Kinetic studies on the interaction of streptokinase and other plasminogen activators with plasminogen and fibrin

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The activation of Lys-plasminogen to plasmin by streptokinase was promoted by soluble fibrin such that K_m was decreased and V_{max} increased. Enhancement was also observed when Glu-plasminogen was the substrate and was shared by the pre-formed streptokinase–plasminogen activator complex, indicating that the stimulation was not exerted primarily on the rate of active site formation.

Plasminogen activators are serine proteinases with restricted specificity, catalysing the hydrolysis of the Arg⁵⁶⁰–Val⁵⁶¹ bond in plasminogen. The resultant plasmin, which is also a serine proteinase, has a relatively broad, trypsin-like specificity so that the selective physiological function, fibrinolysis, requires some localization of plasminogen activation within the thrombus. One approach to distinguish between the various classes of plasminogen activator involves quantification of their interaction with fibrin. The vascular, or tissue, activator, t-PA, forms a ternary complex with fibrin and plasminogen which is reflected in a reduction of K_m for plasminogen (Hoylaerts *et al.*, 1982) and may endow relative specificity for the lysis of fibrin. The kinetics of plasminogen turnover by two-chain urokinases are not similarly affected so that activation occurs systemically in addition to within the thrombus. The activation of plasminogen by the bacterial protein streptokinase (which itself has no proteolytic activity but must form an activator species by stoichiometric complex formation with plasminogen) has been shown to be potentiated by plasma protein(s), specifically fibrinogen and its degradation fragments (Takada & Takada, 1978; Camiolo *et al.*, 1980). There is limited evidence, albeit unquantified, that fibrin can also enhance the activity of streptokinase (Camiolo *et al.*, 1980; English *et al.*, 1981). It was the purpose of the present kinetic studies to attempt to elucidate the mechanism of the en-

hancement of streptokinase by human fibrinogen and fibrin. Our assays examined the activation of both Glu- and Lys-plasminogens; Glu-plasminogen is normally the predominant circulating form whereas Lys-plasminogen may be the principal fibrin-bound species (Thorsen *et al.*, 1984).

Materials and methods

Materials

Human Glu- and Lys-plasminogens were obtained from KabiVitrum (Stockholm, Sweden); residual plasmin was removed by treatment with aprotinin–agarose (500mg of suction-dried gel/10mg of protein were incubated at 4°C for 2–3 h), lysine and 6-aminohexanoic acid were removed by dialysis against 100-fold excess 0.1 M-Tris/0.15 M-NaCl/20% (w/v) glycerol, pH 7.4. Human fibrinogen (grade L) was also obtained from KabiVitrum and was freed from contaminating plasminogen by treatment with L-lysine–Sephadex. The concentration of coagulable protein was measured by the spectrophotometric method described previously (Dupe *et al.*, 1981). The purity and molecular weight of each protein was confirmed by SDS/polyacrylamide-gel electrophoresis (4% stacking gel, 10% separating gel); the concentration of activatable plasminogen was determined by cross-titration using excess streptokinase and *p*-nitrophenyl *p*'-guanidinobenzoate (McClintock & Bell, 1971).

The compound S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide.2HCl) was obtained from Flow Laboratories (Rickmansworth, Herts. WD3 1PQ, U.K.), streptokinase was obtained from KabiVitrum and urokinase (M_r 54000, two-chain, of urinary origin) from Serono (Freiburg, Germany). t-PA (two-chain) was provided by Dr. J. Robinson,

Abbreviations used: Glu-plasminogen, human plasminogen with *N*-terminal glutamic acid (residues 1–790); Lys-plasminogen, human plasminogen with *N*-terminal lysine (residues 77–790); SDS, sodium dodecyl sulphate; t-PA, tissue plasminogen activator.

purified from Bowes melanoma cells according to the method of Browne *et al.* (1985). Type 2 streptokinase-plasminogen activator complex was prepared according to the method described previously (Dupe *et al.*, 1981) and was used within 30 min of formation.

Two types of soluble fibrin preparation were used in the plasminogen activation assay. One was prepared by a method based on the procedure of Smith (1980) as modified by Garman & Smith (1982), stopping the fibrin polymerization reaction with hirudin after 90 min, and comprised a mixture of oligomers (dimers and other polymers). The second type of soluble fibrin was prepared by an adaptation of the method of Verheijen *et al.* (1982) and involved NaBr digestion, without denaturation, of a fibrin clot. Approx. $7\mu\text{M}$ -fibrinogen in 0.5M -NaBr, pH 5.5, was clotted with bovine thrombin (10 NIH units/ml) at room temperature and the clot was allowed to mature for 60 min. The fibrin clot was then dissolved in 3.0M -NaBr, pH 5.5, by sonication. Both preparations were used within 3 h of formation (stored at 4°C); the concentration of soluble fibrin was calculated from the starting fibrinogen concentration.

