Krstenansky, J. L., Owen, T. J., Yates, M. T., & Mao, S. J. T. (1987) *J. Med. Chem. 30*, 1688-1691.
Laidler, K. J. (1987) *Chemical Kinetics*, pp 191-202, Harper and Row, New York.
Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593-626.
Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., & Wieczorek, M. (1987) *Biochemistry 26*, 202-221.
Mann, K. G., & Lundblad, R. L. (1982) in *Haemostasis and Thrombosis* (Colman, R. W., Hirsh, J., Maeder, V. J., & Salzman, E. W., Eds.) pp 112-126, J. B. Lippincott, Philadelphia, PA.
Markwardt, F. (1970) *Methods Enzymol. 19*, 924-932.
Owen, M. C., Brennan, S. O., Lewis, J. H., & Carrell, R. W. (1983) *N. Engl. J. Med. 309*, 694-698.
Read, R. J., Fujinaga, M., Sielecki, A. R., & James, M. N. G. (1983) *Biochemistry 22*, 4420-4433.
Rink, H., Liersch, M., Sieber, P., & Meyer, F. (1984) *Nucleic Acids Res. 12*, 6369-6387.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157-162.
Southan, C., Lane, D. A., Bode, W., & Henschen, A. (1985) *Eur. J. Biochem. 147*, 593-600.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622-4628.
Stone, S. R., Braun, P. J., & Hofsteenge, J. (1987) *Biochemistry 26*, 4617-4624.
Sukumaran, D. K., Clore, G. M., Preuss, A., Zarbock, J., & Gronenborn, A. M. (1987) *Biochemistry 26*, 333-338.
Takahara, M., Hibler, D. W., Barr, P. J., Gerlt, J. A., & Inouye, M. (1985) *J. Biol. Chem. 260*, 2670-2674.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655-709.
Walker, B., Wikstrom, P., & Shaw, E. (1985) *Biochem. J. 230*, 645-650.
Walsmann, P., & Markwardt, F. (1981) *Pharmazie 36*, 633-660.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468-500.

Effectors of the Activation of Human [Glu^1]plasminogen by Human Tissue Plasminogen Activator†

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Received December 28, 1987; Revised Manuscript Received April 28, 1988

ABSTRACT: The activation of human [Glu^1]plasminogen ([Glu^1]Pg) by human recombinant (rec) two-chain tissue plasminogen activator (t-PA) is inhibited by Cl^-, at physiological concentrations, and stimulated by ϵ-aminocaproic acid (EACA), as well as fibrin(ogen). Chloride functions as a result of its binding to [Glu^1]Pg, with a K_i of approximately 9.0 mM, thereby rendering [Glu^1]Pg a less effective substrate for two-chain rec-t-PA. EACA stimulates the activation in Cl^--containing solutions, with a K_a of approximately 4.0 mM, primarily by reversal of the Cl^--inhibitory effect. Fibrinogen appears to exert its stimulatory properties mainly through effects on the enzyme, two-chain rec-t-PA, with a K_a of approximately 3.7 μM in activation systems containing physiological levels of Cl^-. Analysis of the results of this paper reveals that normal plasma components, Cl^- and fibrinogen, exert major regulatory roles on the ability of [Glu^1]Pg to be activated by two-chain rec-t-PA, in in vitro systems. The presence of Cl^- inhibits the stimulation of [Glu^1]Pg activation that would normally occur in the presence of fibrinogen, a result of possible importance to the observation that some degree of systemic fibrinogenolysis accompanies therapeutic use of tissue plasminogen activator.

Human plasminogen ([Glu^1]Pg), the precursor of the fibrinogenolytic and fibrinolytic enzyme plasmin, is a single-chain plasma glycoprotein containing 791 amino acids in known sequence (Wiman, 1973, 1977; Sottrup-Jensen et al., 1977; Malinowski et al., 1984; Forsgren et al., 1987). Activation of [Glu^1]Pg is concomitant with cleavage of the Arg^{561}–Val^{562} peptide bond in the zymogen and is catalyzed by a variety of proteins, such as urokinase (u-PA), streptokinase (SK), and tissue plasminogen activator (t-PA) [for a review, see Castellino (1981)].

As synthesized, t-PA is a single-chain protein containing 527 amino acid residues (Pennica et al., 1983). This protein is also found in a two-chain form, as a result of cleavage of the Arg^{275}–Ile^{276} peptide bond by plasmin (Pennica et al., 1983) and other such enzymes (Ichinose et al., 1984). t-PA is a serine protease, containing the catalytic triad at sequence positions His^{322} (Pennica et al., 1983), Asp^{371} (Pennica et al., 1983), and Ser^{478} (Pennica et al., 1983; Pohl et al, 1984). The single-chain form of t-PA is less active than the two-chain form toward small substrates (Wallen et al., 1982), but apparently, both forms of this enzyme possess similar plasminogen activator activities (Rijken et al., 1982). Whereas t-PA is a poor activator of plasminogen in the absence of certain effector molecules, its activity is greatly stimulated in the presence of

† These studies were supported by Grant HL-13423 from the National Institutes of Health.
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0006-2960/88/0427-6522$01.50/0

fibrin (Hoylaerts et al., 1982) and cyanogen bromide fragments of fibrinogen (CNBr-Fg) (Nieuwenhuizen et al., 1983). The different functions of t-PA have been assigned to domain regions present within its molecular structure, with fibrin binding properties believed to exist within its "finger" domain (Banyai et al., 1983; Mattler et al., 1985; Zonneveld et al., 1986), composing residues 1–43 (Banyai et al., 1983), and its "kringle" 2 region (Zonneveld et al., 1986), consisting of residues 180–261 (Pennica et al., 1983). The protease portion of the molecule is contained within residues 276–527 (Pennica et al., 1983). Other domains with hereto uncertain functions are the growth factor region, consisting of residues 44–91 (Banyai et al., 1983), and the "kringle" 1 portion, containing residues 92–173 (Pennica et al., 1983).

