Kinetic studies on novel plasminogen activators

Demonstration of fibrin enhancement for hybrid enzymes comprising the A-chain of plasmin (Lys-78) and B-chain of tissue-type plasminogen activator (Ile-276) or urokinase (Ile-159)

Robin FEARS, Ian DODD, Harry FERRES and Jeff. H. ROBINSON

Beecham Pharmaceuticals Research Division, Great Burgh, Epsom, Surrey KT18 5XQ, U.K.

The activation of plasminogen by two novel hybrid enzymes, constructed from the A-chain of plasmin and the B-chains of tissue-type plasminogen activator (t-PA) or urokinase, was compared with the activation by the parent enzymes. Basal kinetic constants for 'Lys-plasminogen' (human plasminogen with *N*-terminal lysine) and 'Glu-plasminogen' (human plasminogen with *N*-terminal glutamic acid) activation were similar to those of the parent activators. The K_m for plasminogen turnover for both hybrid enzymes was considerably decreased in the presence of both soluble fibrin and a mimic, a CNBr digest of fibrinogen. These enhancements and the related apparent negative co-operativity are similar to the behaviour of t-PA itself. The results are discussed with regard to the molecular features involved in the mechanism of fibrin stimulation.

INTRODUCTION

Serine proteinases are important regulators of many biological processes, particularly the coagulation and fibrinolytic pathways. Plasminogen activators are serine proteinases with restricted specificity, cleaving the Arg⁵⁶¹–Val⁵⁶² peptide bond in plasminogen to form the active two-chain enzyme plasmin (EC 3.4.21.4). Plasmin has a relatively broad trypsin-like specificity, and its fibrin substrate acts as a cofactor in regulating the interaction between plasminogen and plasminogen activators. Thus one approach to the classification of plasminogen activators involves quantification of their interaction with fibrin, for it is recognized that t-PA (tissue-type plasminogen activator; EC 3.4.21.-), but not u-PA (two-chain urokinase; EC 3.4.21.31), forms a ternary complex with fibrin and plasminogen (Hoyleaerts *et al.*, 1982).

We and others recently showed that it was possible to construct hybrid enzymes comprising the A-chain of Lys-plasmin and the B-chain of either t-PA (Ile²⁷⁶-t-PA) or u-PA (Ile¹⁵⁹-u-PA) (Robinson & Dodd, 1985; Nakayama, 1986; Robbins & Tanaka, 1986; Robbins & Boreisha, 1987; Robinson *et al.* 1988). The hybrids bind to fibrin, but less is known about the effect of fibrin on enzyme activity. In the present studies we have determined kinetic constants and characterized other kinetic properties of the hybrids relative to the parent activators. Our studies have examined the activation of both the native zymogen, Glu-plasminogen, and a partly-degraded form, Lys-plasminogen, produced by proteolytic loss of the *N*-terminal peptide. Use of Lys-plasminogen facilitates the assays and may also simulate the conformation of the zymogen bound to the fibrin matrix (Fears *et al.*, 1985; Cassels *et al.*, 1987; Fears, 1988).

MATERIALS AND METHODS

Materials

The synthesis and characterization of the plasmin A-chain/t-PA B-chain hybrid (apparent M_r 86000) and the plasmin A-chain/u-PA B-chain hybrid (apparent M_r 86000) were described elsewhere (Robinson *et al.*, 1988). t-PA was purified from the recombinant Bowes-melanoma cell line TRBM6 (Browne *et al.*, 1985; Dodd *et al.*, 1986b); the major species had an apparent M_r of 63000. The origin and purification of the other proteins used, and the preparation of soluble fibrin by digestion of clotted human fibrin using NaBr, have been described previously (Fears *et al.*, 1985). The preparation and characterization of a CNBr digest of fibrinogen, simulating the effect of soluble fibrin (Zamarron *et al.*, 1984) has also been described previously (Fears, 1988).

