Rees, M. W. (1946) *Biochem. J. 40*, 632–635.
Schneppenheim, R., & Theede, H. (1980) *Comp. Biochem. Physiol. B 67B*, 561–568.
Slaughter, D., Fletcher, G. L., Ananthanarayanan, U. S., & Hew, C. L. (1981) *J. Biol. Chem. 256*, 2022–2026.
Summers, M. R., Smythers, G. W., & Onoszlan, S. (1973) *Anal. Biochem. 53*, 624–628.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yeh, Y., & Feeney, R. E. (1978) *Acc. Chem. Res. 11*, 129–35.

Enhancement of the Streptokinase-Catalyzed Activation of Human Plasminogen by Human Fibrinogen and Its Plasminolysis Products†

Dudley K. Strickland, Joseph P. Morris, and Francis J. Castellino*

ABSTRACT: The effects of human fibrinogen, and several plasmin-derived fragments of fibrinogen, on the streptokinase-induced activation of human plasminogen (Pg) have been investigated. Fibrinogen stimulates the rate of activation of human Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg. The cofactor activity of fibrinogen appears to reside mainly in the D-domain region, since purified fragment D is active in this system. Fibrinogen fragment E was not active in this regard. The cofactor activity of fragment D was partially dependent on the presence of Ca^{2+}. This effect of Ca^{2+} was likely due to its stabilizing influence on fragment D, as revealed by studies employing differential scanning calorimetry. Conversion of fragment D_1 to fragments D_{2-5} did not alter the cofactor activity. Steady-state kinetic analysis of the activation of Val_{442}-Pg by the streptokinase–Val_{442}-plasmin complex demonstrated that the K_m decreased approximately 2-fold, in the presence of fragment D_1. Very little change in the steady-state kinetic parameters for Glu_1-Pg and Lys_{77}-Pg, when activated by the streptokinase–Lys_{77}-plasmin complex, was noted in the presence of fragment D_1. It was also found that both fibrinogen and fibrinogen fragment D_1 increased the rate of formation of the active site in the streptokinase–plasminogen complex, of all forms of plasminogen, and that this effect was sufficient to explain the overall stimulation of the activation of plasminogen by fibrinogen and fibrinogen fragment D_1.

Activation of the zymogen plasminogen (Pg)[1] to the active enzyme plasmin occurs in the presence of urokinase (Kjeldgaard & Ploug, 1957) and a variety of tissue activators (Binder et al., 1979; Allison et al., 1980; Bobbitt et al., 1980) as well as the bacterial protein streptokinase (SK) (Milstone, 1941; Taylor & Botts, 1968; McKee et al., 1971; Sodetz et al., 1972). The activation of plasminogen by SK has been the subject of intensive investigation in recent years and has been shown to occur through the formation of an equimolar complex of human plasminogen and SK (McClintock & Bell, 1971; Reddy & Markus, 1972), which generates an active center in the plasminogen portion of this complex (Schick & Castellino, 1974). Intramolecular cleavage of the SK–plasminogen complex generates the SK–plasmin complex (Kosow, 1975; Bajaj & Castellino, 1977). Catalytic levels of either the SK–plasminogen or the SK–plasmin complex are capable of rapid activation of plasminogen to plasmin (Markus & Werkheiser, 1964; Ling et al., 1965; Gonzalez-Gronow et al., 1978).

Recently, it has been noted by several investigators that a plasma protein appears to potentiate SK activity toward plasminogen (Takada et al., 1970, 1972; Chesterman et al., 1977). The SK cofactor activity is associated with the fibrinogen molecule (Violand et al., 1980; Camiolo et al., 1980; Takada et al., 1980), a 330000 molecular weight plasma protein whose structure has been extensively investigated by several laboratories. Studies on the degradation products of fibrinogen generated during proteolysis by plasmin have indicated that the process is systematic and sequential, producing a series of well-defined derivatives (Pizzo et al., 1972; 1973; Ferguson et al., 1975). The D and E fragments represent terminal digestion products that are relatively resistant to further degradation by plasmin. Since detailed studies on the mechanism of these fragments, and the intact molecule, in the activation of plasminogen by SK have not been reported, an investigation was undertaken to examine this potentially significant involvement of fibrinogen and several fibrinogen-derived fragments in the SK-induced activation of plasminogen.

Materials and Methods

Proteins. Human fibrinogen was prepared from fresh plasma according to the methods of Blomback & Blomback (1959) and Longas et al. (1980), as described by Morris et al. (1981). The final product was plasminogen free and >95% clottable. $NaDodSO_4$ gel electrophoresis, under reducing conditions, revealed very little degradation of the fibrinogen α chain.

Human Glu_1-Pg was prepared by affinity chromatography (Deutsch & Mertz, 1970), as modified by Brockway & Castellino (1972). Affinty chromatography variant 2 was used in all studies.

Lys_{77}-Pg was prepared by incubation of Glu_1-Pg with urokinase-free plasmin, followed by removal of plasmin with insolubilized soybean trypsin inhibitor and chromatography of Lys_{77}-Pg on a Sepharose 4B–L-lysine column, essentially as

† From the Department of Chemistry, The University of Notre Dame, Notre Dame, Indiana 46556. *Received March 31, 1981.* This work was supported by Grant HL-13423 from the National Institutes of Health. D.K.S. is a postdoctoral fellow of the National Institutes of Health (HL-05900).

[1] Abbreviations: Pg, plasminogen; Pm, plasmin; Glu_1-Pg, native human plasminogen; Lys_{77}-Pg, human plasminogen representing Lys_{77}–Asn_{791}; Val_{442}-Pg, human plasminogen representing Val_{442}–Asn_{791}; SK, streptokinase; HPLC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The nomenclature of Marder et al. (1969) is used for the degradation products of fibrinogen.

0006-2960/82/0421-0721$01.25/0

described by Violand & Castellino (1976).

Val_{442}-Pg was prepared and purified as previously described (Powell & Castellino, 1980). Streptokinase was purified from Kabikinase (A. B. Kabi) according to procedures previously published (Castellino et al., 1976), while urokinase was a gift from Dr. W. H. Holleman (Abbott Laboratories).

Urokinase-free plasmin was prepared by activation of plasminogen with urokinase, followed by chromatography of plasmin on Sepharose 4B–L-lysine, as in the preparation of plasminogen.

