Kinetics of the inhibition of plasminogen activators by the plasminogen-activator inhibitor

Evidence for 'second-site' interactions

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The reactions between plasminogen-activator inhibitor (PAI) and different plasminogen activators were studied in the presence of chromogenic peptide substrates for the enzymes. Our findings suggest that the rate constants for the reactions of PAI with single-chain tissue plasminogen activator (tPA), two-chain tPA, high- M_r urokinase and low- M_r urokinase are high and quite similar (1.6×10^7 – $3.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$). A free active site in the enzymes seems to be necessary for their reaction with PAI. Amino acids with antifibrinolytic properties did not interfere with the reactions. However, di-isopropyl phosphorofluoridate-inactivated tPA inhibited the reaction between PAI and all plasminogen activators in a similar way. These findings clearly demonstrated that a 'second-site' interaction, in addition to that between the enzyme active site and the inhibitor 'bait' peptide bond, is of importance for the high reaction rate. The reaction rate between PAI and single-chain tPA in the presence of an activator substrate (D-Ile-Pro-Arg *p*-nitroanilide) was decreased in the presence of fibrin. Fibrin caused a decrease in the K_m for the single-chain tPA–substrate reaction. As a consequence, the 'free' concentration of single-chain tPA in the system decreased in the presence of fibrin, affecting the reaction rate between PAI and single-chain tPA. The phenomenon might be of physiological relevance, in the sense that single-chain tPA bound to fibrin in the presence of plasminogen would be protected against inactivation by PAI.

INTRODUCTION

During the last few years a number of proteinase inhibitors have been described with a possible action against plasminogen activators. Of these, only one inhibits single-chain tissue plasminogen activator (sc-tPA) rapidly. This inhibitor has tentatively been called plasminogen-activator inhibitor 1 [1] and in the present paper is denoted PAI. Preliminary data from measuring the decline of t-PA activity after addition to plasma have suggested that the reaction rate between 'native' PAI and sc-tPA is about $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [2–4].

The amino acid sequence of PAI has been deduced from its cDNA sequence, and homologies with other plasma proteinase inhibitors have recognized PAI as a member of the serpin superfamily of proteins [5,6]. In analogy with other proteinase–inhibitor reactions, tPA or urokinase (EC 3.4.21.31) forms stable stoichiometric 1:1 complexes with PAI [3,4,7].

Unfortunately, so far PAI has not been purified in an active 'native' form from any source, since it is rapidly inactivated after the production by certain cell lines in culture [8,9]. The most successful purified preparations seem to be at the most 10% active [10]. However, the inactive form of PAI can be re-activated by treatment of the molecule by certain denaturing agents, such as SDS or guanidinium chloride [9,11].

In the present investigation we have studied the kinetics of the reaction between re-activated purified PAI and the plasminogen activators tPA or urokinase. We have used systems in which the residual plasminogen-activator activity was directly and continuously determined by the use of chromogenic substrates. With these systems it was possible to evaluate the influence of certain ligands on the reaction rate and to draw conclusions on the role of 'second-site' interactions for the reaction.

MATERIALS AND METHODS

Determination of PAI activity and antigen

The PAI activity was measured by addition of excess sc-tPA and subsequently determining the residual tPA activity with a coupled spectrophotometric assay [12] with some minor modifications. The chromogenic substrate used in the procedure was D-aminobutyrylcyclohexyltyrosyl-lysine *p*-nitroanilide (Biopool AB, Umeå, Sweden). The PAI activity is expressed in arbitrary units, and 1 arbitrary unit is defined as the amount of PAI that inhibits 1 unit of sc-tPA.

PAI antigen was determined by a double-antibody radioimmunoassay with rabbit antiserum against human plasma/serum PAI essentially as described previously

Abbreviations used: DFP, di-isopropyl phosphorofluoridate; $H M_r$ uPA, high- M_r urokinase; $L M_r$ uPA, low- M_r urokinase, a degraded form of the enzyme, lacking the 135 *N*-terminal amino acid residues of the A-chain; PAI, plasminogen-activator inhibitor 1; sc-tPA, single-chain tissue plasminogen activator; tc-tPA, two-chain tissue plasminogen activator; <Glu (in peptide sequence), pyroglutamic acid (5-oxopyrrolidone-2-carboxylic acid); -NH-Np, *p*-nitroanilide.