Plasminogen activation

The procedure for the calculation of kinetic constants was the same as that described previously (Fears, 1988). In summary, plasmin generation was monitored, using the substrate Glu- or Lys-plasminogen, at 1 min intervals for 5–7 min at 37 °C by using the chromogenic substrate S-2251 (*H*-D-Val-Leu-Lys *p*-nitroanilide dihydrochloride). Initial rates of plasminogen activation were obtained by plotting $A_{405}^{1\text{cm}}$ versus (time)²: this relationship was linear for both hybrids, as it was for t-PA (Fears *et al.*, 1985), indicating that new binding sites were not revealed on the effector during the course of the assay.

The concentration of activator in the assay was 10^{–10} M for t-PA and its hybrid and 3 × 10^{–11} M for u-PA and its hybrid.

Abbreviations used: Glu-plasminogen (the native zymogen), human plasminogen with *N*-terminal glutamic acid (residue 1); Lys-plasminogen, partially degraded human plasminogen with *N*-terminal lysine (originally residue 78 of the native zymogen); t-PA, tissue-type plasminogen activator; u-PA, two-chain urokinase (M_r 54000); t-PA hybrid, A-chain of Lys-plasmin (two-chain serine proteinase produced by activation of plasminogen) linked by a disulphide bridge to the B-chain of t-PA (Ile²⁷⁶-t-PA); u-PA hybrid, A-chain of Lys-plasmin linked by a disulphide bridge to the B-chain of urokinase (Ile¹⁵⁹-u-PA); IC₅₀, concentration giving 50% inhibition.

Table 1. Kinetics of plasminogen activation: t-PA and its hybrid

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.

Activator	Substrate	Potentiator	<i>n</i>	Apparent K_m (μ M)	Apparent k_{cat} (s^{-1})
t-PA	Lys-plasminogen	—	5	17.7 ± 1.5	1.1 ± 0.30
		0.05 μ M Digested fibrin clot	5	0.044 ± 0.003	0.78 ± 0.28
		0.1 μ M CNBr-digested fibrinogen	4	0.025 ± 0.005	1.3 ± 0.28
	Glu-plasminogen	0.1 μ M CNBr-digested fibrinogen	4	0.22 ± 0.04	0.42 ± 0.10
t-PA hybrid	Lys-plasminogen	—	3	15.8 ± 2.0	0.61 ± 0.24
		0.05 μ M Digested fibrin clot	3	0.036 ± 0.002	0.41 ± 0.17
		0.1 μ M CNBr-digested fibrinogen	3	0.013 ± 0.002	0.98 ± 0.04
	Glu-plasminogen	0.1 μ M CNBr-digested fibrinogen	3	0.17 ± 0.04	0.11 ± 0.04

RESULTS AND DISCUSSION

Activation of Lys-plasminogen

In agreement with previous results (Fears *et al.*, 1985; Cassels *et al.*, 1987; Fears, 1988), Lys-plasminogen activation by t-PA was enhanced by fibrin and by CNBr-digested fibrinogen. The effect was expressed as a considerable decrease in apparent K_m with little effect on apparent k_{cat} for the reaction system (Table 1). As described previously (Fears, 1988), the analysis of t-PA enzyme kinetics in the presence of fibrin is complicated by behaviour consistent with negative co-operativity, and the kinetic constants are derived from an analysis

Fig. 1. Lineweaver-Burk plot for plasmin A-chain/t-PA B-chain hybrid

Lys-plasminogen was activated in the presence of 0.1 μ M CNBr-digested fibrinogen. Results are means of four determinations, and the line drawn is the best fit for initial velocities over the range of substrate concentrations 0.01–1.0 μ M.