Preparation of Fragments D and E. Fibrinogen fragments D_1 and E were prepared essentially as described by Pizzo et al. (1973). Specifically, fibrinogen was dissolved in 50 mM Tris-HCl/0.1 M NaCl/1 mM $CaCl_2$, pH 7.4, at a concentration of 6 mg/mL. Urokinase-free plasmin was added (1:100 mol/mol), and the digestion was carried out at room temperature. The progress of the digestion was monitored by HPLC using a Beckman Spherogel TSK 3000 SW gel filtration column (7.5 mm × 600 mm), equilibrated with 50 mM Hepes/200 mM Na_2SO_4, pH 7.4 (Morris et al., 1981). The reaction was terminated by the addition of diisopropyl fluorophosphate (DFP) to a final concentration of 20 mM. The digest was dialyzed overnight against 0.05 M Tris-HCl/1 mM $CaCl_2$, pH 8.9, and placed on a DEAE-Sephadex column (2.5 × 20 cm), equilibrated with 0.05 M Tris-HCl/1 mM $CaCl_2$, pH 8.9. Fragment D_1 was eluted with a linear gradient containing 400 mL each of 50 mM Tris-HCl/1 mM $CaCl_2$, pH 8.9 (starting buffer), and 50 mM Tris-HCl/1 mM $CaCl_2$/200 mM NaCl, pH 8.9 (limit buffer), while fragment E was eluted with a buffer of 50 mM Tris-HCl/1 mM $CaCl_2$/500 mM NaCl, pH 8.9. Fragment D_1 was further purified by gel filtration on a Sephacryl S-200 column (2.5 × 100 cm), equilibrated with 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$, pH 7.4. The isolated fragments were examined by $NaDodSO_4$ gel electrophoresis, in the presence and absence of β-mercaptoethanol (Weber & Osborn, 1969), in order to establish their purity.

Assay for SK Cofactor Activity. The activation of plasminogen by catalytic levels of SK was carried out in 50 mM Tris-HCl/100 mM NaCl, pH 7.4, at 30 °C in a final volume of 400 μL. Streptokinase (0.004 μM) was incubated with fibrinogen, fragment D_1, or fragment E for 1 min at 30 °C. Plasminogen was then added to a final concentration of 1 μM. The initial rate of plasminogen conversion to plasmin was measured by removing 40-μL aliquots at various time intervals and quantitating the plasmin activity with the peptide substrate D-Val-Leu-Lys-*p*-nitroanilide (S-2251, Kabi). The assay was carried out at 30 °C in a buffer consisting of 50 mM Tris-HCl/100 mM NaCl, pH 7.4, with a substrate concentration of 0.5 mM. Hydrolysis of the substrate was monitored at 405 nm, utilizing an ϵ^{1M}_{405nm} value of 9620 for *p*-nitroanilide (Pfleiderer, 1970). The rate enhancement of plasminogen activation in the presence of fibrinogen or fragment D is defined as the rate of plasminogen activation in the presence of these proteins divided by the rate in the presence of SK alone. Using these activation conditions (1 μM plasminogen and 0.004 μM SK), we determined the rate enhancement to be linearly dependent upon the concentration of fibrinogen or fragment D up to concentrations of 0.5 μM.

Steady-State Kinetics. Steady-state kinetic analysis of the activation of various plasminogens by the SK*–plasmin (SK* represents SK modified by incorporation in the equimolar complex with Pg or Pm) complex was carried out at 30 °C in a buffer consisting of 50 mM Tris-HCl/100 mM NaCl, pH 7.4. Preformed SK*–plasmin complexes were prepared by incubating SK and either Glu_1-Pg, Lys_{77}-Pg, or Val_{442}-Pg in a 1:1 (mol/mol) ratio for 15 min at 30 °C. Catalytic levels of the desired complex (4.0 nM) were then added to the plasminogen solution of interest, present at concentrations ranging from 0.1 to 2.0 μM, in a final volume of 0.4 mL. After 30 s, an aliquot of this solution (0.2 mL) was removed, and the amount of plasmin generated was determined by rate analysis of the hydrolysis of the chromogenic peptide substrate, D-Val-Leu-Lys-*p*-nitroanilide (S-2251). The substrate was present at a concentration of 0.5 mM, and a Cary 219 recording spectrophotometer, temperature controlled at 30 °C, was employed for measurements. The rate of hydrolysis of this substrate was converted into plasmin concentration by use of a standard curve generated with a standard plasmin solution. Each data point represents the average of triplicate analysis.

Pseudo-First-Order Kinetic Studies on the Rate of Active-Site Formation in the Plasminogen Activator Complex. This was accomplished by examining rates of active-site formation, under various conditions, by using the fluorescent active-site titrant 3′,6′-bis[(4-guanidinobenzoyl)oxy]-5-[*N*′-(4-carboxyphenyl)thioureido]spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (FDE). FDE was synthesized according to the procedure described by Mangel et al. (1980). The kinetics of hydrolysis of FDE by plasmin have been determined (Mangel et al., 1980), and the parameters obtained suggest that FDE is a very effective active-site titrant for plasmin. The kinetics of active-site formation were measured in a buffer consisting of 50 mM Hepes/100 mM NaCl/1 mM $CaCl_2$, pH 7.2, and were carried out at 30 °C. Plasminogen (0.075 μM) was incubated in the presence or absence of various concentrations of fibrinogen or fragment D for 1 min. FDE was then added to a final concentration of 10 μM. The reaction was initiated by the addition of streptokinase (2.0 μM) and the fluorescence monitored with a Perkin-Elmer MP44A fluorescence spectrophotometer equipped with a thermostated cell holder. The final volume of the reaction mixture was 2.0 mL. The excitation and emission wavelengths were 491 and 514 nm, respectively, both with a bandwidth of 10 nM. The parameter ΔF_0 represents the maximum fluorescence change and was determined by titrating 0.075 μM plasmin (prepared as a 1:1 plasmin–streptokinase complex) with FDE. The data are plotted as log $(\Delta F_0 - \Delta F_{(t)})$ vs. time, and the pseudo-first-order rate constants were determined from the slopes of the lines.

For all results reported here, sodium dodecyl sulfate gel electrophoretic analysis clearly demonstrated that the active site in the streptokinase–plasminogen complex was indeed being monitored, and no conversion to streptokinase–plasmin was observed.

Plasmin Digestion of Fragment D_1. Fragment D_1 (1 mg/mL) was dialyzed against 50 mM Tris/100 mM NaCl, pH 7.4, containing either 1 mM $CaCl_2$ or 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA). The digestion with plasmin (1:40 mol/mol) was carried out at 37 °C, and the progress of digestion was monitored by $NaDodSO_4$ gel electrophoresis, in the presence and absence of β-mercaptoethanol. Cofactor activity measurements were performed at various time intervals, employing the assay described above.

Protein Concentrations. These were determined spectrophotometrically, using the following $\epsilon^{1\%,1cm}_{280nm}$ and molecular weight values, repsectively: Glu_1-Pg, 17.0 and 92000 (Violand & Castellino, 1976); Lys_{77}-Pg, 17.0 and 84000 (Barlow et al., 1969); Val_{442}-Pg, 16.0 and 38000 (Sottrup-Jensen et al., 1977); SK, 9.5 and 45000 (Brockway & Castellino, 1974); fibrinogen, 16.2 and 330000 (Blomback & Blomback, 1959; Scheraga