We have shown previously that the activation of [Glu^1]Pg by SK (Chibber et al., 1985, 1986), u-PA (Urano et al., 1987a,b), and t-PA (Castellino et al., 1988) is inhibited by physiological levels of Cl^-. We believe it important to establish the relationship between inhibition by this anion and stimulation by positive effectors, in order that the physiological relevance of normal plasma components toward activation of [Glu^1]Pg by t-PA can be more fully understood. This information may be of value in consideration of the efficacy of t-PA as a clinical agent for clot dissolution.

Materials and Methods

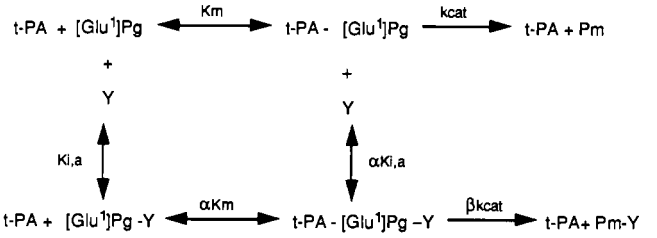
Proteins. Affinity chromatography forms 1 and 2 of [Glu^1]Pg were purified by our previously described modification (Brockway et al., 1972) of the Deutsch and Mertz (1970) affinity chromatography technique. The lyophilized protein was dissolved in a buffer consisting of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH, pH 7.4, and exhaustively dialyzed against that same buffer. For determination of the concentrations of [Glu^1]Pg, an ϵ (1%, 1 cm, 280 nm) of 17.0 and a molecular mass of 94 kDa per mole were employed (Violand & Castellino, 1976). Affinity form 1 was employed for the investigations described herein.

Recombinant (rec) single-chain t-PA was a gift of Genentech, Inc., South San Francisco, CA. The protein obtained was approximately 70–80% in the one-chain form. The material was reconstituted in 50 mL of water and converted to the two-chain form with Sepharose–plasmin, at a 10:1 (mol/mol) ratio of single-chain rec-t-PA to plasmin. Pilot gel studies, on $NaDodSO_4$/PAGE under reducing conditions, were employed to evaluate the most suitable reaction conditions, which were approximately 1 h at room temperature. The concentration of active two-chain rec-t-PA in the stock solution was determined by spectrofluorometric titration with 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) at 4 °C, as we have described (Urano et al., 1988).

A genetic variant of t-PA ($Cys_{264} \rightarrow$ Gly) was provided by the Monsanto Co. This protein was converted to the two-chain form and titrated, as above. The strategy behind the use of this mutant is that the two chains are no longer covalently coupled due to replacement of the Cys residue from the heavy chain that is involved in the covalent binding between the component t-PA polypeptide chains.

Human fibrinogen was a product of Kabivitrum, Stockholm, Sweden. The material was reconstituted with water according to the directions of the manufacturer and percolated through the column of Sepharose–lysine to remove plasminogen and plasmin. A stock solution was prepared by dialysis against a buffer containing 10 mM Hepes–NaOH, pH 7.4. Analysis of the material by $NaDodSO_4$/PAGE gels, under reducing conditions, revealed an intact α-chain, as well as the expected

Scheme I



β- and γ-chains. The resulting fibrinogen was 94% clottable.

CNBr-Fg was prepared by digestion of fibrinogen (10 mg/mL) with CNBr (100-fold molar excess over the methionine content of fibrinogen) in a solution of 70% formic acid. The reaction was allowed to occur for 17 h at 25 °C, under a N_2 atmosphere, after which the solution was dialyzed against water.

In order to prepare soluble fibrin, fibrinogen, at a concentration of 5.5 mg/mL, was first dialyzed against a buffer consisting of 10 mM Hepes–NaOH, pH 7.4. After this, a total of 2.5 international units of ancrod (International Reference preparation 74/581) was added, and the solution was incubated at 37 °C for 2 h. The clot was removed, washed extensively with 10 mM Hepes–NaOH, pH 7.4, and solubilized by addition of a measured volume of 5.7 M urea. This was employed as the stock solution.

[Glu^1]Pg Activation Assays. Continuous coupled assays for evaluating the activation rate of [Glu^1]Pg under differing conditions of Cl^- and protein promoter concentrations were based upon the temporal development of amidolytic activity of the plasmin that is generated as a result of the activation (Chibber et al., 1986). The assays were performed at 37 °C in a buffer containing 10 mM Hepes–NaOH, pH 7.4, plus other desired factors. The components present were [Glu^1]Pg (0.5 μM) and the substrate D-Val-Leu-Lys-*p*-nitroanilide (S-2551), at a concentration of 0.5 mM, in a final volume of 0.8 mL. The activation reaction was accelerated by addition of a known level of two-chain rec-t-PA. The effects of CNBr-Fg and fibrin on these same activation rates were conducted at a single concentration (100 mM) of NaOAc or NaCl.

For titration of the effects of Cl^- and OAc^- on this activation reaction, these same assays were performed with levels of these anions ranging from 0 to 150 mM. The stimulatory effects of EACA and fibrinogen were evaluated by addition of the desired component to the cuvette prior to initiation of the assay with two-chain rec-t-PA. In these latter cases, the total concentration of Cl^- + OAc^- was maintained at 150 mM.

Absorbancies resulting from appearance of *p*-nitroanilide were monitored with time (t) at 405 nm on a Cary 219 recording spectrophotometer and were converted to the concentration of substrate hydrolyzed by employing an ϵ (1 M, 1 cm, 405 nm) of 10 000. Initial velocities were calculated from absorbance (A) versus t^2 plots, as previously described (Chibber et al., 1986).