Plasminogen activation

Plasminogen activation was measured by a modification of the method of Wohl *et al.* (1980) using 1.0 ml semi-micro-cuvettes at 37°C in a double-beam recording spectrophotometer (SP8-100; Pye Unicam, Cambridge, U.K.). The activity of each activator was determined using plasminogen concentrations in the range $0.05\text{--}3 \times K_m$ in a total volume of $500\mu\text{l}$ of 0.05M -Tris/ 0.1M -NaCl, pH 7.4, containing 0.5 mM -S-2251. Where appropriate, fibrinogen or soluble fibrin was also added and the contents of the cuvette allowed to equilibrate before the reaction was initiated by addition of the activator; reference samples contained the same additions except activator. The absorbance versus time curves for *p*-nitroaniline release were analysed by using the integrated form of the Michaelis-Menten equation (Wohl *et al.*, 1978, 1980); initial rates of plasminogen activation were obtained by plotting $A_{405}^{60\text{m}}$ versus (time)². In preliminary experiments, this relationship was found to be linear up to 10 min even when Glu-plasminogen was used as substrate, indicating that there was no appreciable conversion of Glu- to Lys-plasminogen in the assay. This lack of conversion was confirmed by analysis of the products of the reaction (up to 30 min incubation) by SDS/polyacrylamide-gel electrophoresis.

Kinetic constants were obtained from the averages of quadruplicate determinations using the Lineweaver-Burk plot and the results were confirmed using the Hanes plot, where the error

Fig. 1. Dependence of enhancement on fibrin concentration. Glu-plasminogen ($0.1\mu\text{M}$) was activated by streptokinase-plasminogen pre-formed activator complex ($1 \times 10^{-10}\text{M}$). Results are means $\pm$ S.E.M. for six determinations.

span is not distorted by reciprocation. It was observed that the double reciprocal plots were linear throughout the range of plasminogen concentrations used for each activator, except for t-PA. This discrepancy has been noted by other workers and, although it can be resolved by application of the general Wong-Hanes equation (Rånby, 1982) the problem was circumvented in the studies by slightly limiting the range of plasminogen concentrations used ($0.2\text{--}2 \times K_m$).

In preliminary studies the relationship between concentration of fibrinogen or fibrin and degree of enhancement of plasminogen activation was studied. Fig. 1 shows the results from a typical experiment using pre-formed streptokinase-plasminogen activator complex and Glu-plasminogen substrate. Maximum enhancement was obtained at $0.1\text{--}0.2\mu\text{M}$ soluble fibrin; the relative diminution in stimulation at higher concentrations of fibrin, and fibrinogen, might be attributable to the effect of viscosity on diffusion of reactants (Rånby, 1982) but also to competition by fibrin and fibrinogen with S-2251 for plasmin. The activity of free plasmin, measured using 0.5 mM -S-2251, was decreased by 15% at $0.68\mu\text{M}$ -fibrin and by 20% at $0.68\mu\text{M}$ -fibrinogen. A lack of effect of lower concentrations of fibrin(ogen) on plasmin/S-2251 activity confirms the results of other workers (Wiman *et al.*, 1979; Hoylaerts *et al.*, 1982; Rånby, 1982).

Results and discussion

Fibrinogen decreased the K_m of streptokinase for Lys-plasminogen and increased V_{max} (Table 1). When 6-aminohexanoic acid was present at a

Table 1. Kinetics of plasminogen activation by streptokinase and the pre-formed activator complex
Results are means $\pm$ s.e.m., where indicated, for the designated number of separate experiments. Each experiment comprised quadruplicate assays for a range of plasminogen concentrations as described in the Materials and methods section.

Activator	Plasminogen substrate	Enhancer	n	K _m (μ M)	V _{max.} (nmol·min ⁻² ·fibrinolytic unit ⁻¹)	V _{max.} /K _m
Streptokinase	Lys	—	5	0.33 $\pm$ 0.07	8.8 $\pm$ 0.9	27
	Lys	0.1 μ M-Fibrinogen	3	0.18 $\pm$ 0.02	12.3 $\pm$ 0.3	68 ($\times$ 2.5)
	Lys	0.1 μ M-Fibrin oligomers	3	0.054 $\pm$ 0.017	13.9 $\pm$ 0.8	257 ($\times$ 10)
	Lys	0.05 μ M-Digested fibrin clot	2	0.034	22.5	662 ($\times$ 25)
Streptokinase-Lys-plasminogen activator complex (pre-formed)	Lys	—	4	0.26 $\pm$ 0.03	5.6 $\pm$ 0.4	22
	Lys	0.1 μ M-Fibrinogen	2	0.17	7.3	43 ($\times$ 2.0)
	Lys	0.1 μ M-Fibrin oligomers	2	0.077	12.2	158 ($\times$ 7.2)
	Lys	0.05 μ M-Digested fibrin clot	2	0.025	8.6	344 ($\times$ 16)
	Glu	—	2	0.42	1.0	2.4
	Glu	0.05 μ M-Digested fibrin clot	2	0.18	2.8	15.6 ($\times$ 6.5)

Table 2. Kinetics of Lys-plasminogen activation by urokinase and t-PA
Results are means $\pm$ s.e.m. for the designated number of separate experiments. Each experiment comprised quadruplicate assays for a range of plasminogen concentrations as described in the Materials and methods section.