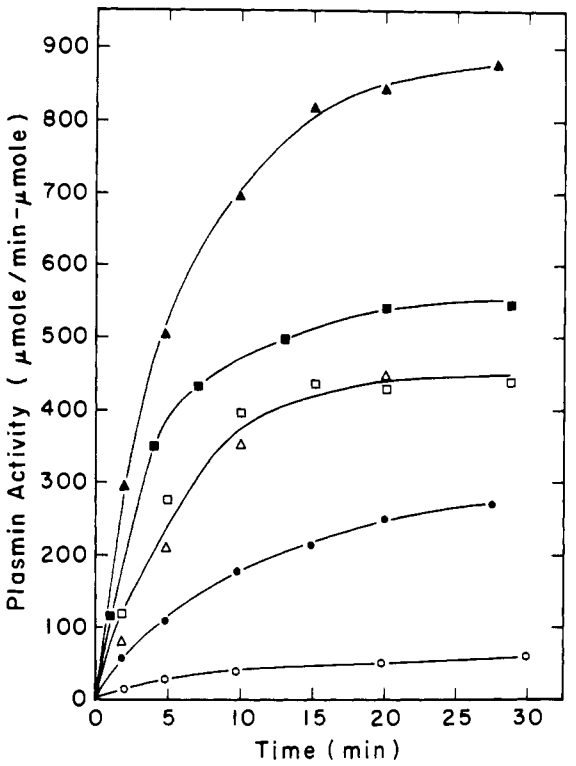

FIGURE 1: Effect of fibrinogen on the rate of plasminogen activation in the presence of catalytic amounts of streptokinase. Streptokinase (0.004 μM) was incubated in the presence and absence of fibrinogen (4 μM), and the activation was initiated by the addition of plasminogen (1 μM). (○) Glu_1-Pg; (●) Glu_1-Pg + fibrinogen; (□) Lys_{77}-Pg; (■) Lys_{77}-Pg + fibrinogen; (△) Val_{442}-Pg; (▲) Val_{442}-Pg + fibrinogen. Aliquots (40 μL) were removed at various time intervals and assayed for plasmin activity by using the peptide substrate D-Val-Leu-Lys-*p*-nitroanilide (S-2251, Kabi).

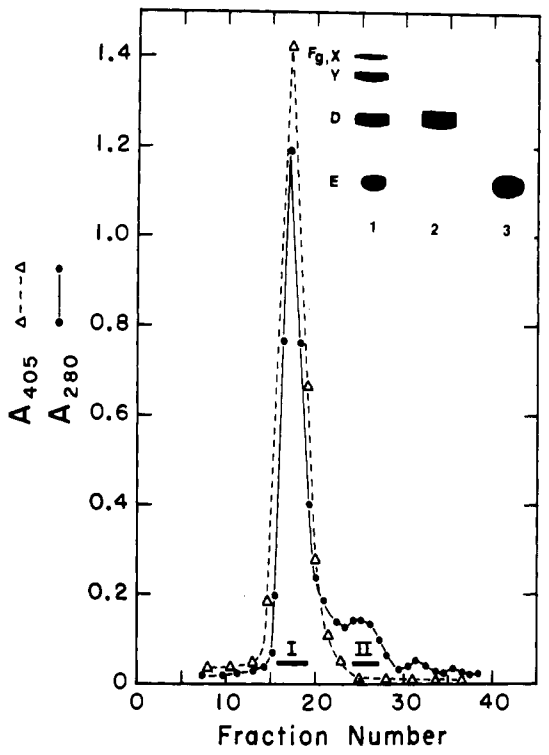

FIGURE 2: Separation of fragments D and E by gel-permeation chromatography using HPLC. An aliquot (1 mL) of a plasmin digest of fibrinogen was injected on a Spherogel TSK 3000 SW column (7 mm × 600 mm) equilibrated with 50 mM Hepes/200 mM Na_2SO_4/1 mM $CaCl_2$, pH 7.4. Fractions were assayed for cofactor activity by using Val_{442}-Pg as described under Materials and Methods. Also shown is $NaDodSO_4$ gel electrophoretic analysis of (1) fibrinogen digest, (2) fragment D_1 (pool I), and (3) fragment E (pool II).

& Laskowski, 1957); fragment D, 19.9 and 94 000 (Ferguson et al., 1975); fragment E, 10.2 and 50 000 (Marder et al., 1969).

Differential Scanning Calorimetry. The MC-1 scanning calorimeter (Microcal, Amherst, MA) was used to obtain the thermograms. Scan rates of 60 °C/h were used, and the enthalpy values were determined as previously described (Ploplis et al., 1981). The samples were prepared by extensive dialysis against 50 mM Hepes/100 mM NaCl/1 mM $CaCl_2$, pH 7.4, for the data obtained in the presence of calcium and against 50 mM Hepes/100 mM NaCl/1 mM EGTA, pH 7.4, for the data obtained in the absence of calcium.

Atomic Absorption. Atomic absorption was performed on a Varian Model AA 775 with a CRA 90 attachment. A standard $CaCl_2$ solution (0.1 M) was purchased from Radiometer and was used to generate standard curves.

Results

Figure 1 shows the activation rates of Gly_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg with catalytic amounts of streptokinase in the presence and absence of saturating amounts of fibrinogen. The data demonstrate that fibrinogen, in all cases, increases the rate of plasminogen activation. The same rate increase could be observed when plasmin was assayed for active-site concentration by *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride. Determination of the initial rate revealed that fibrinogen induces a 4-fold increase in the activation rate of Glu_1-Pg, a 2-fold increase in the activation rate of Lys_{77}-Pg, and a 4-fold increase in the activation rate of Val_{442}-Pg, a plasminogen derivative greatly reduced in lysine-binding capacity. At various time intervals during the activation, aliquots were removed and examined by $NaDodSO_4$ gel electrophoresis to ensure that normal activation of plasminogen had occurred. The rate enhancement was shown to be SK dependent, and preincubation of fibrinogen with plasminogen for up to 24 h did not lead to activation of plasminogen. This rules out the possibility of a plasminogen activator contamination in the fibrinogen.

In order to determine which domain of the fibrinogen molecule was responsible for the cofactor activity, fibrinogen was subjected to plasmin digestion. When the fibrinogen digest was fractionated by gel-permeation chromatography, it was observed that the cofactor activity was located in the fractions containing fragment D (Figure 2). No activity was found in the pool containing fibrinogen fragment E, or the other lower molecular weight peptides resulting from plasmin cleavage. In repeated experiments, it was discovered that the activity recovered in fragment D varied greatly. Subsequent studies revealed that Ca^{2+} was essential in order to retain full activity. Thus, Ca^{2+} was included in all buffers during fragment D isolation.

Figure 3 shows the effect of fragment D_1 on the activation of Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg by SK. Saturating levels of fragment D_1 result in a 2-fold, 3-fold, and 5-fold increase in the activation rate of each plasminogen, respectively, under the conditions employed. These results differ from the results obtained when using fibrinogen. In the case of Glu_1-Pg activation, fragment D is not as effective as fibrinogen, while in the activation of both Lys_{77}-Pg and Val_{442}-Pg, fragment D is slightly more effective than fibrinogen.