Analysis of Activation Rate Data with Anions and EACA. The rate data of [Glu^1]Pg activation by rec-t-PA, in the absence and presence of EACA and various anions, were analyzed by standard Lineweaver–Burk plots. The mechanistic treatment, shown in Scheme I, was utilized to derive kinetic equations and was based upon the known ability of the effectors, i.e., EACA and anions, to bind to and cause observable conformational alterations in [Glu^1]Pg (Chibber et al., 1985, 1986; Urano et al., 1987a). In Scheme I, Y is the effector, i.e., anion and/or EACA. The positive effector, EACA, possess a dissociation constant K_a to [Glu^1]Pg and αK_a to the

Table I: Effect of Different Salts on the Initial Activation Rate of Human [Glu¹]plasminogen by Recombinant Two-Chain Tissue Plasminogen Activator[a]

salt	activation rate[b] −Fg	activation rate[b] +Fg[c]
NaI	2.9 ± 0.2	23 ± 2
LiCl	4.3 ± 0.3	48 ± 5
KCl	3.8 ± 0.2	39 ± 4
NaCl	3.8 ± 0.2	40 ± 4
$NaHCO_3$	7.5 ± 0.3	151 ± 10
NaF	14.0 ± 0.4	177 ± 11
KF	13.2 ± 0.4	233 ± 13
LiOAc	13.7 ± 0.4	220 ± 11
NaOAc	14.4 ± 0.4	181 ± 10

[a] The assay components were as follows: [Glu¹]Pg, 0.5 μM; two-chain rec-t-PA, 4.0 nM; S-2251, 0.5 mM; Hepes–NaOH, 10 mM; desired salt present at 100 mM. The pH was 7.4. [b] The units are picomoles of plasmin formed per second. [c] The concentration of fibrinogen (Fg) was 2 μM.

rec-t-PA–[Glu¹]Pg complex. Similarly, the negative effector, Cl^-, possesses a dissociation constant K_i to [Glu¹]Pg and αK_i to the rec-t-PA–[Glu¹]Pg complex. In Scheme I, K_m and k_{cat} have their usual meanings. Pm represents human plasmin.

In order to obtain the kinetic constants summarized in Scheme I, we employed the iterative procedure described in detail in a previous publication (Urano et al., 1987b).

Results

We have shown previously that an anion inhibitory effect exists in the activation of human [Glu¹]Pg by SK and u-PA (Chibber et al., 1985, 1986; Urano et al., 1987a,b) and that the order of effectiveness of anions approximates their placement in the Hofmeister ion series. The data of Table I establish that a similar effect exists for the initial activation rate of [Glu¹]Pg by two-chain rec-t-PA. Alteration of the cation, with Cl^-, F^-, or OAc^- as the anion, does not significantly affect the initial activation rate of [Glu¹]Pg by this enzyme. However, the nature of the anion does influence this same rate. An approximate 4-fold rate increase is found through the series $I^- > Cl^- > HCO_3^- > F^- > OAc^-$. The presence of fibrinogen serves to stimulate these rates but does not lead to an alteration of the order of anion effectiveness in this regard.

The effect of various promoters on the long-term activation of human [Glu¹]Pg by two-chain rec-t-PA, in the presence of 10 mM Hepes–NaOH/100 mM NaCl, pH 7.4, is shown in Figure 1. For the property of interest, these agents are ordered as fibrin > CNBr-Fg > fibrinogen. While this same activation of [Glu¹]Pg by two-chain rec-t-PA with all promoters is much more rapid when 100 mM NaOAc is substituted for NaCl (data not shown), their order of effectiveness remains essentially the same. The plot in Figure 1 is shown for appreciation of the overall phenomonological effect only and cannot readily be employed for a kinetic analysis of [Glu¹]Pg activation by two-chain rec-t-PA, since during the long-term activation, Na $DodSO_4$/PAGE experiments clearly demonstrate the occurrence of the conversion of [Glu¹]Pg to [Lys⁷⁸]Pg, the latter protein possessing different kinetic properties with this activator than the former. Further, hydrolytic action by the generated plasmin on the promoters might also influence their stimulatory properties. The importance of such events are made obvious by the data of Figure 2, wherein the hydrolysis of S-2251 resulting from activation of [Glu¹]Pg by two-chain rec-t-PA, in the presence of fibrin and Cl^-, is plotted against time squared. Here, a biphasic curve is seen. When this same experiment is performed with [Glu¹]Pg in OAc^-, or with [Lys⁷⁸]Pg in either Cl^- or OAc^-, a linear plot is obtained. These results are clearly consistent with the slower reaction

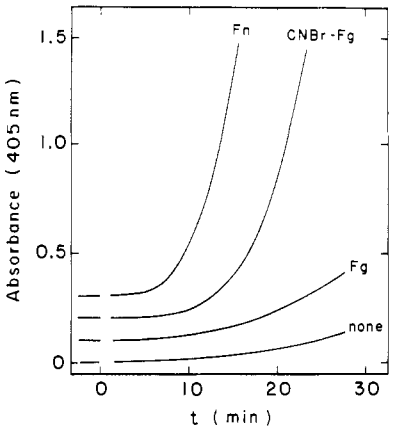


FIGURE 1: Effect of various promoters on the activation of human [Glu¹]Pg by two-chain rec-t-PA. The activation mixture consisted of 0.5 μM [Glu¹]Pg, the desired promoter, referred to 50 μg/mL fibrinogen, and the chromogenic substrate S2251 present at 0.5 mM. The buffer was 10 mM Hepes–NaOH/100 mM NaCl, pH 7.4, at 37 °C. The reactions were accelerated with two-chain rec-t-PA at 0.4 nM. The promoters are human fibrinogen (Fg), CNBr fragments of fibrinogen (CNBr-Fg), and soluble fibrin (Fn).