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[13]. In this system ^{125}I -labelled purified 'latent' inhibitor from HT 1080 fibrosarcoma cells was used as tracer. Goat anti-(rabbit IgG) antibody was employed as the second antibody. The concentration of PAI was expressed in $\mu\text{g/l}$, with non-labelled purified 'latent' material as standard.

Purification of PAI

The inactive 'latent' form of PAI was purified from medium of the human fibrosarcoma cell line HT 1080 by affinity chromatography on concanavalin A-Sepharose and gel filtration on Sephacryl S-300 as described previously [14]. The inactive PAI was subsequently activated by treatment with 4 M-guanidinium chloride by addition of the solid substance followed by incubation at 4 °C for 16 h and dialysis against 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl. In order to control the degree of re-activation, PAI activity and antigen were assayed as described above. Batches of PAI with a specific activity higher than 700 000 arbitrary units/mg were employed in the present experiments.

For calculation of molar concentrations of PAI an M_r of 50 000 and a specific activity of 1×10^6 arbitrary units/mg [13] were used.

Enzymes

Tissue plasminogen activator (tPA). Pure sc-tPA (lot no. 063 6133) and tc-tPA (lot no. 063 6134) were from Biopool AB. For calculations of molar concentrations an M_r of 65 000 and a specific activity of 650 000 international units/mg were used for both forms of tPA (see below).

Urokinase. The low- M_r urokinase (LM_r uPA) was purchased from Leo (Copenhagen, Denmark; lot no. A 75A). An M_r of 32 000 and a specific activity for pure urokinase of 100 000 CTA units/mg were used in calculating the molar concentrations, where 1 CTA unit equals that of 1 international unit of uPA (see below). High- M_r urokinase (HM_r uPA) was from Institute Choay (Paris, France). An M_r of 54 000 and the same specific activity as for LM_r uPA were used for calculating its molar concentration.

Thus all enzyme and inhibitor concentrations were estimated from activity measurements, reported specific activities and known M_r values. This gives a small uncertainty with regard to the enzyme concentrations, which is also transferred to the estimates of absolute rate constants. However, the relative differences in reaction rates obtained by different ligands are not at all affected by this fact. The specific activities of tPA and uPA were checked against the W.H.O. international standards for these proteins.

Fibrin

The soluble fibrin was prepared exactly as described previously [12] by digestion of human fibrinogen with Bathroxobin and dissolving the formed clot in 3.5 M-urea (final concentration) to give a fibrin concentration of 30 μM . When required the fibrin was diluted with freshly prepared 3.5 M-urea.

Reagents

Chromogenic peptide substrates for determination of the amidolytic activity of uPA (<Glu-Gly-Arg-NH-Np, S-2444) and of tPA (D-Ile-Pro-Arg-NH-Np, S-2288) and CNBr-cleavage fragments of fibrinogen [15] were from Kabi Vitrum Diagnostica (Mölnådal, Sweden, courtesy of Dr. Steffen Rosén). Fibrinogen was from IMCO, Stockholm, Sweden. Lysine, arginine, guanidinium chloride and di-isopropyl phosphorofluoridate (DFP) were from Sigma Chemical Co., St Louis, MO, U.S.A., and 6-aminohexanoic acid was from Carl Roth KG (Karlsruhe, Germany). Heparin was from Kabi Vitrum (Stockholm, Sweden) and Bathroxobin was from Pentapharm (Basel, Switzerland). Other chemicals were of analytical grade and mostly purchased from Merck, Darmstadt, Germany.

Inactivation of tPA with DFP

sc-tPA or tc-tPA (1.8 mg/ml in 1 M- NH_4HCO_3) was incubated with 50 mM-DFP (final concentration) for 6 h at 20 °C and subsequently dialysed for 18 h against 1 M- NH_4HCO_3 . No amidolytic activity towards D-Ile-Pro-Arg-NH-Np could be detected after this treatment for any of the enzymes.

Determination of Michaelis constants for the different enzymic systems

The Michaelis constants, K_m , of tPA (sc-tPA and tc-tPA) and uPA (HM_r uPA and LM_r uPA) for the substrates D-Ile-Pro-Arg-NH-Np and <Glu-Gly-Arg-NH-Np respectively were determined in 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl. The enzyme concentrations used were about 80–100 nM for all the enzymes. The substrate concentrations used were 0.5–3.0 mM- and 0.17–1.0 mM-D-Ile-Pro-Arg-NH-Np for sc-tPA and tc-tPA respectively. K_m determinations were also performed with sc-tPA in the presence of soluble fibrin (0–300 nM). In this case the Tris buffer was supplemented with 10 g of bovine serum albumin/ml in order to keep the fibrin in solution. For the HM_r and LM_r forms of uPA the <Glu-Gly-Arg-NH-Np substrate was used at concentrations of 0.02–0.30 mM. The analyses were performed at least in triplicate.

