are SK and Glu_1-Pg.

Human plasminogen is the plasma protein zymogen of the fibrinolytic and fibrinogenolytic protease, plasmin. Activation of plasminogen to plasmin by all known activators is accomplished by cleavage of one peptide bond, i.e., Arg_{560}-Val_{561}, in the single-chain precursor molecule (Robbins et al., 1967).

In addition to serving as a substrate for plasmin action, fibrinogen and its cleavage products have been shown in recent years to be positive effectors of plasminogen activation by streptokinase (Camiolo et al., 1980; Takada et al., 1980; Violand et al., 1980). Employing human uterine tissue plasminogen activator, Hoylaerts et al. (1982) demonstrated that the stimulation of plasminogen activation in the presence of fibrinogen and fibrin followed an ordered and sequential mechanism, wherein the activator first bound to the effector, followed by plasminogen. With human urokinase as the activator of plasminogen, Lucas et al. (1983) have shown that fibrinogen and fragment D stimulated the k_{cat}/K_m for Glu_1-Pg[1] activation by approximately 4-fold and that fibrinogen fragment E elevated the k_{cat}/K_m for this activation by approximately 2-fold.

Since the active participation of fibrinogen and fibrin in reactions that lead to their proteolysis has been established to be of primary importance in the overall mechanism of fibrinolysis, we wished to understand more thoroughly the mechanism by which these effectors functioned in this regard. Unlike tissue plasminogen activator, which has been demonstrated to bind to fibrinogen and fibrin (Thorsen et al., 1972), streptokinase has not been shown to possess this property. Thus, elucidating the mechanism whereby fibrinogen is capable of stimulating activation of plasminogen by streptokinase is particularly pertinent. This paper represents a report of our studies in this latter area.

Materials and Methods

Proteins. Native Glu_1-Pg was purified from human Cohn fraction III by using a gradient elution modification (Brockway & Castellino, 1972) of the Deutsch & Mertz (1970) affinity chromatography technique. Plasminogen variant 1 was used for all studies described herein. Urokinase-free plasmin and Lys_{77}-Pg were prepared as described by Strickland et al. (1982). Val_{442}-Pg was generated by partial elastolytic digestion of Glu_1-Pg and purified by affinity chromatography according to published procedures (Powell & Castellino, 1980). SK was purified from Kabikinase (AB Kabi) as reported previously (Castellino et al., 1976), and UK was a gift of Dr. W. H. Holleman of Abbott Laboratories.

Human fibrinogen was purified from fresh human plasma as described earlier (Morris et al., 1981). Fibrinogen fragments D and E were obtained essentially as described by Pizzo et al. (1973). In this procedure we found it necessary to repetitively pass the products over the DEAE-cellulose column described in that report in order to remove all traces of fragment D from fragment E, and vice versa.

Protein concentrations were determined spectrophotometrically by using the following $\epsilon_{280nm}^{1\%,1cm}$ and molecular weight values, respectively: Glu_1-Pg, 17.0 and 92 000 (Violand &

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[1] Abbreviations: Glu_1-Pg, native circulating human plasminogen containing glutamic acid at the NH_2 terminus; Lys_{77}-Pg, a human plasminogen form containing lysine-77 as the NH_2-terminal amino acid; Val_{442}-Pg, a human plasminogen fragment containing valine-442 at the NH_2 terminus. SK, UK, Fg, and Pm refer to streptokinase, urokinase, fibrinogen, and plasmin, respectively.

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Castellino, 1976); Lys_{77}-Pg, 17.0 and 84000 (Violand & Castellino, 1976); Val_{442}-Pg, 16.0 and 38000 (Sottrup-Jensen et al., 1977); SK, 9.5 and 45000 (Brockway & Castellino, 1974); Fg, 16.2 and 330000 (Blomback & Blomback, 1959; Scheraga & Laskowski, 1957); fragment D, 19.9 and 94000 (Ferguson et al., 1975); fragment E, 10.2 and 50000 (Marder et al., 1969).

Polyacrylamide Gel Electrophoresis. Protein samples were denatured in sodium dodecyl sulfate ($NaDodSO_4$)–6 M urea solutions at 37° C, with or without β-mercaptoethanol. Electrophoresis was conducted according to published procedures (Weber & Osborn, 1969).