of the linear region of the double-reciprocal plot (0.01–1.0 μ M), possibly corresponding to the hyperbolic saturation of a first binding site. A similar downward deviation in the double-reciprocal plot was obtained for the plasmin/t-PA hybrid (Fig. 1) and the plasmin/u-PA hybrid (result not shown) in the presence of fibrin or CNBr-digested fibrinogen. The kinetic constants in the presence of effector (Tables 1 and 2) are derived for the same limited range of Lys-plasminogen concentrations (0.01–1.0 μ M). For both hybrids, the basal kinetic constants for Lys-plasminogen activation were similar to those for the parent activators (Tables 1 and 2). The responses by both the t-PA hybrid and the u-PA hybrid to CNBr-digested fibrinogen and to soluble fibrin were similar to the response by t-PA; there was a marked decrease in apparent K_m and little effect on k_{cat} .

A preparation of t-PA reconstituted from the isolated A- and B-chains of the enzyme was also enhanced in activity by CNBr-digested fibrinogen (result not shown). There was no effect of CNBr-digested fibrinogen on the activation of Lys-plasminogen by parent u-PA (Table 2), a finding in agreement with the lack of effect of soluble fibrin (Fears *et al.*, 1985). In preliminary studies (results not shown) there were only small effects of native fibrinogen on the activities of the hybrids. The effect of fibrinogen on t-PA itself is also known to be modest (Fears *et al.*, 1985).

Activation of Glu-plasminogen

The kinetic constants for the activation of Glu-plasminogen in the presence of CNBr-digested fibrinogen were also relatively similar for t-PA and the t-PA hybrid (Table 1). The values for K_m were considerably decreased from the basal values, but it is not possible to calculate the relative degrees of enhancement, because the enzymes were not saturated, even by 50 μ M-Glu-plasminogen, in the absence of effector. The K_m of the u-PA hybrid for Glu-plasminogen was decreased approx. 50-fold in the presence of CNBr-digested fibrinogen; there was little effect on k_{cat} (Table 2).

Table 2. Kinetics of plasminogen activation: u-PA and its hybrid

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.

Activator	Substrate	Potentiator	<i>n</i>	Apparent K_m (μ M)	Apparent K_{cat} (s^{-1})
u-PA	Lys-plasminogen	—	5	5.7 ± 0.3	9.4 ± 1.1
		0.1 μ M CNBr-digested fibrinogen	3	6.0 ± 0.5	8.8 ± 1.0
u-PA hybrid	Lys-plasminogen	—	3	6.8 ± 1.2	19.2 ± 2.2
		0.1 μ M CNBr-digested fibrinogen	3	0.020 ± 0.002	21.8 ± 2.8
	Glu-plasminogen	—	3	10.4 ± 2.1	0.72 ± 0.10
		0.1 μ M CNBr-digested fibrinogen	3	0.20 ± 0.02	0.88 ± 0.22

Fig. 2. Activation of Glu-plasminogen by plasmin A-chain/t-PA B-chain hybrid

Glu-plasminogen was activated in the presence of 0.1 μ M CNBr-digested fibrinogen. Results are means of four determinations.

There was some evidence for substrate inhibition at high concentrations of Glu-plasminogen for both the t-PA hybrid (Fig. 2) and the u-PA hybrid (result not shown). Therefore the kinetic constants in the presence of effector (Tables 1 and 2) were derived from an analysis over the substrate range 0.05–1 μ M. Such inhibition may represent competition for the fibrin-binding sites between the domains on plasminogen (in 10^4 -fold excess) and plasmin A-chain of the hybrids.

Effect of 6-aminohexanoic acid on plasminogen activation

The novel activators were characterized further in terms of their response to the antifibrinolytic lysine analogue 6-aminohexanoic acid. We observed an inhibition of the CNBr-digested-fibrinogen enhancement effect using either Glu- or Lys-plasminogen, and the effect could be described in terms of simple binding

curves (Petersen *et al.*, 1985). The effect was similar for the hybrids and t-PA. For example, using 0.1 μ M-Lys-plasminogen, the concentration of 6-aminohexanoic acid to give 50% inhibition of the CNBr-digested fibrinogen stimulation (IC_{50}) was 2.5×10^{-5} M for t-PA and the u-PA hybrid and 1.3×10^{-5} M for the t-PA hybrid. With 0.5 μ M-Glu-plasminogen the IC_{50} was 2.0×10^{-5} M for t-PA, 2.1×10^{-5} M for the t-PA hybrid and 3.1×10^{-5} M for the u-PA hybrid.