The effect of various concentrations of purified fragment D_1 on the overall rate of formation of Val_{442}-Pm from Val_{442}-Pg, in the presence of catalytic levels of SK, is shown in Figure 4. Fragment D_1 increases the initial rate of formation of Val_{442}-Pm, dependent upon its concentration, to a

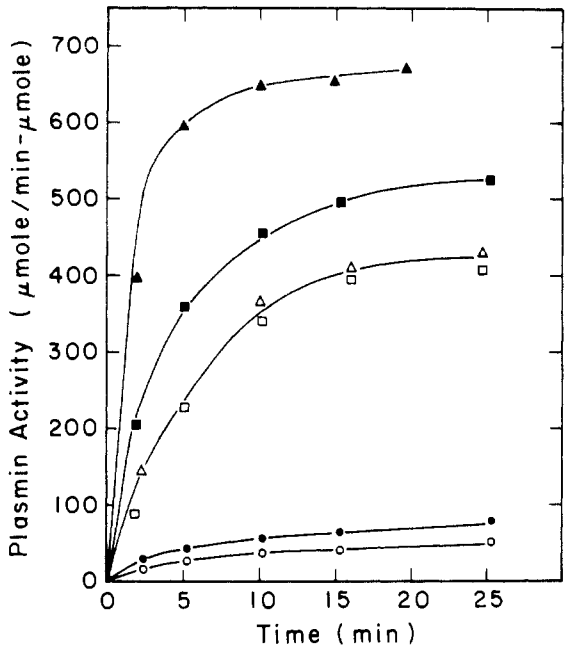

FIGURE 3: Effect of fragment D_1 on the rate of activation of plasminogen. Streptokinase (0.004 μM) was incubated with fragment D_1 (3 μM) and the activation initiated by the addition of plasminogen (1 μM). (○) Glu_1-Pg; (●) Glu_1-Pg + fragment D_1; (□) Lys_{77}-Pg; (■) Lys_{77}-Pg + fragment D_1; (△) Val_{442}-Pg; (▲) Val_{442}-Pg + fragment D_1. Aliquots (40 μL) were removed at various time intervals and assayed for plasmin activity as described under Materials and Methods.

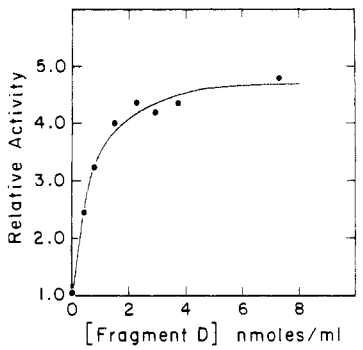

FIGURE 4: Effect of fragment D_1 concentration (0.2–7.5 μM) on the rate of streptokinase-induced activation of Val_{442}-Pg. Streptokinase (0.004 μM) was incubated with fragment D_1 at 30 °C, and the activation was initiated by the addition of Val_{442}-Pg (1 μM). After 2 min, the amount of plasmin generated was assayed as described under Materials and Methods. The data are expressed as the rate of plasminogen activation relative to that of the sample containing no fragment D_1, and a relative activity of 1.0 represents the activation rate of plasminogen in the absence of fragment D_1.

maximum of 4–5-fold. The total concentration of fragment D_1 required for half-maximal stimulation, as obtained from this figure, is approximately 0.5 μM.

Figure 5 shows the effect of Ca^{2+} on the cofactor activity of fragment D_1. Removal of Ca^{2+} from fragment D_1 was accomplished by extensive dialysis vs. EGTA. Analysis of the calcium-free protein by atomic absorption indicated that the Ca^{2+} content was less than 0.2 mol of Ca^{2+}/mol of protein. The Ca^{2+} concentration available for binding to fragment D was regulated by using EGTA–Ca^{2+} buffers (Portzell et al., 1964), and the results show that in the absence of Ca^{2+} the cofactor activity of fragment D_1 is markedly reduced. Full cofactor activity can be restored by the addition of low levels of Ca^{2+} (<100 μM).

The effect of calcium on the structure of fragment D_1 was examined by the use of differential heat capacity scanning

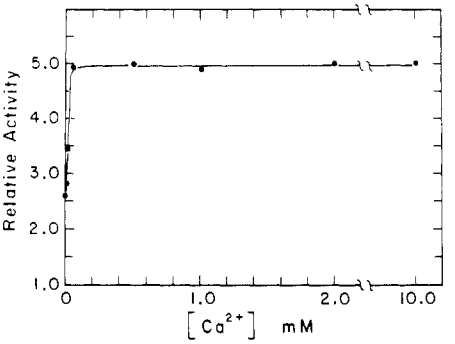

FIGURE 5: Effect of calcium concentration on the activity of fragment D_1. Calcium-free fragment D_1 was prepared by extensive dialysis against 1 mM EGTA. Aliquots were then removed, and calcium was added back to fragment D_1. The assay for cofactor activity was carried out under the following conditions: SK, 0.004 μM; Val_{442}-Pg, 1 μM; fragment D_1, 0.5 μM. Data are expressed as the rate of plasminogen activation relative to the rate obtained in the absence of fragment D_1, and a relative activity of 1.0 is defined as the activation rate of plasminogen in the absence of fragment D_1.

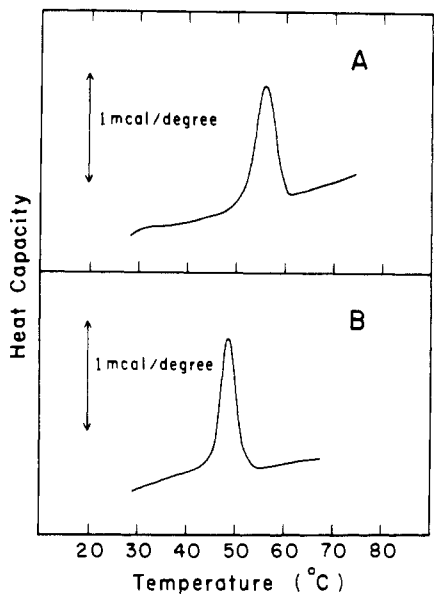

FIGURE 6: Thermal denaturation of fragment D_1. (A) Fragment D_1 (1.42 mg/mL) in the presence of 1 mM $CaCl_2$. (B) Fragment D_1 (1.78 mg/mL) in the presence of 1 mM EGTA. In both cases, the scan rate was 60 °C/h.

calorimetry, and the results are shown in Figure 6. In the presence of 1 mM Ca^{2+} (Figure 6A), single thermal transition is observed, with a T_m of 56 ± 0.5 °C and a ΔH value of 373 kcal/mol. This ΔH value is in agreement with that obtained by Donovan & Mihalyi (1974). Removal of Ca^{2+} from fragment D_1 caused a 7 °C downward shift in the T_m of the transition, from 56 ± 0.5 °C to 49 ± 0.5 °C, and a significant reduction in the ΔH value to 267 kcal/mol. This suggests that binding of Ca^{2+} by fragment D_1 results in a marked stabilization of the protein structure toward thermal denaturation and implies that Ca^{2+} induces a structural alteration in fragment D_1.