Activation Assays. The SK-mediated activation of plasminogen was monitored spectrophotometrically in a coupled assay employing a chromogenic substrate, D-Val-Leu-Lys-*p*-nitroanilide (S-2251, Kabi), to measure the activity of generated plasmin. Prior to assay, purified fragment D and fragment E were exhaustively dialyzed against the assay buffer, 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl)/0.1 M NaCl, pH 7.6 at 25 °C (pH 7.4 at the assay temperature, 37 °C), overnight in the cold. Lyophilized plasminogen and SK were dissolved in assay buffer. The substrate was reconstituted in H_2O and its concentration determined spectrophotometrically ($\epsilon^{1cm,1M}_{316nm}$ = 13000). All solutions were preserved on ice.

Assays were performed in a final volume of 0.8 mL, constituted as follows: water and assay buffer (2-fold concentrated) were added to a cuvette, followed by aliquots of the stock substrate, Fg, fragment D, or fragment E (when desired), and plasminogen such that the volume at this point was 0.79 mL. The cuvette was then equilibrated at 37 °C in the thermostated sample compartment of a Cary 219 recording spectrophotometer. A quantity of 10 μL of SK solution (diluted appropriately from a stock solution at >1 mg/mL) was added, and the increase in absorbance at 405 nm, corresponding to *p*-nitroanilide liberation from S-2251, was continuously recorded. The final concentrations of the components of the assay were as follows: Tris·HCl, 0.05 M; NaCl, 0.1 M; S-2251, 0.5 mM; SK, 1 nM; Fg, fragment D, or fragment E, 0–2 μM; plasminogen, 0.1–1 μM.

Formation of SK–Activator Species. SK at a final concentration of 80 nM in the above assay buffer was incubated at 4 °C for 1 h in the following mixtures separately: (a) 82 nM Fg, (b) 79 nM Glu_1-Pg, and (c) 82 nM Fg and 79 nM Glu_1-Pg. Following incubation, 10-μL aliquots of these solutions were employed separately, as replacements for SK, in the above activation mixture.

Time-Dependent Alterations in the Equimolar Streptokinase–Plasminogen Activator Complex. SK and Glu_1-Pg, both at final concentrations of 0.8 μM in assay buffer, were incubated at 4 °C. At various times after mixing of the two proteins, aliquots (0.01 mL) were withdrawn and mixed with 0.09 mL of assay buffer. An aliquot (0.01 mL), corresponding to a final concentration of 1 nM SK, was used in the above assay in lieu of SK at 4 °C. Control activations were performed with SK alone (without preincubation with Glu_1-Pg) and with or without Fg (1.1 μM). At times corresponding to the preincubation periods of SK and Glu_1-Pg, samples were removed from the stock solution and analyzed by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis.

Results

Activation Assays. Our methods for calculating rates of plasminogen activation, and their justification, are detailed in the Appendix. Activation rates were reproducible within an error range of 5% in all studies, and this is approximately

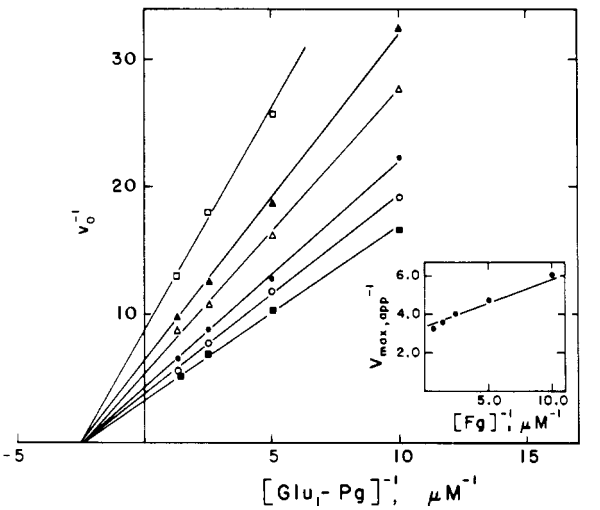

FIGURE 1: Lineweaver–Burk plots of the reciprocal of the initial activation rate v_0^{-1} of Glu_1-Pg, expressed as seconds per nanomolar Pm, against the reciprocal concentration of Glu_1-Pg, using the continuous S-2251 assay described under Materials and Methods, at various levels of Fg. The Fg concentrations were (□) 0, (▲) 0.1, (△) 0.2, (●) 0.4, (○) 0.8, and (■) 1.6 μM. (Inset) The $V_{max,app}$ values, obtained from the *y* intercepts of the above plot, are plotted against the concentrations of Fg.

the error in the steady-state parameters. All data points represent the average of three replicate experiments.