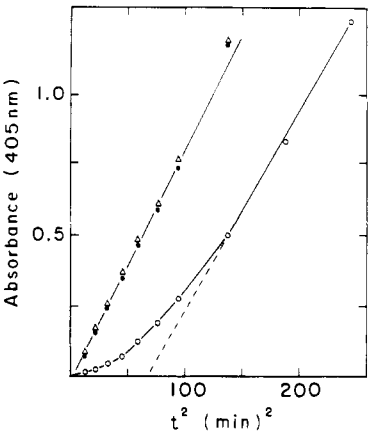


FIGURE 2: Plots of activation rate against t^2. (○) Activation of [Glu¹]Pg by two-chain rec-t-PA in the presence of Fn, in NaCl. The conditions are as in Figure 1. (●) Activation of [Glu¹]Pg by two-chain rec-t-PA in the presence of Fn, in NaOAc. The conditions are as in Figure 2. (△) Activation of [Lys⁷⁸]Pg by two-chain rec-t-PA in the presence of Fn, in NaOAc. The conditions are as in (○) or (●).

phase in [Glu¹]Pg/Cl^- being due to activation of [Glu¹]Pg by two-chain rec-t-PA and the more rapid phase being due to the faster activation of [Glu¹]Pg subsequent to its enzymatic conversion to [Lys⁷⁸]Pg, or subsequent to [Glu¹]Pg adapting the conformation of [Lys⁷⁸]Pg on the fibrin surface. In addition, exposure of new and more effective sites on the promoter molecule, as a result of proteolytic action of the generated plasmin, might also explain the biphasic nature of the reaction. The linear plot noted in Figure 2, with fibrin as the promoter for the two-chain rec-t-PA-catalyzed activation of [Lys⁷⁸]Pg, is also seen under these same conditions with fibrinogen as the positive effector (Figure 3). In the case of stimulation by CNBr-Fg, a trend toward curvature is noted at high concentrations (Figure 3), suggesting the existence of a more complex stimulatory mechanism for this agent.

We have shown previously (Urano et al., 1987b) that Cl^- places [Glu¹]Pg in an unfavorable conformation for activation by u-PA and that this conformational alteration does not occur with [Glu¹]PA in OAc^- or with [Lys⁷⁸]Pg in Cl^- or OAc^-, the latter three of which are in similar conformationally favorable activation states. Thus, in order for us to evaluate the kinetics of activation of [Glu¹]Pg by two-chain rec-t-PA and the kinetic properties of the promoter, we chose to employ initial rate

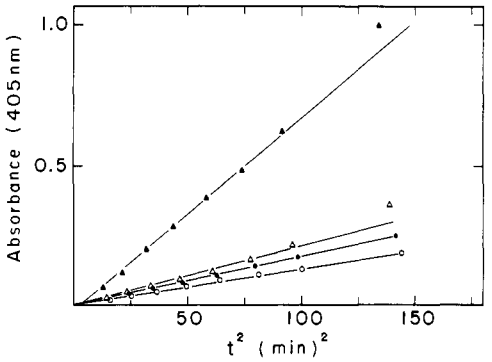


FIGURE 3: The activation promoters are as follows: (○) none; (●) fibrinogen; (△) CNBr-Fg; (▲) Fn. The experimental conditions were 0.5 μM [Lys[78]]Pg, the desired promotor, referred to 50 μg/mL fibrinogen, and the chromogenic substrate S2251 present at 0.5 mM. The buffer was 10 mM Hepes–NaOH/100 mM NaCl, pH 7.4, at 37 °C. The reaction were initiated with two-chain rec-t-PA at 0.4 nM.

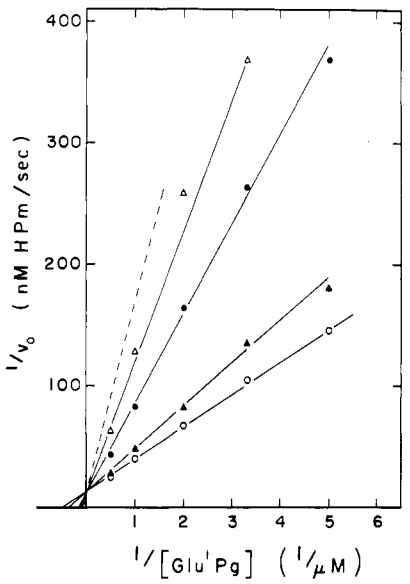


FIGURE 4: Lineweaver–Burk analysis of the effect of Cl^- on the initial rate of activation (v_0) of [Glu[1]]Pg by two-chain rec-t-PA at 37 °C. The concentration of [Glu[1]]Pg was varied between 0.2 and 2.0 μM, and the Cl^- concentrations were (○) none and (▲) 5, (●) 35, and (△) 100 mM. The calculated rate of activation of [Glu[1]]Pg at saturating Cl^- is also shown (--). The lines drawn through the data points were calculated from the iterative procedure described under Materials and Methods. In the experiments, the ionic strength was maintained constant at 150 mM by substitution of an equivalent amount of NaOAc with the desired concentration of NaCl. Activations were initiated by addition of two-chain rec-t-PA (4.4 nM final concentration). The buffer was 10 mM Hepes–NaOH, pH 7.4.

kinetics at very short reaction times (<4 min) and with soluble well-defined promoters. These latter results were compared to those found with the small molecule positive effector EACA. Under these conditions, in all cases to be described below, $NaDodSO_4$/PAGE demonstrated that at the reaction times employed both [Glu[1]]Pg and the effector molecules retained their respective structural integrities and that, consequently, we were indeed analyzing the reaction of choice, i.e., the activation of native [Glu[1]]Pg by two-chain rec-t-PA, with the promoter that was initially presented to the activation system.

Figure 4 illustrates a Lineweaver–Burk plot of the effect of various concentrations of NaCl on the initial rates of activation of [Glu[1]]Pg by two-chain rec-t-PA. Such plots were not linear at [Glu[1]]Pg concentrations greater than approximately 2 μM (data not shown), indicating that the inhibition by NaCl is overcome at high concentrations of [Glu[1]]Pg and