In order to examine the effect of removal of the carboxy-terminal region of the γ' chain of fragment D_1 on the cofactor activity, fragment D_1 was digested with plasmin in the presence of 1 mM EGTA. The presence of calcium prevents conversion of D_1 to D_{2-5} (Haverkate & Timan, 1977; Purves et al., 1978), but in the absence of Ca^{2+}, conversion of D_1 to D_{2-5} does occur. As a result of this digestion, complete conversion of D_1 to D_{2-5} occurred (data not shown), and the cofactor activity was similar to fragment D_1, both in the presence and absence of

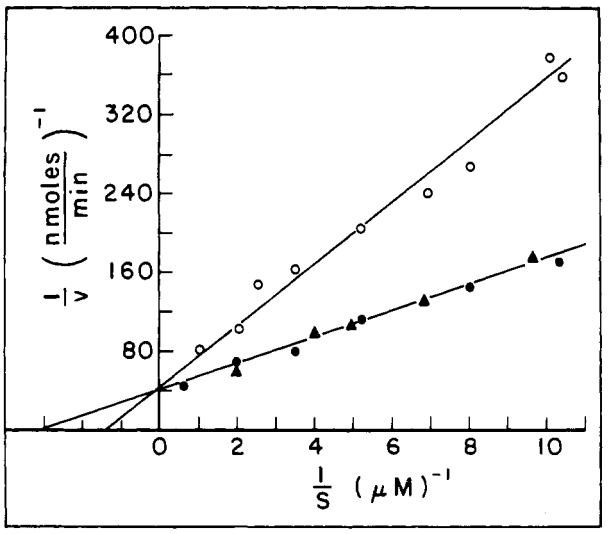

FIGURE 7: Lineweaver–Burk plots of the activation Val_{442}-Pg by SK*–Val_{442}-plasmin in the absence and presence of fragment D_1. In a total volume of 0.4 mL, the substrate concentration was varied between 0.1 and 2 μM. The lines drawn through the data points represent the best fit determined by least-squares linear regression analyses of the data. (○) Val_{442}-Pg; (●) Val_{442}-Pg + 2 μM fragment D_1; (▲) Val_{442}-Pg + 4 μM fragment D_1.

Ca^{2+}, demonstrating that removal of the carboxy-terminal region of the γ' chain does not alter its stimulatory activity.

In order to investigate the mechanism of stimulatory action of fibrinogen or fragment D on the overall activation of plasminogen, we first examined the effect of fragment D_1 on the steady-state kinetic parameters of Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg toward their respective SK–plasmin complexes. Here, the SK*–Lys_{77}-plasmin and SK*–Val_{442}-plasmin complexes were first formed, the former by incubating either Glu_1-Pg or Lys_{77}-Pg with stoichiometric levels of SK and the latter by incubating Val_{442}-Pg with a stoichiometric level of SK. Reduced $NaDodSO_4$ electrophoretic gels of the SK*–Lys_{77}-plasmin and SK*–Val_{442}-plasmin equimolar complexes clearly showed the presence of the component peptide chains of Lys_{77}-plasmin or Val_{442}-plasmin, as well as the slightly degraded SK (SK*) component (Brockway & Castellino, 1974). Catalytic levels of the SK*–Lys_{77}-plasmin equimolar complex were then incubated with various levels of either Glu_1-Pg or Lys_{77}-Pg, and fragment D_1, if desired, and the initial rates of generation of Lys_{77}-plasmin were determined, as described under Materials and Methods. A similar experiment was performed with the SK*–Val_{442}-plasmin equimolar complex, as an activator for Val_{442}-Pg, again in the presence and absence of fragment D_1. Typical data, presented in the form of a Lineweaver–Burk plot, are shown in Figure 7. In all assays of this type, a time course of activation at each plasminogen levels was obtained, assuring that all rate data were initial rates of activation. The kinetic constants, in the presence and absence of saturating levels of fragment D_1, for the activation of each human plasminogen derivative are listed in Table I. As can be seen, in the case of Glu_1-Pg and Lys_{77}-Pg, there is very little effect of fragment D_1 on the steady-state kinetic parameters for activation. On the other hand, a 2-fold decrease in the K_m for activation of Val_{442}-Pg by SK*–Val_{442}-Pg, is observed, in the presence of fragment D_1. However, this effect cannot account for the entire stimulation of Val_{442}-Pg activation, by SK, in the presence of fragment D_1.

Our K_m values obtained for activation of Glu_1-Pg and Lys_{77}-Pg by SK*–Lys_{77}-Pm in the absence of fragment D_1 are in reasonable agreement with the values reported by Wohl et al. (1980), in which a different assay was employed. However, our value of k_{cat} for Glu_1-Pg is considerably higher than the value reported by these investigators. No reason for this discrepancy can be forwarded.

Table I: Steady-State Kinetic Parameters for Activation of Human Plasminogen by the Streptokinase–Plasmin Activator Complex in the Absence and Presence of Fibrinogen Fragment D_1

substrate[a]	$K_{m,app}$ (μM)	k_{cat} (min^{-1})	$k_{cat}/K_{m,app}$ (μM^{-1} min^{-1})
Glu_1-Pg	0.61 ± 0.15	27.8 ± 1.5	45.7
Glu_1-Pg + fragment D_1	0.90 ± 0.2	36.8 ± 1.5	40.8
Lys_{77}-Pg	0.16 ± 0.05	68.7 ± 2.5	429
Lys_{77}-Pg + fragment D_1	0.17 ± 0.05	71.2 ± 2.5	414
Val_{442}-Pg	0.71 ± 0.20	14.2 ± 1.0	20
Val_{442}-Pg + fragment D_1	0.33 ± 0.10	15.6 ± 1.0	47.3

[a] For Glu_1-Pg and Lys_{77}-Pg, the activator was SK*–Lys_{77}-Pm, whereas for Val_{442}-Pg, the activator was SK*–Val_{442}-Pm. The concentration of fragment D_1 was 3.0 μM, when present.

Table II: Effect of Fibrinogen and Fibrinogen Fragment D_1 on the Rate of Formation of the Active Site in the Streptokinase–Plasminogen Equimolar Complex

plasminogen employed	k_{obsd} (ms^{-1})	$[C]_{0.5}$ (μM)[a]
Glu_1-Pg	9.0 ± 1.5	
Glu_1-Pg + fragment D_1	44.1 ± 3.0	0.67 ± 0.1
Glu_1-Pg + fibrinogen	67.2 ± 4.0	0.25 ± 0.05
Lys_{77}-Pg	23.5 ±2.0	
Lys_{77}-Pg + fragment D_1	73.9 ± 4.5	0.50 ± 0.1
Lys_{77}-Pg + fibrinogen	59.0 ± 3.0	0.07 ± 0.01
Val_{442}-Pg	15.3 ± 2.0	
Val_{442}-Pg + fragment D_1	102.2 ± 7.0	0.27 ± 0.1
Val_{442}-Pg + fibrinogen	134.3 ± 7.5	0.30 ± 0.15

[a] Represents the concentration of fibrinogen or fragment D_1 required for half-maximal stimulation of active-site formation.