Determination of reaction rates for the tPA-PAI and the uPA-PAI reactions

The reaction rates between the different plasminogen activators (tPA and uPA) and PAI were studied in the presence of activator-specific chromogenic substrates. The absorbance at 405 nm was continuously recorded during the reaction. The following schematic model describes the system:

where E, I, EI, S, ES and P are free activator (enzyme), free PAI (inhibitor), activator-inhibitor complex, activator-specific substrate, activator-substrate complex and substrate-cleavage product respectively. The second-order rate constant for the activator-inhibitor reaction is k_1 . The model and the subsequent mathematical treatment are based on the assumptions: (1) only free

enzyme, E, reacts with the inhibitor; (2) the Michaelis constant K_m is approximately equal to the dissociation constant of the enzyme-substrate complex; (3) the substrate concentration is constant during the reaction time studied.

The differential describing the rate of activator/inhibitor complex formation is:

$$d[EI]/dt = k_1[E][I] \quad (1)$$

By use of the defined initial concentrations $[E]_0$ and $[I]_0$, the stoichiometric relationships $[EI] = [E]_0 - [E] - [ES]$ and $[I] = [I]_0 - [EI]$ and the equilibrium expression $[E][S]/[ES] = K_m$, eqn. (1) can be transformed into the readily integrated differential:

$$d[E]/dt = -k_1[E](a + b[E])/b \quad (2)$$

where a is $([I]_0 - [E]_0)$ and b is $(1 + [S]/K_m)$. Integration of eqn. (2) from $(0, [E]_0)$ to $(t, [E])$ yields:

$$k_1 t = (-b/a) \cdot \ln \{ (a[E]_0 + b[E]_0[E]) / (a[E] + b[E]_0[E]) \} \quad (3)$$

When the substrate concentration $[S]$ is zero, b becomes 1 and eqn. (3) reduces to:

$$k_1 t = (-1/a) \cdot \ln \{ (a[E]_0 - [E]_0[E]) / (a[E] - [E]_0[E]) \}$$

which is the classical rate equation for second-order reactions:

$$k_1 t = \{ -1/([I]_0 - [E]_0) \} \cdot \ln \{ [E]_0([I]_0 - [E]_0[E]) / [I]_0[E] \} \quad (4)$$

Experimentally, PAI and the activator substrate (at several different concentrations) were mixed in 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl, and 590 μ l of this was transferred to a cuvette in the sample holder of the spectrophotometer. At time zero the activator solution (10 μ l) was added and the absorbance was continuously recorded graphically. Tangents, T , along the curve were determined at various time points. To determine the tangent at time zero, T_0 , separate experiments were carried out where the inhibitor was omitted but with other experimental conditions unchanged.

The activator concentration at any given time point was calculated from the slope, T , at that point, from the known total concentration of activator added, $[E]_0$, from the slope obtained in the corresponding experiment without inhibitor, T_0 , and the relevant Michaelis constant, K_m , by using the following equation:

$$[E] = T[E]_0/bT_0 \quad (5)$$

where b , as before, is $(1 + [S]/K_m)$ with $[S]$ being the substrate concentration.

For experiments with sc-tPA the tPA and PAI concentrations were 9.6 nM and 12.8 nM respectively and the substrate (D-Ile-Pro-Arg-NH-Np) concentration in the range 0.50–2.0 mM. The corresponding values for tc-tPA were 8.3 nM for tPA, 8.7 nM for PAI and 0.17–1.0 mM for D-Ile-Pro-Arg-NH-Np.

The uPA-PAI reaction was studied with the substrate < Glu-Gly-Arg-NH-Np. For LM_r uPA the initial concentrations were 11.7 nM for the enzyme, 17.3 nM for PAI and 0.080–0.50 mM for the substrate. For HM_r uPA the concentrations of uPA, PAI and substrate were 7.8 nM, 11.3 nM and 0.067–0.33 mM respectively.