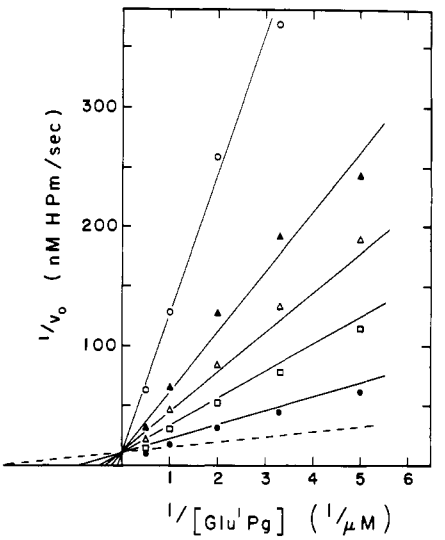


FIGURE 5: Lineweaver–Burk analysis of the effect of EACA on the initial rate of activation (v_0) of [Glu[1]]Pg by two-chain rec-t-PA at 37 °C in a Cl^--containing buffer. The concentration of [Glu[1]]Pg was varied between 0.2 and 2.0 μM, and the EACA concentrations were (○) none and (▲) 0.2, (△) 0.4, (□) 0.7, and (●) 2.0 mM. The calculated rate of activation of [Glu[1]]Pg at saturating EACA is also shown (--). The lines drawn through the data points were determined by the iterative procedure described under Materials and Methods. Activations were accelerated by addition of two-chain rec-t-PA (4.4 nM final concentration). The buffer was 10 mM Hepes–NaOH/100 mM NaCl/50 mM NaOAc, pH 7.4.

suggesting that a second reaction occurs at a higher K_m value. However, at zymogen concentrations below 2 μM the iterative plots fit the experimental points very well with the kinetic constants listed. Thus, our conclusions are relevant to the activation reaction that occurs at the lower K_m.

In all experiments, typified by the data of Figure 4, the ionic strength was maintained constant at 150 mM, by substitution of the desired level of Cl^- for a corresponding amount of OAc^-; this latter anion at this concentration exhibits a much smaller inhibition of the activation of [Glu[1]]Pg, as compared to Cl^-. Over the range of [Cl^-] that we have examined, i.e., 0–100 mM, a progressive inhibition occurs, primarily due to an effect on the K_m of the reaction.

The influence of EACA on the initial activation rate of [Glu[1]]Pg with two-chain rec-t-PA, in 10 mM Hepes–NaOH/100 mM NaCl, pH 7.4, is illustrated in Figure 5. A progressive increase in this reaction occurs as the concentration of EACA is altered, from 0 to 2 mM. Once again, the influence of EACA is exerted on the K_m of the activation. EACA did not stimulate the activation of [Glu[1]]Pg when NaOAc was substituted for NaCl under the above conditions.

The lines drawn through the experimental points of Figures 4 and 5 were calculated by the iterative procedure described under Materials and Methods. In all cases, the lines show an excellent fit with the data. The constants provided in Figure 6 were calculated with use of the kinetic scheme described under Materials and Methods.

We have also examined the effect of fibrinogen (Fg) on the initial activation rate of [Glu[1]]Pg by two-chain rec-t-PA in 10 mM Hepes–NaOH/100 mM NaCl, pH 7.4. Lineweaver–Burk plots of the data obtained are shown in Figure 7. Over the range of [fibrinogen] employed, from 0 to 1.7 μM, a progressive increase in the reaction rate was obtained, due primarily to a decrease in the apparent K's of the reaction. A double-reciprocal plot of the change in slopes of the lines in Figure 7 against the concentration of fibrinogen yielded a linear plot. The plots are consistent with a mechanism of

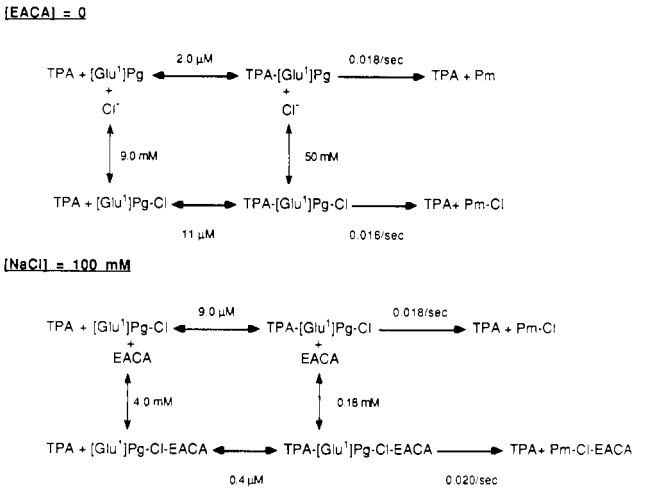
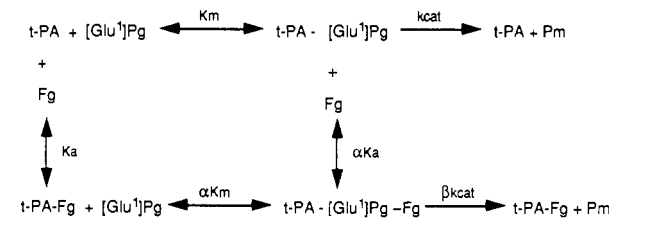


FIGURE 6: Kinetic constants for the Cl^- inhibition and EACA stimulation of [Glu¹]Pg by two-chain rec-t-PA (TPA). Pm refers to human plasmin and EACA to ϵ-aminocaproic acid.

Scheme II



nonessential enzyme activation by fibrinogen, with $\alpha = 0.13$ and $\beta = 1.35$ (Segel, 1975), according to Scheme II.

From the data of Figure 7, and appropriate replots (Segel, 1975), the various kinetic constants have been calculated and are presented in Figure 8. Lineweaver–Burk plots (data not shown) of the stimulation by various levels of fibrinogen of the two-chain rec-t-PA-catalyzed activation of [Glu¹]Pg in 10 mM Hepes–NaOH/150 mM NaOAc, pH 7.4, were qualitatively similar to those of Figure 7, except that their intersection point was found to be in the second quadrant, suggesting that both the K's and the k_{cat} had been altered by the promoter. Double-reciprocal plots of the changes in slopes and intercepts of these lines, against the concentration of fibrinogen, were linear. These observations are consistent with fibrinogen serving as a mixed-type nonessential activator, with $\alpha = 0.16$ and $\beta = 3.0$ (Segel, 1975), consistent with Scheme II. From the Lineweaver–Burk plots of the activation data in the presence of OAc^- and suitable replots (Segel, 1975), the kinetic constants presented in Figure 8 were obtained.