Activator	Enhancer	n	K _m (μ M)	V _{max.} (nmol·min ⁻² ·amidolytic unit ⁻¹)	V _{max.} /K _m
Urokinase	—	5	5.6 $\pm$ 0.7	4.0 $\pm$ 0.9	0.71
	0.1 μ M-Fibrinogen	3	5.3 $\pm$ 0.6	3.7 $\pm$ 0.5	0.68
	0.1 μ M-Fibrin oligomers	4	5.8 $\pm$ 0.6	3.9 $\pm$ 0.4	0.67
t-PA	—	4	19.1 $\pm$ 2.4	0.32 $\pm$ 0.03	0.017
	0.1 μ M-Fibrinogen	3	1.9 $\pm$ 0.4	0.30 $\pm$ 0.04	0.15 ($\times$ 8.8)
	0.1 μ M-Fibrin oligomers	3	0.04 $\pm$ 0.01	0.40 $\pm$ 0.05	10 ($\times$ 588)

concentration capable of saturating the high-affinity lysine binding sites (1 mM) the stimulatory effect was reduced (result not shown). The effect of two different soluble fibrin preparations on the K_m was consistently and markedly greater than the effect of equimolar fibrinogen (Table 1). This observation is novel but it supports the semi-quantitative differences noted previously using purified rabbit proteins (English *et al.*, 1981).

The molecular events involved in streptokinase activation comprise the following:

where streptokinase* is a proteolytically degraded form. When streptokinase alone is added to the reaction mixture, derived kinetic parameters relate, therefore, to the streptokinase-plasminogen complex, at least at early times. We have also examined the kinetic properties of the pre-formed activator complex (Table 1). Although the K_m of the Type 2 complex (Dupe *et al.*, 1981) for Lys-plasminogen is similar to that for streptokinase

(plasminogen) itself, V_{max.} is less, probably because of the progressive degradation of the streptokinase moiety during aging of the complex (Markus *et al.*, 1976). The relative effect of fibrinogen on the activation of Lys-plasminogen by the pre-formed complex is similar to that shown for streptokinase (plasminogen) and is modest in extent. Both types of soluble fibrin again markedly decreased the K_m and the net increase in catalytic efficacy was similar to that observed for streptokinase (plasminogen). The K_m for the complex using Glu-

plasminogen as substrate was similar to that for Lys-plasminogen as substrate but V_{max.} was much less, in agreement with other published results (Robbins *et al.*, 1983). The addition of soluble fibrin again decreased the K_m and increased V_{max.}

The other major classes of activator were assayed by using the same procedure. The kinetic constants of urokinase were not affected by fibrinogen or soluble fibrin (Table 2). The K_m of t-

PA for Lys-plasminogen was decreased by fibrinogen and by fibrin, to a greater extent, (Table 2) with little effect on $V_{\max}$, in agreement with other published results (Hoylaerts *et al.*, 1982).

Kinetic analysis of plasminogen activation by t-PA suggests that the enhancement of fibrin occurs through binding of the activator to fibrin with subsequent addition of plasminogen (sequential ordered mechanism) to form a 'cyclic' ternary complex (Hoylaerts *et al.*, 1982). We propose that the enhancement phenomenon for streptokinase may also be described in terms of complex formation, although our experiments do not identify the order of addition, which is probably rapidly-reversible [well-characterized ternary complexes have not been isolated, at least using fibrinogen (Camiolo *et al.*, 1980)]. It has been asserted (Strickland *et al.*, 1982) that the stimulatory effect of fibrinogen on streptokinase resides mainly in the D domain and is mediated by an increased rate of formation of the active site in the streptokinase-plasminogen complex rather than by subsequent expression of that catalytic activity. In our experiments, enhancement of streptokinase activity by fibrinogen is small by comparison with the response to soluble fibrin and the effect of the latter on the pre-formed complex is only slightly diminished. Thus, a promotion in the rate of active-site formation is not the only, or the principal, explanation for the kinetic effect of fibrin and, presumably, the fibrin-enhancement of streptokinase-plasminogen will be shared by the acylated pro-drugs of the activator complex such as BRL 26921 (Smith *et al.*, 1981) when these agents deacylate within a thrombus. The present results, and others demonstrating fibrin-binding for agents like BRL 26921 (Fears *et al.*, 1985; Green *et al.*, 1985), tend to contradict the traditional assumption that streptokinase-plasminogen has little or no specific affinity for fibrin (Collen & Verstraete, 1983; Fox *et al.*, 1984). The clinical consequences of this fibrin-binding and enhancement have yet to be established, particularly by comparison with t-PA where enhancement is considerable (Table 2). It should be noted, however, that the expression of such kinetic attributes in terms of net thrombolytic efficacy may depend critically on the ability of the plasminogen activators to penetrate organized natural thrombi and thus to achieve equilibration with the fibrin network. This requirement implies an important role for pharmacokinetic properties in the assessment of thrombolytic enzymes, particularly when agents such as t-PA, which are rapidly metabolized (Korninger *et al.*, 1981; Nilsson *et al.*, 1984) are compared with the more

slowly cleared acyl-enzymes (Dupe *et al.*, 1984; Esmail *et al.*, 1984).

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