Effects of Fg, Fragment D, and Fragment E on Plasminogen Activation. It has been shown in a previous report that Fg and fragment D stimulated the rates of activation of SK of Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg (Strickland et al., 1982). In that same report, we demonstrated that fragment E was refractive in that regard. In light of the fact that recent studies by Takada et al. (1982) suggested that fragment E did stimulate Pg activation by SK, we thoroughly repeated our previous studies under a wide range of Pg, SK, and fragment E concentrations. Once again, we were not able to demonstrate any enhancing ability of fragment E. In view of these results, no further kinetic studies were undertaken of the effects of fragment E on Pg activation.

Effect of Fg on Glu_1-Pg Activation. A Lineweaver–Burk plot of the effect of Fg on the initial rate of Glu_1-Pg activation by SK, at various initial concentrations of Glu_1-Pg, is shown in Figure 1. Clearly, Fg enhances this reaction and exerts its influence by increasing the $V_{max,app}$ of the activator. There is no detectable change in the K_m of the reaction, which has a value of 0.40 μM. The inset of Figure 1 illustrates the results of a replot of the dependency of the V_{max} of this reaction on the concentration of Fg. Upon extrapolation of these data to the *y* intercept, a k_{cat} of 0.29 s^{-1} is obtained in the presence of saturating levels of Fg and Glu_1-Pg. This is contrasted to a k_{cat} of 0.12 s^{-1} for Glu_1-Pg activation in the absence of Fg. From the values of the k_{cat}/K_m listed in Table I for activation of Glu_1-Pg, the presence of Fg results in approximately a 2.5-fold stimulation of the efficiency of the activator complex, mainly due to effects of Fg on the V_{max} of the enzyme. The dissociation constant for Fg (K_{Fg}) in the activation of Glu_1-Pg by SK has been determined from a replot of the data of Figure 1. Figure 2 represents a replot of the data in Figure 1 to reflect the dependence of activation rates at various Fg concentrations. The results show that K_{Fg} is constant at various levels of Glu_1-Pg, with a value of 0.08 μM. The inset of Figure 2 illustrates a plot of the V_{max} values, obtained from the *y* intercepts of Figure 2, against the concentration of Glu_1-Pg. The V_{max} for the reaction at infinite concentrations of Fg and

Table I: Influence of Fibrinogen and Fragment D on Steady-State Kinetic Parameters for Activation of Human Plasminogen by Streptokinase

conditions	parameter	Glu_1-Pg	Lys_{77}-Pg	Val_{442}-Pg
fibrinogen absent	K_m (μM)	0.40	0.50	2.0
	k_{cat} (s^{-1})	0.12	0.33	1.0
	k_{cat}/K_m (s^{-1} μM^{-1})	0.30	0.66	0.5
fibrinogen present	K_m (μM)	0.40	0.09	0.40
	k_{cat} (s^{-1})	0.29	0.31	1.0
	k_{cat}/K_m (s^{-1} μM^{-1})	0.73	3.67	2.5
	K_{Fg} (μM)	0.08	0.14	0.17
fragment D present	K_m (μM)	0.40	0.16	0.25
	k_{cat} (s^{-1})	0.30	0.30	1.0
	k_{cat}/K_m (s^{-1} μM^{-1})	0.75	2.06	5.0
	K_{FD} (μM)[a]	0.25	2.0	1.7

[a] These experiments were performed in the presence of 5.0 mM $CaCl_2$.

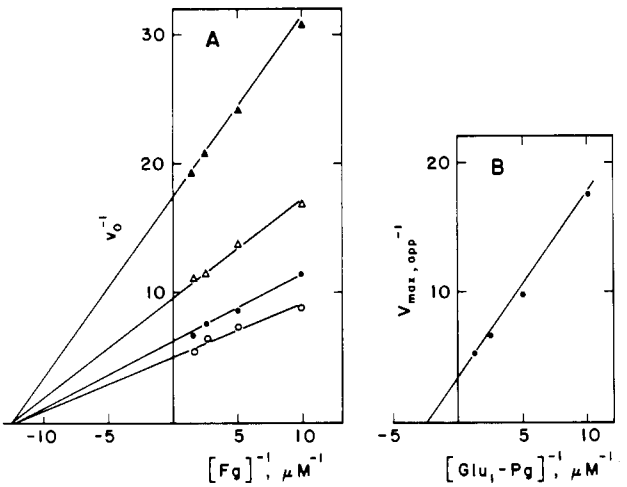

FIGURE 2: (A) As in Figure 1, except that the v_0^{-1} values have been plotted against the reciprocal concentration of Fg, at various levels of Glu_1-Pg. The Glu_1-Pg concentrations were (▲) 0.1, (△) 0.2, (●) 0.4, and (○) 0.8 μM. (B) The $V_{max,app}$ values, obtained from the y intercepts of (A), are plotted against the reciprocal concentrations of Glu_1-Pg.