Discussion

The purpose of this study was to determine whether the activation of [Glu¹]Pg by two-chain rec-t-PA is influenced by the normal plasma components, Cl^- and fibrinogen. It has been shown previously that a similar activation of [Glu¹]Pg by two other important activators, SK and u-PA, is strongly influenced by these same components (Chibber et al., 1985, 1986; Urano et al., 1987a,b; Castellino et al., 1988). The results of such an investigation with t-PA are necessary in considering the manner of regulation of its activity in plasma, which has possible important implications in the production of systemic fibrinogenolysis in patients undergoing therapeutic thrombolytic treatment with t-PA.

The data of Table I show that anions inhibit the overall activation of [Glu¹]Pg by two-chain rec-t-PA according to their relative positions in the Hofmeister anion series. This suggests

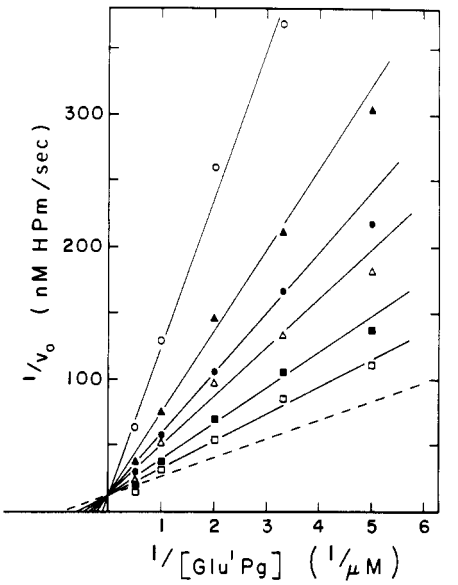


FIGURE 7: Lineweaver–Burk analysis of the effect of fibrinogen on the initial rate of activation (v_0) of [Glu¹]Pg by two-chain rec-t-PA at 37 °C in a Cl^--containing buffer. The concentration of [Glu¹]Pg was varied between 0.2 and 2.0 μM, and the fibrinogen concentrations were (○) none and (▲) 0.33, (●) 0.6, (△) 0.85, (■) 1.2, and (□) 1.7 μM. The calculated rate of activation of [Glu¹]Pg at saturating fibrinogen is also shown (--). The lines drawn through the data points were determined by linear least-squares analysis of the data. Activations were initiated by addition of two-chain rec-t-PA (4.4 nM final concentration). The buffer was 10 mM Hepes–NaOH/100 mM NaCl/50 mM NaOAc, pH 7.4.

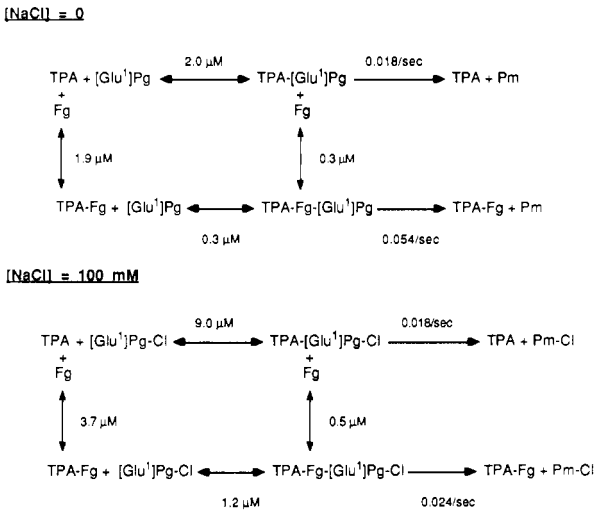


FIGURE 8: Kinetic constants for the Cl^- inhibition and fibrinogen stimulation of [Glu¹]Pg by two-chain rec-t-PA (TPA). Pm refers to human plasmin and Fg to human fibrinogen.

that the anion site(s) responsible for this effect is (are) of a general nature and of sufficient flexibility to accommodate a variety of anions, as was the case for the anion inhibitory site in the u-PA-catalyzed activation of [Glu¹]Pg. The simplest kinetic mechanism that is consistent with the steady-state initial velocity data that are illustrated in Figure 6 involves anion binding to [Glu¹]Pg, with provision of a substrate–anion complex that possesses a higher K_m for the activating enzyme, two-chain rec-t-PA. A considerable amount of physical data is in accord with this view. We have shown previously that anion binding by [Glu¹]Pg results in a large conformational alteration in this protein, which is measurable by sedimentation velocity ultracentrifugation (Urano et al., 1987b) and intrinsic fluorescence enhancement (Urano et al., 1987a). This conformationally altered [Glu¹]Pg is less activatible by u-PA

(Urano et al., 1987b). Since a distinct conformational alteration is produced in the substrate, [Glu^1]Pg, consequent to anion binding, and since anion inhibition of activation is present with two separate activators, neither of which display anion inhibition toward small synthetic peptide substrates, it is most reasonable to suggest that anion interaction with the substrate is responsible for the inhibitory effects observed. In accord with this view, other forms of plasminogen which do not undergo a conformational alteration upon anion binding, i.e., [Lys^{77}]Pg and [Val^{442}]Pg, do not show this same inhibitory effect with Cl^-. In fact, an opposite trend is noted with these latter forms of plasminogen, an observation which is the subject of continuing investigations. Therefore, while it is probable that anions interact separately with the activating enzyme, two-chain rec-t-PA, we do not feel that such an equilibrium is important to the kinetic rate-determining step that describes the anion inhibitory effects seen with [Glu^1]Pg as the substrate, and it has not included these interactions in Figure 7. A kinetically important anion–t-PA interaction in complex with [Glu^1]Pg is not ruled out by the mechanism described.