Since the stimulation of the SK-induced activation of plasminogen by fragment D_1 (as well as fibrinogen) could not be accounted for by the action of the preformed activator complex on plasminogen, we have studied the effect of fibrinogen and fragment D_1 on the rate of formation of the activator complex. In order to accomplish this, SK and the appropriate plasminogen, in the presence or absence of fibrinogen or fragment D, were incubated in the presence of the active-site titrant, FDE. In all experiments reported herein, this procedure allowed analysis of the rate of formation of the active site in the SK–plasminogen complex. The rate of reaction of FDE with this initial active site is so rapid as to preclude any further reactions within the SK–plasminogen complexes. The choice of FDE over *p*-nitrophenyl *p*-guanidinobenzoate for these experiments was governed by the fluorescence properties of the released product, *p*-[(*p*-guanidinobenzoyl)fluorescyl-6-thioureido]benzoic acid, thereby allowing very low concentrations of active sites to be monitored. For all experiments, reduced $NaDodSO_4$ electrophoretic analysis of the complexes formed showed only SK and the initial plasminogen present at all time points, attesting to the fact that the rate of active-site formation in the SK–plasminogen complex was indeed being monitored. The data obtained were plotted in the form of a first-order plot, and the pseudo-first-order rate constant were determined from the slope of each line and replotted against the concentration of fibrinogen or fragment D_1. These results are shown in Figure 8. The data derived from these plots are listed in Table II. Here, k_{obsd} was determined by extrapolation of the rate constants in Figure 8 to infinite levels of fibrinogen or fragment

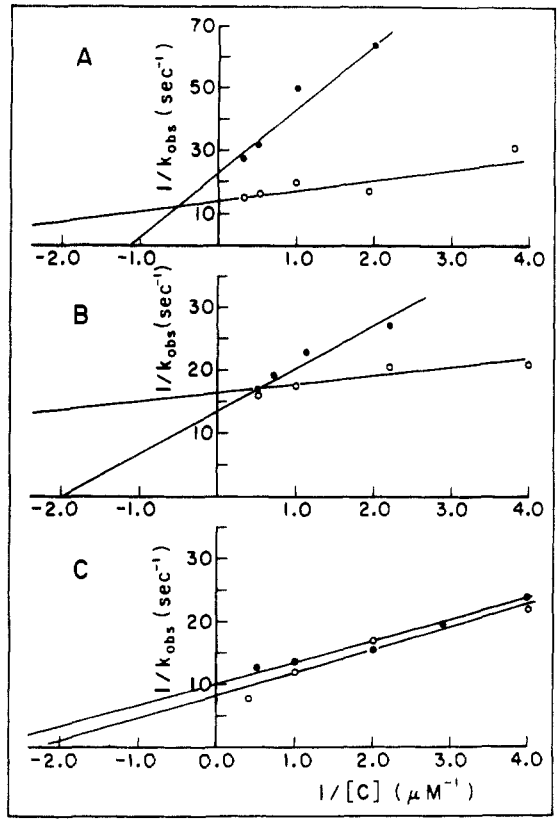

FIGURE 8: Double-reciprocal plots of the effect of varying fibrinogen (○) or fragment D (●) concentration on the observed pseudo-first-order rate constant of formation of the active site in the SK–plasminogen complex. The pseudo-first-order rate constant was determined as described under Materials and Methods. The conditions used to monitor the fluorescence were as follows: excitation, 491 nm; emission, 514 nm; slits, 10 nm; temperature, 30 °C. The lines drawn were generated by least-squares linear regression analysis of the data. (A) Glu_1-Pg; (B) Lys_{77}-Pg; (C) Val_{442}-Pg.

D_1, and $[C]_{0.5}$, the concentration of fibrinogen or fragment D_1 required to produce half-maximal stimulation, was obtained from the respective slopes of the lines in Figure 8 (slope = $[C]_{0.5}/k_{obsd}$).

From the data presented in Table II, it is clear tht fibrinogen or fragment D_1 increases the rate of active-site generation in Glu_1-Pg, in the presence of SK, by 5–7-fold. From the values of $[C]_{0.5}$ for each, it appears as though fibrinogen exhibits its effect at approximately 3-fold lower concentrations than fragment D_1. For generation of the active site in the equimolar SK–Lys_{77}-Pg complex, fibrinogen or fragment D_1 increases the k_{obsd} approximately 2–3-fold, with fibrinogen possessing a 7-fold lower $[C]_{0.5}$ then fragment D_1. The k_{obsd} for formation of the active site in the equimolar SK–Val_{442}-Pg complex is greatly stimulated by both fibrinogen and fragment D_1, resulting in a 9-fold and 7-fold increase in this parameter, respectively. Here, however, the $[C]_{0.5}$ is approximately the same for both fibrinogen and fragment D_1.

Discussion

The present study was aimed at an investigation of the effect of human fibrinogen and some important fibrinogen fragments on the SK-induced activation of human plasminogen. In previous studies, a substance in plasma has been shown to be a potentiator of the activation of plasminogen by SK (Takada et al., 1970, 1972). This activity has also been shown to be present in fibrinogen (Chesterman et al., 1977; Camiolo et al., 1980), fibrin (Camiolo et al., 1980), and in fibrinogen degradation products (Violand et al., 1980). However, the region of the fibrinogen molecule responsible for this effect has not been forwarded, and the mechanism of action of fibrinogen and fibrinogen degradation products in potentiating SK activity in plasminogen activation has not been investigated in detail. In the present study, we have localized the region of fibrinogen that is mainly responsible for its SK-potentiator activity, and we have examined the mechanism of this effect.

The presence of fibrinogen increases the overall rate of streptokinase-induced activation of Glu_1-Pg, Lys_{77}-Pg, and Val_{442}-Pg, with a more effective rate increase observed in the activation of Glu_1-Pg and Val_{442}-Pg. Proteolytic cleavage of fibrinogen by plasmin yielded a product that apparently remained an effective cofactor in the SK-induced activation of plasminogen. In the present study, cofactor activity was localized in the D-domain region of fibrinogen. Differences in the degree of enhancement by fibrinogen and fragment D do exist, indicating that fibrinogen has slightly different activating properties than fragment D.

The presence of Ca^{2+} is required for maximal expression of cofactor activity by fragment D, and removal of Ca^{2+} by exhaustive dialysis against EGTA reduced the activity by 50%. Full activity could be restored by adding calcium to the calcium-free protein at calcium concentrations sufficient to saturate the calcium binding site of fragment D_1 (Lindsey et al., 1978). The structure of fragment D_1 is markedly affected by calcium, as revealed by its stabilization toward thermal denaturation. When fragment D_1 was subjected to thermal denaturation by using differental scanning calormetry, the presence of Ca^{2+} caused at 7 °C shift in the T_m and a 40% increase in the ΔH value. Additionally, the presence of Ca^{2+} prevents degradation of fragment D_1 to fragments D_2–D_5 by plasmin. These data suggest that the conformation of fragment D is altered in the presence of calcium, producing a more effective activator. It should be noted, however, that Ca^{2+} interactions with other proteins, such as SK or Pg, or the complexes generated during the activation of Pg, may be important in this phenomenon. Conversion of fibrinogen fragment D_1 to D_2–D_5 did not affect the cofactor activity. These results suggest that the carboxyl-terminal portion of the γ' chain of fragment D_1, the region involved in the cross-linked site (Ferguson et al., 1975; Pizzo et al., 1973) and the region cleaved by plasmin in the conversion of D_1 to D_5 (Ferguson et al., 1975; Haverkate & Tinan, 1977), is probably not involved in the expression of SK-potentiator activity of fragment D.