Glu_1-Pg, obtained from the y intercept of this plot, is calculated to be 0.3 s^{-1} at saturating levels of Fg and Glu_1-Pg, in agreement with the same value calculated from the inset of Figure 1.

Effect of Fg on Lys_{77}-Pg Activation. Similar kinetic experiments have been conducted on the activation of Lys_{77}-Pg by catalytic levels of SK. Lineweaver–Burk plots of the dependence of the initial activation rate on the concentration of this zymogen, at different concentrations of Fg, are presented in Figure 3A. The graphs are distinctly different from those obtained for Glu_1-Pg activation in Figure 1. Here the $k_{cat,app}$ for the activation of Lys_{77}-Pg, 0.33 s^{-1}, is not affected by the concentration of Fg; the stimulating ability of Fg is due to its lowering the $K_{m,app}$ for this process. Figure 3B illustrates the results of a replot of the dependence of the $K_{m,app}$, obtained from the x intercept of Figure 3A, upon the concentration of Fg. At saturating levels of Fg, the K_m for activation of Lys_{77}-Pg by SK is calculated to be 0.09 μM. This is compared to the K_m value of 0.50 μM obtained for activation of Lys_{77}-Pg by SK in the absence of Fg, as obtained from the data of Figure 3A. The K_{Fg} has been calculated from the x intercept of Figure 3B and found to be 0.14 μM. The method for this later calculation is based upon the assumption (vide infra) of an ordered bireactant mechanism for the effect of Fg on the activation of Lys_{77}-Pg by SK. Overall, the k_{cat}/K_m for activation of Lys_{77}-Pg by SK in the presence of Fg is 3.67 s^{-1} μM^{-1}, a value that is compared to 0.66 s^{-1} μM^{-1} in the absence of

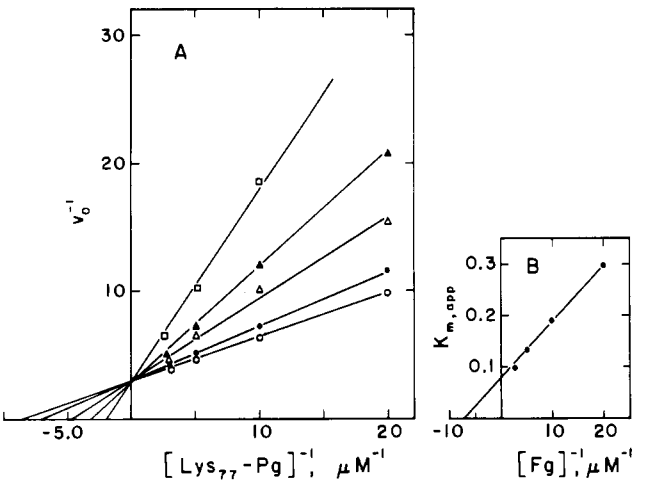

FIGURE 3: (A) Lineweaver–Burk plots of the reciprocal of the initial activation rate v_0^{-1} of Lys_{77}-Pg, expressed as seconds per nanomolar Pm, against the reciprocal concentration of Lys_{77}-Pg, at various levels of Fg. The Fg concentrations were (□) 0, (▲) 0.05, (△) 0.1, (●) 0.2, and (○) 0.4 μM. (B) The $K_{m,app}$ values, obtained from the x intercepts of the graph in Figure 3A, are plotted against the concentrations of Fg.

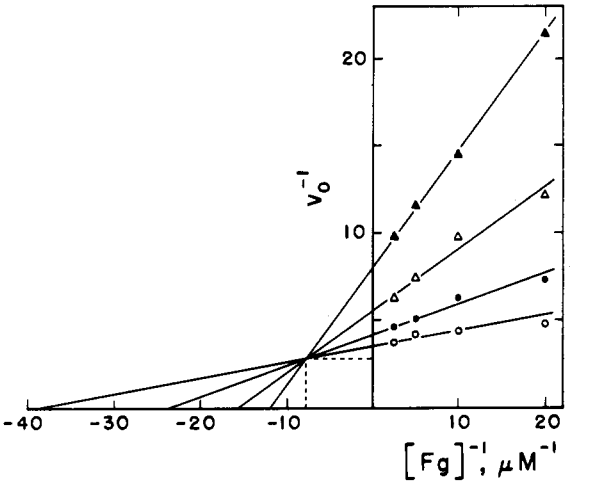

FIGURE 4: As in Figure 3A, except that the v_0^{-1} values have been plotted against the reciprocal concentration of Fg, at various levels of Lys_{77}-Pg. The Lys_{77}-Pg concentrations were (▲) 0.05, (△) 0.1, (●) 0.2, and (○) 0.4 μM.