A similar experimental system was also used to study the effect of certain ligands on the reaction rate between PAI and the different plasminogen activators. The different ligands studied were 6-aminohexanoic acid,

lysine, arginine and guanidinium chloride at final concentrations of 1 mM and heparin at a concentration of 10 mg/l. In addition, human fibrinogen (0.3–3.0 μ M), CNBr-digested fibrinogen (33 mg/l), soluble fibrin (0–300 nM), DFP-inactivated sc-tPA (42–840 nM) or DFP-inactivated tc-tPA (134–2230 nM) were also studied. The substrate concentrations used in these experiments were about 1.5 times the K_m .

In the experiments with soluble fibrin, the Tris buffer was supplemented with 10 g of bovine serum albumin/l, in order to keep the fibrin in solution. Furthermore, in the series of experiments with fibrin, including the control experiments without fibrin, the final experimental mixture also contained 0.029 M-urea. The DFP-inactivated tPA was not stable in the Tris buffer and these experiments were instead performed in 0.04 M-sodium phosphate buffer, pH 7.8, containing 0.10 M-NaCl.

In the experiments with different ligands the apparent rate constants, $k_{1(app.)}$, were determined by using the classical equation for second-order reactions (eqn. 4) and compared with the results obtained in the absence of ligand.

The spectrophotometric determinations were performed at 25 °C on a Kontron Uvikon 810 spectrophotometer equipped with a Uvikon 21 recorder. The recorder scale mostly used was 0–0.02 A. All solutions had to be dust-free and were therefore prepared from freshly sterile-filtered buffers.

RESULTS

Reaction rates of the inhibition of tPA and uPA by PAI

The reactions between PAI and the different plasminogen activators were evaluated in the presence of activator-specific chromogenic substrates. The reaction rates obtained were highly dependent on the concentration of these substrates. The apparent rate constants, $k_{1(app.)}$, decreased with increasing substrate concentrations, especially at concentrations at and below the K_m (results not shown). The different Michaelis constants were determined in separate experiments, with higher enzyme concentrations (Table 1).

In contrast with the $k_{1(app.)}$ obtained at different substrate concentrations, the values for the different rate constants as calculated by eqn. (3) (see the Materials and methods section) were not significantly different at different substrate concentrations. The mean values $\pm$ S.D. are given in Table 2, and as can be seen all constants are high and quite similar (1.6×10^7 – 3.9×10^7 M⁻¹·s⁻¹) for the different plasminogen activators studied.

Table 1. Michaelis constants, K_m , for the different enzymic systems studied

The values are given as means $\pm$ S.D. ($n = 3$ –6).

Enzyme	Substrate	K_m (mM)
sc-tPA	D-Ile-Pro-Arg-NH-Np	1.2 ± 0.13
tc-tPA	D-Ile-Pro-Arg-NH-Np	0.20 ± 0.01
HM_r uPA	< Glu-Gly-Arg-NH-Np	0.059 ± 0.008
LM_r uPA	< Glu-Gly-Arg-NH-Np	0.061 ± 0.009

Table 2. Second-order rate constants, k_1 , for the reactions between PAI and different plasminogen activators

The values are given as means $\pm$ S.D. ($n = 3-7$).

Enzyme	$10^{-7} \times k_1$ ($M^{-1} \cdot s^{-1}$)
sc-tPA	1.6 ± 0.5
tc-tPA	3.9 ± 0.5
HM_r uPA	1.9 ± 0.4
LM_r uPA	1.8 ± 0.5

Effect of certain non-protein ligands on the PAI-tPA reactions

The effect of different ligands on the PAI-tPA reaction was investigated in the presence of D-Ile-Pro-Arg-NH-Np at about 1.5 times the K_m value for sc-tPA and tc-tPA in these systems (1.5 mM and 0.33 mM respectively). The following ligands were studied: 6-aminohexanoic acid, lysine, arginine, guanidinium chloride and heparin. The results, as summarized in Table 3, demonstrated that none of these ligands had any significant effect on the apparent rate constant of the reaction, neither with sc-tPA nor with tc-tPA.

Effect of DFP-inactivated tPA on the PAI-tPA reaction

The effect of DFP-inactivated sc-tPA and DFP-inactivated tc-tPA on the reaction between sc-tPA and PAI is demonstrated in Fig. 1. An inhibitory effect was observed with both types of tPA, but sc-tPA was more effective: a 50% decrease in the $k_{1(app.)}$ was obtained with 180 μM DFP-inactivated sc-tPA as compared with 1.30 μM for DFP-inactivated tc-tPA. The influence of DFP-inactivated tPA was also studied on the reactions of PAI with tc-tPA, HM_r uPA and LM_r uPA. The results were very similar, as demonstrated for the PAI-sc-tPA reaction in Table 4.