The K_m for the activation of [Glu^1]Pg or [Glu^1]Pg–Cl by two-chain rec-t-PA found in this study is considerably lower than that published in some previous works (Rijken et al., 1982; Hoylaerts et al., 1982). During the course of this investigation, we found that Lineweaver–Burk plots of the initial activation rates of [Glu^1]Pg, as a function of the concentration of [Glu^1]Pg, are distinctly nonlinear at levels of [Glu^1]Pg greater than approximately 2 μM. Thus, we did not employ zymogen concentrations higher than this level, in contrast to previously published investigations (Rijken et al., 1982; Hoylaerts et al., 1982). The K_m does depend upon the nature of the anion but appears to be in the range of 1–10 μM at the usual levels of Cl^- present in most studies to date. The low K_m for this activation reaction which we obtain in the absence of effectors is more in agreement with the K_m value of approximately 7 μM published by Ranby (1982).

We show herein that EACA greatly stimulates the initial activation rate of [Glu^1]Pg by two-chain rec-t-PA in the presence of anions that have first depressed this same activation rate. The simplest kinetic mechanism, in 100 mM NaCl, consistent with the effect of this stimulator that is derived from application of the iterative procedure to the initial rate plots of Figure 5 is shown in Figure 6. In this mechanism, we have invoked the presence of a [Glu^1]Pg–Cl–EACA complex, since we have shown in a previous study (Urano et al., 1987b) that EACA does not displace Cl^- from [Glu^1]Pg. In Figure 6, we propose that the major influence of EACA is to induce a large decrease in the K_m for the [Glu^1]Pg–Cl complex to approximately that of [Glu^1]Pg, in the absence of inhibiting anions. The K_m for the reaction of the [Glu^1]Pg–Cl–EACA complex is lower than that for [Glu^1]Pg, suggesting that a true reversal by EACA of the kinetic inhibition by Cl^- may not be the exact operative mechanism. However, it should be recalled that OAc^- is present in the experiments in the absence of Cl^-, in order to maintain the ionic strength of the assay constant, and unpublished experiments in our laboratory show that OAc^- does affect two-chain rec-t-PA activity. If all anions are omitted from the assay, except for the 10 mM Hepes–NaOH, pH 7.4, buffer, the K_m for [Glu^1]Pg decreases to 0.7 μM, very nearly the same as that of 0.4 μM found for the [Glu^1]Pg–Cl–EACA complex. A virtual reversal of anion inhibition by EACA was found when u-PA was employed as the activator (Urano et al., 1987b).

Physical–chemical studies of the EACA effect on [Glu^1]Pg are in accord with the kinetic mechanism derived from the initial rate data. We have shown previously (Urano et al., 1987b), by sedimentation velocity analysis, that EACA reverses the conformational change produced in [Glu^1]Pg by anions, a process that yields a conformational state similar to that of [Glu^1]Pg, in the absence of inhibiting anions. In addition, [Lys^{77}]Pg, which exists in a conformation similar to that of [Glu^1]Pg, in the absence of inhibiting anions, or the [Glu^1]Pg–Cl–EACA complex, was activated with two-chain rec-t-PA at a rate similar to that of plasminogen, under the latter two sets of conditions. Importantly, EACA neither shows a significant ability to alter the conformation of [Lys^{78}]Pg in the absence or presence of Cl^- (Violand et al., 1975, 1978) nor stimulates the activation rate of this protein (Claeys & Vermylen, 1974). Thus, the great amount of information accumulated suggests that a conformation exists (high S) for [Glu^1]Pg, in the presence of anions, such as Cl^-, that is poorly activatible by two-chain rec-t-PA. A large conformational alteration (low S) occurs as a result of EACA binding to the [Glu^1]Pg–anion complex, which is similar to that of [Glu^1]Pg, in the absence of anions, and to [Lys^{77}]Pg in the presence or absence of anions and EACA. The low S conformation, readily observable by sedimentation velocity ultracentrifugation, is a highly activatible form of the protein. It has been shown that two-chain rec-t-PA also interacts with lysine and EACA (Ichinose et al., 1986), but since these latter agents do not stimulate the activation of [Lys^{77}]Pg, which also binds EACA (Markus et al., 1978), or [Val^{442}]Pg, which shows a greatly diminished capacity for interaction with EACA (Sottrup-Jensen et al., 1977), we do not believe that the recTCTPA–EACA equilibrium is important to the rate-determining step that describes the kinetic mechanism and have not included this reaction in Figure 7. Again, however, the kinetic mechanism does not rule out an EACA–t-PA interaction in complex with [Glu^1]Pg-Cl.

The effect of fibrinogen on the initial rates of activation of [Glu^1]Pg activation, in a Cl^--containing buffer, is shown in Figure 7. Similar, but not identical results were obtained in OAc^--containing buffers (described under Results). From these plots and appropriate replots (Chibber et al., 1986), the kinetic mechanism for the fibrinogen stimulatory effects, found in the presence of both anions, is provided in Figure 8. The key feature of this mechanism is the presence of a rec-t-PA–fibrinogen complex that is responsible for the rate-determining step in the kinetic stimulation shown by this positive effector. Since it is well-known that [Glu^1]Pg binds very weakly to fibrin (Rakozci et al., 1978), and also presumably to fibrinogen, we do not believe that this equilibrium is important to the rate-limiting kinetic step and have not included such an interaction in Figure 8. In addition, our evidence demonstrates that fibrinogen stimulates the activation of [Val^{442}]Pg by two-chain rec-t-PA, despite the relative inability of this form of plasminogen to interact with fibrin, suggesting that the plasminogen–fibrin (and likely fibrinogen) interaction is not essential for stimulation by this promoter. Finally, a genetic variant of rec-t-PA, wherein Cys^{264} is altered to Gly, providing a t-PA wherein the two polypeptide chains are not stabilized by a disulfide bond, activates [Glu^1]Pg at a rate similar to that of two-chain rec-t-PA but does not show as significant a stimulation by fibrinogen or fibrin (S. Urano, unpublished experiments) as does native two-chain rec-t-PA. This strongly suggests that the fibrinogen–rec-t-PA equilibrium is much more important to the rate-determining step that describes the kinetic mechanism than the fibrinogen–[Glu^1]Pg equilibrium, and this is reflected in the scheme presented in Figure 8. Once again, however, the mechanism described does not rule out

a [Glu^1]Pg interaction in complex with two-chain rec-t-PA. The mechanism of Figure 8 is in essential agreement with the work of Hoylaerts et al. (1982), which showed that the kinetic mechanism that best describes the activation of [Glu^1]Pg by t-PA in the presence of fibrin films was sequential and ordered, with t-PA first binding to fibrin followed by binding of the zymogen. While our mechanism is somewhat similar to that proposed by Hoylaerts et al., (1982), it (as well as theirs) cannot be described as an obligatory ordered reaction, since activation does occur in the presence of fibrinogen and fibrin.