Fg. This 5.6-fold stimulation of the reaction by Fg is due exclusively to the lowering of K_m by Fg.

When the initial rate data of Figure 3 are replotted against the fibrinogen concentrations at different fixed levels of Lys_{77}-Pg, a series of straight lines interacting in the second quadrant are obtained, as seen in Figure 4. The nature of this plot indicates that the reaction mechanism is consistent with a rapid equilibrium ordered bireactant system, where Fg adds to the activator prior to Lys_{77}-Pg. A replot from Figure 4 (not shown) of reciprocal $V_{max,app}$ values against the reciprocal of the Lys_{77}-Pg concentrations yields a k_{cat} of 0.34 s^{-1} and a K_m of 0.091 μM, values that are in agreement with those calculated from the graphs of Figure 3. A similar replot (not shown) of the reciprocals of the $K_{Fg,app}$ values of Figure 4 against the reciprocals of the Lys_{77}-Pg concentrations yields a K_{Fg} of 0.12 μM and a K_m of 0.11 μM, again in agreement with the values of these same parameters as calculated from plots of Figures 3 and 4. A summary of all kinetic constants is provided in Table I.

The results illustrated in Figure 4 are consistent with a kinetic reaction mechanism involving nonessential enzyme activation via a rapid equilibrium ordered bireactant sysem

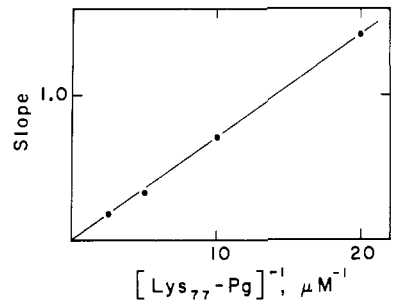

FIGURE 5: Replot of the slopes of the lines of Figure 4 against the concentrations of Lys_{77}-Pg.

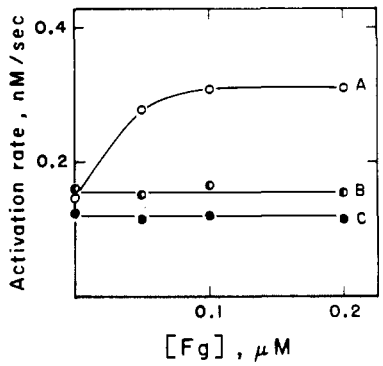

FIGURE 6: Effect of preincubation of SK and Glu_1-Pg on the activity of the activator complex to be stimulated by Fg. The preincubated activator species were (○) SK and Fg, (◐) SK and Glu_1-Pg, and (●) SK, Fg, and Glu_1-Pg. The experimental details are provided under Materials and Methods.

(Segel, 1975). We have tested this hypothesis by generating a plot (Figure 5) of the slopes of the lines of Figure 4 against the reciprocal concentrations of Lys_{77}-Pg. The resulting graph is a straight line that passes through the origin, verifying that the reaction is ordered with Fg adding to the Lys_{77}-Pg·SK activator prior to its substrate, Lys_{77}-Pg (Segel, 1975).

Effects of Fg on Val_{442}-Pg Activation. The kinetic behavior of Val_{442}-Pg toward activation by SK, as well as the effect of Fg on this process, is qualitatively very similar to that of Lys_{77}-Pg, and plots similar to those presented in Figures 3–5 are obtained. Values for the K_m, k_{cat}, and K_{Fg} for Val_{442}-Pg activation are summarized in Table I. Again, the kinetic behavior is in accordance with an ordered mechanism (vide supra).

Effect of Fragment D on Plasminogen Activation. All kinetic plots for the effect of fragment D on the rates of activation by SK of Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg are qualitatively identical with those for Fg, presented in Figures 1-5. Values for K_m, k_{cat}, and the dissociation constant for fragment D (K_{FD}) from these data for each plasminogen are listed in Table I.