Mechanism of fibrin enhancement

It has been demonstrated previously (Robbins & Tanaka, 1986; Robbins & Boreisha, 1987) that the hybrids comprising plasmin A-chain and the B-chain of u-PA or t-PA can bind to fibrin. We now show that the activity of the t-PA hybrid is promoted by both soluble fibrin and CNBr-digested fibrinogen. The activity of the u-PA hybrid is also promoted by CNBr-digested fibrinogen (soluble fibrin was not tested). For both novel agents we have characterized the enhancement as mediated by an effect on K_m , and it is remarkable that the extent of enhancement of the t-PA hybrid is similar to that of parent t-PA. Although the relative enhancement of the u-PA hybrid is less, the basal activity is high and the catalytic efficiency in the presence of fibrin (second-order rate constant) is greater than that of t-PA and its hybrid.

Our results are not entirely compatible with those obtained by Robbins and co-workers (Robbins & Tanaka, 1986; Robbins & Boreisha, 1987; Lee *et al.*, 1988), using Glu-plasminogen as substrate, for what appear to be similar hybrid proteins. The results obtained by Robbins and co-workers for the u-PA hybrid are not themselves consistent, because enzyme activity was found to be markedly stimulated by soluble fibrin (Robbins & Tanaka, 1986), but little affected by CNBr-digested fibrinogen (Lee *et al.*, 1988). The effect of CNBr-digested fibrinogen on Glu-plasminogen activation by the t-PA hybrid that was observed by Lee *et al.* (1988) exceeded the effect with t-PA, but the values derived for the kinetic constants for the standard plasminogen activators (t-PA, u-PA and streptokinase) varied considerably from the values calculated by other workers.

It is conceivable that there are structural differences between our hybrid preparations and those used by Robbins and co-workers, reflecting heterogeneity in the

parent proteins. Variations in assay conditions may also contribute to the disparity between the present results and those obtained by Robbins and co-workers. For example, buffer composition has differed, even within studies (Lee *et al.*, 1988), and it is known that different anions influence plasminogen activation. The higher concentration of CNBr-digested fibrinogen ($0.217 \mu\text{M}$) used by Lee *et al.* (1988) is known to inhibit plasmin (Lee *et al.*, 1988) and to increase background absorbance in the assay (Fears, 1988). Furthermore, Lee *et al.* (1988) used a maximum concentration of Glu-plasminogen that was less than the derived K_m for some of the plasminogen activators, so that their calculation of kinetic constants may have been relatively imprecise.

Thus on the basis of the present results, with both Glu- and Lys-plasminogen as substrate, we deduce, by contrast with previous work (Lee *et al.*, 1988), that the second-order rate constants for the hybrids in the absence of fibrin are reasonably similar to those of the parent activators and that both hybrids are markedly enhanced in activity on binding to fibrin.

These findings were unexpected. The isolated B-chain of t-PA is not stimulated by fibrin (Dodd *et al.*, 1986a), and the affinity of t-PA for fibrin is usually considered to be greater than the affinity of Lys-plasminogen for fibrin. Although part of the binding of t-PA may be mediated by kringle domains of sequence similar to those of structures on the plasmin A-chain, it has been suggested that the initial binding of t-PA is determined by a domain of sequence similar to that of the finger domain on fibronectin (van Zonneveld *et al.*, 1986; Verheijen *et al.*, 1986), in which case a similar interaction could not be simulated by the plasmin A-chain.