In summary, examination of the mechanisms provided in Figures 6 and 8 shows that EACA and fibrinogen stimulate the two-chain rec-t-PA-catalyzed activation of [Glu^1]Pg in different manners, the former via effects on the substrate, [Glu^1]Pg and the latter through effects on the enzyme, two-chain rec-t-PA. These observations are fortified by the results showing that EACA does not stimulate the activation in OAc^--containing solutions, since the conformational alteration that would be produced in [Glu^1]Pg by EACA has already occurred. However, fibrinogen does further stimulate activation of [Glu^1]Pg in these same OAc^- solutions.

Finally, the results of this paper provide important information on the control of the recTCTPA-catalyzed initial activation rate of native circulating [Glu^1]Pg by normal plasma components, Cl^- and fibrinogen. The stimulation of activation of [Glu^1]Pg that would normally be observed in the presence of fibrinogen is severely depressed by Cl^-, in theory allowing a low level of plasmin production to occur in plasma. In a microenvironment wherein Cl^- is depleted, fibrinogen or fibrin would promote activation of [Glu^1]Pg, leading to increased amounts of [Glu^1]Pg activation. This aspect of [Glu^1]Pg activation, and its control by Cl^-, requires attention in consideration of mechanisms of plasminogen activation in plasma.

Registry No. [Glu^1]Pg, 9001-91-6; t-PA, 105913-11-9; Cl^-, 16887-00-6; OAc^-, 71-50-1; I^-, 20461-54-5; HCO_3^-, 71-52-3; F^-, 16984-48-8; ϵ-aminocaproic acid, 60-32-2.

REFERENCES

Banyai, L., Varadi, A., & Patthy, L. (1983) *FEBS Lett. 163*, 37–41.

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

Castellino, F. J. (1981) *Chem. Rev. 81*, 431–446.

Castellino, F. J., Urano, T., & deSerrano, V. S. (1988) *Proceedings of the International Meeting on Plasminogen, Function and Assay, Clinical Significance*, Vienna, Austria, pp 15–23, S. Karger Medical and Scientific, Basel, Switzerland.

Chibber, B. A. K., Morris, J. P., & Castellino, F. J. (1985) *Biochemistry 24*, 3429–3434.

Chibber, B. A. K., Radek, J. T., Morris, J. P., & Castellino, F. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1237–1241.

Claeys, H., & Vermylen, J. (1974) *Biochim. Biophys. Acta 342*, 351–359.

Deutsch, D., & Mertz, E. (1970) *Science (Washington, D.C.) 170*, 195–196.

Forsgren, M., Raden, B., Israelsson, M., Larsson, K., & Heden, L. O. (1987) *FEBS Lett. 213*, 254–260.

Hoylaerts, M., Rijkin, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912–2919.

Ichinose, A., Kisiel, W., & Fujikawa, K. (1984) *FEBS Lett 175*, 412–418.

Ichinose, A., Takio, K., & Fujikawa, K. (1986) *J. Clin. Invest. 78*, 163–169.

Malinowski, D. P., Sadler, J. E., & Davie, E. W. (1984) *Biochemistry 23*, 4243–4250.

Markus, G., Evers, J. L., & Hobika, G. H. (1978) *J. Biol. Chem. 253*, 733–739.

Mattler, L. E., Marks, C. A., & Bang, N. (1985) *Thromb. Haemostasis 54*, 266.

Nieuwenhuizen, W., Verheijen, J. H., Vermond, A., & Chang, G. T. G. (1983) *Biochim. Biophys. Acta 755*, 531–533.

Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V., & Collen, D. (1983) *Nature (London) 301*, 214–221.

Pohl, G., Kallstrom, M., Bergsdorf, N., Wallen, P., & Jornvall, H. (1984) *Biochemistry 23*, 3701–3707.

Rakozci, I., Wiman, B., & Collen, D. (1978) *Biochim. Biophys. Acta 540*, 295–300.

Ranby, M. (1982) *Biochim. Biophys. Acta 704*, 461–469.

Rijken, D. C., Hoylaerts, M., & Collen, D. (1982) *J. Biol. Chem. 257*, 2920–2925.

Segel, I. (1975) *Enzyme Kinetics*, Wiley, New York.

Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1977) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191–209.

Urano, T., Chibber, B. A., K., & Castellino, F. J. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031–4034.

Urano, T., Sator de Serrano, V., Chibber, B. A. K., & Castellino, F. J. (1987b) *J. Biol. Chem. 262*, 15959–15964.

Urano, T., Urano, S., & Castellino, F. J. (1988) *Biochem. Biophys. Res. Commum. 150*, 45–51.

Violand, B. N., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 3906–3912.

Violand, B. N., Sodetz, J. M., & Castellino, F. J. (1975) *Arch. Biochem. Biophys. 170*, 300–305.

Violand, B. N., Byrne, R., & Castellino, F. J. (1978) *J. Biol. Chem. 253*, 5395–5401.

Wallen, P., Bergsdorf, N., & Ranby, M. (1982) *Biochim. Biophys. Acta 719*, 318–328.

Wiman, B. (1973) *Eur. J. Biochem. 39*, 1–9.

Wiman, B. (1977) *Eur. J. Biochem. 76*, 129–137.

Zonneveld, A.-J., Veerman, H., & Pannekoek, H. (1986) *J. Biol. Chem. 261*, 14214–14218.