Generation of a Fg-Enhanceable Active Site in the SK·Pg Activator Complex. During the course of the studies reported in this paper, we observed that, upon prolonged incubation of SK with Pg, the resultant activator complex progressively lost its ability to be enhanced by Fg. In an earlier study (Strickland et al., 1982), we had shown that the activity of the final stable activator complex, SK·Pm, was not enhanced by Fg. The results shown in Figure 6 demonstrate that, upon incubation of SK with Glu_1-Pg in the presence or absence of Fg and assay of its activator activity in the presence of increasing levels of Fg, an activator species is formed that cannot be stimulated by Fg. Stimulation only occurs when SK is added to Glu_1-Pg under otherwise complete assay conditions. These results suggest that Fg only stimulates an early form of the activator

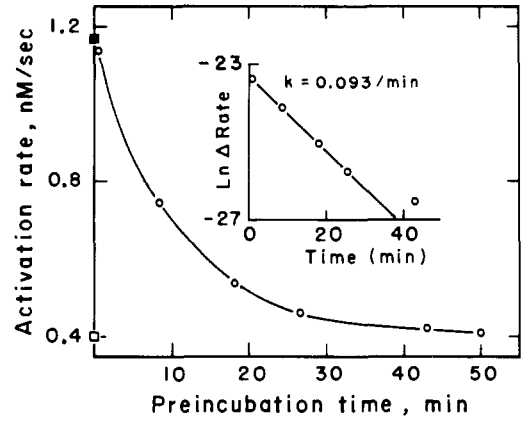

FIGURE 7: Time course of the loss of ability of Fg to stimulate the activity of the SK·Glu_1-Pg activator complex. A preincubation mixture of SK and Glu_1-Pg (both at 0.8 μM) was utilized to activate plasminogen in the presence of Fg (1.1 μM) at 4 °C. Activation rates without preincubation in the absence (□) and presence (■) of Fg are also shown. (Inset) First-order kinetic plot of the data.

complex. This concept is verified by the data in Figure 7, which shows the rate of loss of stimulation by Fg of the preformed SK·Glu_1-Pg activator complex. This process is characterized by a first-order rate constant of 0.093 min^{-1}. Upon concurrent analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of the nature of the proteins that were present during the time period wherein the activator complex lost its ability to be stimulated by Fg, we found that the only molecular components were SK and Glu_1-Pg.

Discussion

The enhancement by Fg of the activation of human plasminogen by SK has been noted in several laboratories (Camiolo et al., 1980; Takada et al., 1980; Violand et al., 1980). However, the mechanism for the augmentation by SK has not been described in detail. In the case of activation of Glu_1-Pg by SK in the presence of Fg or fragment D, the data of Figure 1 show clearly that the effect of Fg is on the V_{max} of the activation reaction. The effect is similar for both Fg and fragment D and results in an approximate 2.5-fold stimulation of Glu_1-Pg activation in the presence of these effector molecules.

For Lys_{77}-Pg and Val_{442}-Pg activation by SK, the effect of Fg and fragment D is quite different from that of Glu_1-Pg activation. As shown in Figure 3 for Lys_{77}-Pg, the effect of Fg is on the K_m of the reaction. Although not presented herein, the data obtained for the stimulation by Fg and fragment D of Val_{442}-Pg activation is qualitatively the same as for the stimulation by Fg of Lys_{77}-Pg activation. From the k_{cat}/K_m values listed in Table I, a 3–10-fold stimulation of the rate of Lys_{77}-Pg and Val_{442}-Pg activation in the presence of Fg and fragment D occurred, depending upon the exact activation conditions examined. In general, from the K_{Fg} and K_{FD} values, it appeared as though Fg was more effective in this effect on plasminogen activation than was fragment D. From the replot of the data of Figure 3, as the reciprocal initial activation rates vs. the reciprocal of the concentrations at different constant levels of Lys_{77}-Pg (Figure 4), the mechanism whereby Fg (or fragment D) exerts its effect in the activation of Lys_{77}-Pg by SK is revealed and interpreted as a rapid equilibrium ordered bireactant system (Segel, 1975). This is confirmed by the plot shown in Figure 5. A similar situation exists for Val_{442}-Pg activation by SK in the presence of Fg or fragment D. Therefore, the reaction sequence for activation of Lys_{77}-Pg and Val_{442}-Pg to their respective plasmins is proposed to be

$$A + Fg \overset{K_{Fg}}{\rightleftharpoons} Fg\text{-}A + Pg \overset{K_{Pg}}{\rightleftharpoons} Fg\text{-}A\text{-}Pg \xrightarrow{k_{Pg}} Fg\text{-}A + Pm$$

where A represents the activator complex (SK·Lys_{77}-Pg or SK·Val_{442}-Pg) and Pg is either Lys_{77}-Pg or Val_{442}-Pg. Fragment D can be substituted for Fg in this scheme. In this system Fg (or fragment D) is a nonessential activator, but when Fg is coupled to A, the complex binds substrate more effectively than A alone. A similar reaction scheme has been proposed for the effect of fibrin on the activation of Lys_{77}-Pg and Val_{442}-Pg by human tissue plasminogen activators (Hoylaerts et al., 1982).