The mechanism of activation of human plasminogen (HPg) by SK has been widely studied, and general agreement exists on the steps involved. Initially, SK and plasminogen interact in a 1:1 stoichiometry, producing the following series of reactions [examples of important references are Hummel et al. (1966), Ling et al. (1967), McClintock & Bell (1971), Reddy & Markus (1972), Kosow (1975), Gonzalez-Gronow et al. (1977), and Bajaj & Castellino (1977)]:

$$SK + HPg \rightleftharpoons SK\text{–}HPg \rightarrow SK\text{–}HPg' \rightarrow SK^*\text{–}HPM \quad (1)$$

$$HPg + SK^*\text{–}HPm(SK\text{–}HPg') \rightarrow HPm \quad (2)$$

Here, the steps in reaction 1 are concerned with formation of the actual activator complex. SK and HPg interact in a 1:1 stoichiometry, yielding SK–HPg. Within the complex, a conformational reorganization occurs, leading to formation of SK–HPg′, possessing an active site in the plasminogen moiety. This latter complex is rapidly altered to a complex of SK and human plasmin (SK*–HPm), in which SK is also modified by proteolysis (SK*). In step 2, either SK*–HPm or SK–HPg′ can act as enzymes in the formation of excess HPg to human plasmin (HPm).

Since the experiments in Figures 1, 3, 4, and 5 were designed to measure the overall rate of activation of plasminogen to plasmin, by SK, the stimulatory effect of fibrinogen or fragment D on the process could be due to effects on reactions 1 or 2 above. Thus, further experiments were necessary to distinguish between their various possible sites of action. In order to examine the effect of fibrinogen and fragment D on step 2 of the above sequence of reactions, we added preformed SK*–Lys_{77}-Pm to Glu_1-Pg and Lys_{77}-Pg and SK*–Val_{442}-Pm to Val_{442}-Pg and determined the resulting steady-state values of $k_{cat}/K_{m,app}$ for each indicated plasminogen, in the presence and absence of fragment D_1. The results obtained, illustrated in Figure 7 and listed in Table I, show that fragment D_1 had no effect on activation of Glu_1-Pg or Lys_{77}-Pg by preformed SK*–Lys_{77}-Pm, as evidenced by the fact that the $k_{cat}/K_{m,app}$ for each plasminogen did not appreciably change in the presence of fragment D_1. The results also indicate that Lys_{77}-Pg is activated much more effectively by SK*–Lys_{77}-Pm than is Glu_1-Pg. The data in Table I also demonstrate that fragment D_1 increased the $k_{cat}/K_{m,app}$ value for activation of Val_{442}-Pg by preformed SK*–Val_{442}-Pg by 2-fold. However, this enhancement was not sufficient to account for the 4–5-fold enhancement of the overall activation rate of Val_{442}-Pg by fragment D_1 (Figure 4).

It is experimentally very difficult to discriminate between the various components of reaction 1 above. However, when SK and plasminogen are incubated in the presence of an effective and rapid active-site titrant, such as FDE, the reaction should not proceed beyond the SK–HPg′ complex, as is the case for experiments of this type performed with other active-site titrants (Reddy & Markus, 1972; Schick & Castellino, 1974). This was confirmed in the present study by reduced $NaDodSO_4$ gel electrophoresis, which showed that only SK and the initial plasminogens were present under all conditions of Figure 8, despite the formation of approximately 1 mol of active site per mol of SK–HPg′ complex, at completion of the reactions. In this study, we determined the rate of formation of the SK–HPg′ complex, in the presence and absence of fibrinogen and fragment D_1, by addition of SK to the desired plasminogen, in the presence of FDE. The reaction was rendered pseudo first order by addition of a large excess of SK to plasminogen. In general terms, the formation of SK–HPg′ (ΔSK–HPg′) with time (t) can be described by

$$\Delta\text{SK–HPg}' = A_1e^{-\tau_1 t} + A_2e^{-\tau_2 t}$$

where A_1 and A_2 are constants, determined by the initial conditions, and τ_1 and τ_2 are a combination of rate constants for all steps leading to the formation of SK–HPg′. This equation predicts that the appearance of SK–HPg′ should be a biphasic process, with τ_1 and τ_2 characterizing each phase. The experimental data obtained fit a first-order rate expression, with no lag phase observed in all cases, suggesting that one of the steps is considerably faster than the other. Variation of the SK concentration (in all cases >10-fold molar excess over plasminogen concentration) had essentially no effect on the rate constant,[2] suggesting that the rate-limiting step in the sequence is SK–HPg → SK–HPg′.

The data of Table II show that both fibrinogen and fibrinogen fragment D_1 had a profound effect on k_{obsd} for generation of the active site in the SK–HPg′ complex. The greatest effect was noted for formation of SK-Val_{442}-Pg′, in which k_{obsd} increased approximately 10-fold when either fibrinogen or fragment D_1 was present. For formation of SK–Glu_1-Pg′, increases in k_{obsd} of 5–7-fold were obtained with fibrinogen and fragment D_1, and for formation of SK–Lys_{77}-Pg′, increases of 2–3-fold in this parameter were obtained. In all cases, the stimulation of formation of SK–Pg′ complexes is more than sufficient to account for the stimulation of the overall SK-induced activation of plasminogen by fibrinogen and fragment D_1. Since autolysis of plasmin is occurring in studies of the effect of fibrinogen and fragment D_1 on the overall activation rate, which may lead to underestimates of the enhancement factors, and since the rate of reaction of SK–HPg′ → SK*–HPm cannot be experimentally measured, it is not surprising that the extent of stimulation of the overall activation rate by fibrinogen and fragment D_1 is not in full agreement with the extent of stimulation of the rate of active-site formation in SK–HPg′. However, the agreement is sufficient to strongly implicate stimulatory effects of fibrinogen and fragment D_1 on ΔSK–HPg′ as the chief contributor to stimulation of the overall activation of plasminogen by streptokinase. It should be emphasized, however, that these same kinetic results would be obtained were fibrinogen or fragment D_1 to increase the rate of SK–HPg formation from SK and HPg, decrease the rate of dissociation of SK–HPg into SK and HPg, or enhance the rate of formation of SK–HPg′ from SK–HPg.

It should also be noted from Table II that $[C]_{0.5}$ is significantly lower for fibrinogen than for fragment D_1, suggesting that fibrinogen is effective at lower levels in enhancement of ΔSK–HPg′ for both SK–Glu_1-Pg and SK–Lys_{77}-Pg. No difference in $[C]_{0.5}$ for fibrinogen and fragment D_1 is observed in the case of Val_{442}-Pg.

Finally, it is believed that lysine binding sites (LBS) on plasminogen are important for interactions between plasmin(ogen) and fibrin(ogen) (Thorsen, 1975; Rakoczi et al., 1978). Since Val_{442}-Pg is also believed to possess greatly reduced lysine binding properties (Wiman et al., 1978), but yet its SK-induced activation rate is still greatly stimulated by fibrinogen, either (1) the LBS are not important to this particular effect of fibrinogen, an observation also forwarded by Camiolo et al. (1980), (2) the LBS are generated in the SK–Val_{442}-HPg′ complex, or (3) some LBS are indeed present in the Val_{442}-Pg, which is consistent with an observation by Castellino et al. (1981).