Not only is the net catalytic efficiency of the hybrids in the presence of fibrin similar to that of t-PA (and unlike that of u-PA), but their response to 6-aminohexanoic acid and the nature of the kinetic transitions are also similar. Furthermore, the location of the binding site on fibrin is probably similar, as it can be uncovered by CNBr digestion of fibrinogen, implicating a site in the region A α 148–A α 207 (Nieuwenhuizen *et al.*, 1983). The only significant difference between the hybrids and t-PA in the present work is the possible susceptibility of the former to inhibition by high concentrations of Glu-plasminogen.

We conclude that the specific domains of the A-chain of t-PA are not necessary for ternary-complex formation between enzyme, substrate and fibrin and promotion of catalytic efficiency. Thus the results obtained for the hybrids substantiate results previously obtained for the

activator complex streptokinase–plasminogen and its stabilized pro-enzyme 'APSAC' [anisoyleated Lys-plasminogen streptokinase activator complex; Eminase (Beecham)], where high fibrin affinity was also associated with fibrin enhancement of activity (Fears *et al.*, 1985; Cassels *et al.*, 1987). Our observations of considerable fibrin enhancement for the plasmin A-chain hybrids linked to the B-chain of u-PA or t-PA, together with the finding of a slow clearance *in vivo* (Robinson *et al.*, 1988), suggest that these enzymes deserve further evaluation as novel thrombolytic agents.

REFERENCES

- Browne, M. J., Dodd, I., Carey, J. E., Chapman, C. G. & Robinson, J. H. (1985) *Thromb. Haemostasis* **54**, 422–424
- Cassels, R., Fears, R. & Smith, R. A. G. (1987) *Biochem. J.* **247**, 395–400
- Dodd, I., Fears, R. & Robinson, J. H. (1986a) *Thromb. Haemostasis* **55**, 94–97
- Dodd, I., Jalalpour, S., Southwick, W., Newsome, P., Browne, M. J. & Robinson, J. H. (1986b) *FEBS Lett.* **209**, 13–17
- Fears, R. (1988) *Biochem. J.* **249**, 77–81
- Fears, R., Hibbs, M. J. & Smith, R. A. G. (1985) *Biochem. J.* **229**, 555–558
- Hoylaerts, M., Rijken, D. C., Lijnen, H. R. & Collen, D. (1982) *J. Biol. Chem.* **257**, 2912–2919
- Lee, P. P., Wohl, R. C., Boreisha, I. G. & Robbins, K. C. (1988) *Biochemistry* **27**, 7506–7513
- Nakayama, Y., Shinohara, M., Tani, T., Kawaguchi, T., Furuta, T., Izawa, T., Kaise, H., Miyazaki, W. & Nakano, Y. (1986) *Thromb. Haemostasis* **56**, 364–370
- Nieuwenhuizen, W., Vermond, A., Voskuilen, M., Traas, D. W. & Verheijen, J. H. (1983) *Biochim. Biophys. Acta* **748**, 86–92
- Petersen, L. C., Brender, J. & Suenson, E. (1985) *Biochem. J.* **225**, 149–158
- Robbins, K. C. & Boreisha, I. G. (1987) *Biochemistry* **26**, 4661–4667
- Robbins, K. C. & Tanaka, Y. (1986) *Biochemistry* **25**, 3603–3611
- Robinson, J. H. & Dodd, I. (1985) *Eur. Patent Appl. EPA* 0155387
- Robinson, J. H., Dodd, I., Esmail, A., Ferres, H. & Nunn, B. (1988) *Thromb. Haemostasis* **59**, 421–425
- Van Zonneveld, A.-J., Vearman, H. & Pannekoek, H. (1986) *J. Biol. Chem.* **261**, 14214–14218
- Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., de Munk, G. A. W., Pouwels, P. H. & Enger-Valk, B. E. (1986) *EMBO J.* **5**, 3525–3530
- Zammaron, C., Lijnen, H. R. & Collen, D. (1984) *J. Biol. Chem.* **259**, 2080–2083