Whereas the tissue plasminogen activator (TPA) is capable of directly binding to fibrin(ogen), SK does not possess this property. Therefore, in contrast to the situation with TPA, SK must bind to Fg (and fragment D) via its interaction with plasminogen. If this indeed occurs, this binding phenomenon does not involve the lysine binding sites of plasminogen, since the stimulation of the SK-mediated plasminogen activation by Fg is not affected by the presence of ϵ-aminocaproic acid (Smith et al., 1984).

A very important finding in this report is the establishment of an SK·Pg species sensitive to Fg stimulation, which is converted with an apparent first-order rate constant of 0.093 min^{-1} at 4 °C into an SK·Pg species not sensitive to Fg (Figures 6 and 7). Thus the steps in the formation of plasminogen activator in mixtures of SK and Pg must be rewritten to include this species. We propose the scheme:

$$SK + HPg \rightleftharpoons SK\cdot HPg \rightleftharpoons SK\cdot HPg^* \rightarrow SK\cdot HPg' \rightarrow SK\cdot HPm$$

where SK·HPg is a stoichiometric and inactive complex of the two proteins, SK·HPg* is the equimolar streptokinase–human plasminogen activator complex, which is stimulated by fibrinogen, and SK·HPg′ is the same complex, presumably conformatively rearranged, which is not stimulated by fibrinogen to the same extent as SK·HPg*. This latter complex is then converted into another activator complex, consisting of equimolar levels of streptokinase and human plasmin, SK·HPm [see review by Castellino (1979)].

APPENDIX

Kinetic Treatment of the Data. The coupled activation assays utilized in the course of the studies described under Materials and Methods result in increasing reaction velocities with time, at each concentration of plasminogen (Pg). For the conversion of Pg to plasmin (Pm) in the presence of an activator (A):

$$\frac{d[Pm]}{dE} = \frac{k_{cat}[A][Pg]}{K_m + [Pg]} \quad (1)$$

where k_{cat} and K_m refer to the steady-state catalytic properties of the activator, A. Further, the amidolytic cleavage of the substrate S-2251 (S) by the generated Pm, yielding the chromophoric product *p*-nitroaniline (P), is represented by

$$\frac{d[P]}{dE} = \frac{k_{cat}'[Pm][S]}{K_m' + [S]} \quad (2)$$

where k_{cat}' and K_m' are the kinetic constants for Pm toward S-2251, at 37 °C in 0.05 M Tris·HCl/0–1 M NaCl, pH 7.4. We have determined these values to be 16 s^{-1} and 0.3 mM, respectively. In all of our experiments, we have utilized a concentration of S-2251 of 0.5 mM. Substituting these parameters into eq 2, we obtain

$$d[P] = \frac{(16\ s^{-1})[Pm](5 \times 10^{-4}\ M)}{(5 \times 10^{-4}\ M) + (3 \times 10^{-4}\ M)}\, dt \quad (3)$$

Upon integration of eq 3 from [P] = 0 at $t = 0$ to [P] = P at $t = t$, the following equation results:

$$[P] = 10 \int_0^t [Pm]\, dt \quad (4)$$

Similarly, upon integration of eq 1 from [Pm] = 0 at $t = 0$ to [Pm] = Pm at $t = t$, we obtain

$$[Pm] = \frac{k_{cat}[A][Pg]}{K_m + [Pg]}\, t \quad (5)$$

Substituting eq 5 into eq 4, we obtain the following equation after rearrangement and substitution of V_{max} for $k_{cat}[A]$ and $A_{405}/10^4$ for [P]:

$$A_{405} = \frac{(5 \times 10^4)(V_{max})[Pg]}{K_m + [Pg]}\, t^2 \quad (6)$$

where A_{405} is the absorbency at 405 nM at time t.