Acknowledgments

The assistance of Dr. Godfrey Amphlett in obtaining atomic absorption analysis is greatly appreciated.

References

Allison, D. M., Dirksen, J. W., Schmelfer, R. F., Makoid, M. C., & Murray, W. J. (1980) *Thromb. Res. 19*, 525–534.

Bajaj, S. P., & Castellino, F. J. (1977) *J. Biol. Chem. 252*, 492–498.

Barlow, G. H., Summaria, L., & Robbins, K. C. (1969) *J. Biol. Chem. 244*, 1138–1141.

Binder, B. R., Spragg, J., & Austen, K. F. (1979) *J. Biol. Chem. 254*, 1998–2003.

Blomback, B., & Blomback, M. (1959) *Ark. Kemi 10*, 415–443.

Bobbit, J. L., Calvin, S. A., Hutchins, J. F., Arnettt, G. C., & Hull, R. N. (1980) *Thromb. Res. 18*, 315–332.

Brockway, W. J., & Castellino, F. J. (1972) *Arch. Biochem. Biophys. 151*, 194–199.

Brockway, W. J., & Castellino, F. J. (1974) *Biochemistry 13*, 2063–2070.

Camiolo, S. M., Markus, G., Evers, J. L., & Hobika, G. H. (1980) *Thromb. Res. 17*, 697–706.

Castellino, F. J., Sodetz, J. M., Brockway, W. J., & Siefring, G. E. (1976) *Methods Enzymol. 45*, 224–257.

[2] D. K. Strickland, unpublished experiments.

Castellino, F. J., Ploplis, V. A., Powell, J. R., & Strickland, D. K. (1981) *J. Biol. Chem. 256*, 4778–4782.
Chesterman, C. N., Cederholm-Williams, S. A., Allington, M. J., & Sharp, A. A. (1977) *Thromb. Res. 10*, 421–426.
Deutsch, D., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 195.
Donovan, J. W., & Mihalyi, E. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4125–4138.
Ferguson, E. W., Fretto, L. J., & McKee, P. A. (1975) *J. Biol. Chem. 250*, 7210–7218.
Gonzalez-Gronow, M., Violand, B. N., & Castellino, F. J. (1977) *J. Biol. Chem. 252*, 2175–2177.
Gonzalez-Gronow, M., Siefring, G. E., & Castellino, F. J. (1978) *J. Biol. Chem. 253*, 1090–1094.
Haverkate, F., & Timan, G. (1977) *Thromb. Res. 10*, 803–812.
Hummel, B. C. W., Buck, F. F., & DeRenzo, E. C. (1966) *J. Biol. Chem. 241*, 3474–3479.
Kjeldgaard, N. O., & Ploug, J. (1957) *Biochim. Biophys. Acta 24*, 283–289.
Kosow, D. P. (1975) *Biochemistry 14*, 4459–4465.
Lindsey, G. G., Brown, G., & Purves, L. R. (1978) *Thromb. Res. 13*, 345–350.
Ling, C., Summaria, L., & Robbins, K. C. (1965) *J. Biol. Chem. 240*, 4213–4218.
Ling, C., Summaria, L., & Robins, K. C. (1967) *J. Biol. Chem. 242*, 1419–1425.
Longas, M. O., Newman, J., & Johnson, A. J. (1980) *Int. J. Biochem. 11*, 559–564.
Mangel, W. F., Livingston, D. C., Brockelhurst, J. R., Liu, H. Y., Peltz, G. A., Cannon, J. F., Leytus, S. P., Wehryly, J. A., Salter, B. L., & Mosher, J. L. (1980) *Cold Spring Harbor Symp. Quant. Biol. 44*, 669–680.
Marder, V. J., Schulman, N. R., & Carroll, W. R. (1969) *J. Biol. Chem. 244*, 2111–2119.
Markus, G., & Werkheiser, W. C. (1964) *J. Biol. Chem. 239*, 2637–2643.
McClintock, D. K., & Bell, P. H. (1971) *Biochem. Biophys. Res. Commun. 43*, 694–702.
McKee, P. A., Lemmon, W. B., & Hampton, J. W. (1971) *Thromb. Diath. Haemorrh. 26*, 512–533.
Milstone, H. (1941) *J. Immunol. 42*, 109–116.
Morris, J. P., Blatt, S., Powell, J. R., Strickland, D. K., & Castellino, F. J. (1981) *Biochemistry 20*, 4811–4816.
Pfleiderer, G. (1970) *Methods Enzymol. 19*, 514–521.
Pizzo, S. V., Schwartz, M. L., Hill, R. L., & McKee, P. (1972) *J. Biol. Chem. 247*, 636–645.
Pizzo, S. V., Taylor, L. M., Schwartz, M. L., Hill, R. L., & McKee, P. A. (1973) *J. Biol. Chem. 248*, 4584–4590.
Ploplis, V. A., Strickland, D. K., & Castellino, F. J. (1981) *Biochemistry 20*, 15–21.
Portzell, H., Caldwell, P. C., & Ruegg, J. C. (1964) *Biochim. Biophys. Acta 79*, 581–591.
Powell, J. R., & Castellino, F. J. (1980) *J. Biol. Chem. 255*, 5329–5335.
Purves, L. R., Lindsey, G. G., Brown, G., & Franks, J. (1978) *Thromb. Res. 12*, 473–484.
Rakoczi, I., Winman, B., & Collen, D. (1978) *Biochim. Biophys. Acta 540*, 295–300.
Reddy, K. N. N., & Markus, G. (1972) *J. Biol. Chem. 247*, 1683–1691.
Scheraga, H. A., & Laskowski, M., Jr. (1957) *Adv. Protein Chem. 12*, 1–131.
Schick, L. A., & Castellino, F. J. (1974) *Biochem. Biophys. Res. Commun. 57*, 47–54.
Sodetz, J. M., Brockway, W. J., & Castellino, F. J. (1972) *Biochemistry 11*, 4451–4457.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Peterson, T. E., & Magnusson, S. (1977) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191–209.
Takada, A., Takada, Y., & Ambrus, J. L. (1970) *J. Biol. Chem. 245*, 6389–6396.
Takada, A., Takada, Y., & Ambrus, J. L. (1972) *Biochim. Biophys. Acta 263*, 610–618.
Takada, A., Mocizuki, K., & Takada, Y. (1980) *Thromb. Res. 19*, 767–773.
Taylor, F. B., & Botts, J. (1968) *Biochemistry 7*, 232–242.
Thorsen, S. (1975) *Biochim. Biophys. Acta 393*, 55–65.
Violand, B. N., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 3906–3912.
Violand, B. N., Castellino, A., & Castellino, F. J. (1980) *Thromb. Res. 19*, 705–710.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wiman, B., Boman, L., & Collen, D. (1978) *Eur. J. Biochem. 87*, 143–146.
Wohl, R. C., Summaria, L., & Robbins, K. C. (1980) *J. Biol. Chem. 255*, 2005–2013.