Therefore, plots of A_{405} vs. t^2 at constant [E] and varied [Pg] produce a series of straight lines, the slopes (B) of which, from eq 6, are given by

$$B = \frac{(5 \times 10^4)(V_{max})[Pg]}{K_m + [Pg]} \quad (7)$$

From eq 7, it is seen that the initial rate of activation of Pg ($d[Pm]/dt$) is given by $B/(5 \times 10^4)$. Lineweaver–Burk plots of the kinetic data are thus obtained by plotting $(5 \times 10^4)/B$ vs. $1/[Pg]$. Values of B were determined at each [Pg] from the slopes of plots of A_{405} vs. t^2.

Registry No. Plasminogen, 9001-91-6; streptokinase, 9002-01-1.

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Clotting of Fibrinogen. 1. Scanning Calorimetric Study of the Effect of Calcium

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ABSTRACT: The denaturation temperature T_d and the enthalpy of thermal denaturation ΔH_d of the D nodules of fibrinogen increase 12-13 °C and 40%, respectively, when fibrinogen is clotted by thrombin in the presence of 10^{-3} M calcium ion. The rate of change of T_d and ΔH_d is first order in thrombin concentration. In the absence of calcium, little change in T_d is observed, but the increase in ΔH_d still occurs. The shift in T_d as a function of logarithm of calcium concentration is sigmoid, with a half-point at 2.5×10^{-5} M calcium for human and 6.0×10^{-5} M calcium for bovine fibrinogens, suggesting that the shift is due to binding of calcium at the high-affinity binding sites of fibrin. The T_d of the D nodule of native fibrinogen also increases, but not as much, on addition of calcium. This increase in T_d is also sigmoid with log calcium, with a half-point of 1.6×10^{-3} M calcium for human and 3.2×10^{-3} M calcium for bovine fibrinogens, and appears to be due to binding of calcium to the low-affinity binding sites of fibrinogen. At calcium concentrations greater than 10^{-4} M, traces of factor XIII in the bovine fibrinogen preparation become activated and cause cross-linking of the fibrin gel. But the changes in T_d and ΔH_d still occur when factor XIIIa is inactivated by iodoacetamide, and the rate of the changes is not altered by addition of large amounts of factor XIIIa. Thus, the changes in T_d and ΔH_d are not due to cross-linking but result from intermolecular interactions of the D nodules in the fibrin clot, which are strengthened by binding of calcium at the high-affinity binding sites of fibrin.

Fibrinogen is an approximately linear array of three globular regions, or nodules, separated by two triple-stranded, coiled-coil connectors. The larger terminal nodules have been designated D nodules, while the smaller central one is called the E nodule, after the globular fragments obtained by proteolysis. Conversion of fibrinogen into fibrin is a multistep reaction. The first step is the enzymatic cleavage of the fibrinopeptides by thrombin from the N-termini of the Aα and Bβ chains of fibrinogen. This is followed by purely physicochemical steps of polymerization. The gel formed may be cross-linked eventually by another enzymatic step performed by factor XIIIa. The molecular structure of fibrinogen remains essentially intact on conversion to fibrin. For reviews, see Doolittle (1973, 1984) and Hermans & McDonagh (1982).

In our previous study using differential scanning calorimetry (DSC), we showed that the D and E nodules of fibrinogen were heat denatured at different temperatures. During formation of the fibrin gel, after addition of thrombin, the thermal stability of the D nodule increased. The rates of increase of the enthalpy of the transition and the increase in its characteristic temperature appeared to be a function of thrombin concentration. The thermal stability of the E nodule was not affected significantly by clotting (Donovan & Mihalyi, 1974). In a continuation of these studies, we observed that the shift of the denaturation temperature of the D nodule that accompanies clotting is markedly affected by calcium ions (Mihalyi & Donovan, 1978). In the present paper we correlate the changes in the thermal stability of the D nodule with the concentration of free calcium ion and the calcium binding studies of Marguerie et al. (1977) and Nieuwenhuizen et al. (1979, 1981a) and our own. Since our preparations were not free of factor XIII, above a certain free calcium concentration factor XIII became activated and caused cross-linking of the gel. Several different approaches were taken to show that the changes in thermal stability were not caused by the cross-linking. Finally, the order of the reaction with respect to thrombin was established.

The denaturation of the D and E nodules of fibrinogen has been investigated at pH 8.5 and 3.5 by Privalov & Medved' (1982) and by Medved' et al. (1983). Their results, which confirm our previous work, also indicate that substructures, or domains, exist in both the D and E nodules.