Effect of fibrinogen/fibrin and CNBr-degraded fibrinogen on the PAI-tPA and PAI-uPA reactions

The reaction between PAI (9.9 nM) and sc-tPA (9.6 nM) was studied in the presence of the substrate D-Ile-Pro-Arg-NH-Np (1.5 mM) and of soluble fibrin (0–300 nM). As shown in Fig. 2, a 5-fold decrease in the reaction rate

Table 3. Effect of different ligands on the apparent rate constants, $k_{1(app.)}$, of the reaction between PAI and sc-tPA or tc-tPA

The data are expressed as percentages of the $k_{1(app.)}$ as found in the control experiments.

Ligand	$k_{1(app.)}$ (% of control $k_{1(app.)}$)	
	sc-tPA	tc-tPA
6-Aminohexanoic acid (1 mM)	112	88
Lysine (1 mM)	92	83
Arginine (1 mM)	93	119
Guanidinium chloride (1 mM)	110	107
Heparin (10 mg/l)	95	117

Fig. 1. Effect of DFP-inactivated sc-tPA (●) and DFP-inactivated tc-tPA (○) on the reaction between PAI and sc-tPA

The apparent k_1 values found are expressed as percentages of the value obtained in the control sample. Each point represents the mean of two determinations.

was obtained at the highest fibrin concentration studied (300 nM) and a decrease in the apparent rate constant $k_{1(app.)}$ to 50% was obtained at about 30 nM soluble fibrin.

The activity of sc-tPA towards the substrate D-Ile-Pro-Arg-NH-Np in the concentration range 0.5–2.0 mM was increased proportionally with increasing fibrin concentration (Fig. 3). As shown in the Figure, the stimulating effect observed on the reaction by fibrin, was exclusively on the K_m .

When the effect of fibrin on the K_m of the reaction between sc-tPA and D-Ile-Pro-Arg-NH-Np was taken into account in the calculations (eqn. 3; see the Materials and methods section) of the second-order rate constant k_1 , the values obtained for the reactions of sc-tPA and PAI carried out in the presence of 30 nM-, 100 nM- and 300 nM-fibrin were 1.2×10^7 , 1.3×10^7 and $1.9 \times 10^7 M^{-1} \cdot s^{-1}$ respectively. Thus the presence of fibrin did not significantly affect the rate constant of the PAI-sc-tPA reaction.

The effect of fibrin on the reaction between PAI (10.4 nM) and tc-tPA (10.2 nM) was also studied. No significant effect was observed at fibrin concentrations up

Table 4. Effect of DFP-inactivated sc-tPA and DFP-inactivated tc-tPA on the reaction between PAI and different plasminogen activators

The results are expressed as the concentration of inactivated tPA at which a decrease in $k_{1(app.)}$ to 50% was obtained. Abbreviation: N.D., not determined.

Enzyme	Concn. of inactivated tPA for 50% decrease in $k_{1(app.)}$ (μM)	
	DFP-inactivated sc-tPA	DFP-inactivated tc-tPA
sc-tPA	0.18	1.3
tc-tPA	0.18	1.1
HM_r uPA	0.16	N.D.
LM_r uPA	0.16	1.1

Fig. 2. Effect of fibrin on the $k_{1(\text{app.})}$ of the reaction between PAI and sc-tPA in the presence of 1.5 mM-D-Ile-Pro-Arg-NH-Np

The data are graphically represented by the slopes of the lines $k_{1(\text{app.})}t$ versus t . ○, Control without fibrin; △, 10 nM-fibrin; □, 30 nM-fibrin; ●, 100 nM-fibrin; ▲, 300 nM-fibrin. Each point represents the mean of two experiments.

Fig. 3. Effect of fibrin on the sc-tPA-catalysed conversion of D-Ile-Pro-Arg-NH-Np

The data are presented as a Lineweaver-Burk plot. ●, Control without fibrin; ▲, 75 nM-fibrin; ■, 150 nM-fibrin. Each point represents the mean of three determinations.

to 300 nM, nor could any effect of fibrin be observed in this case on the tPA-catalysed conversion of the substrate.

Furthermore, no influence of fibrin (300 nM) was observed on the reaction rate between PAI and any types of uPA, studied in the presence of 0.17 mM- < Glu-Gly-Arg-NH-Np. For HM_r uPA and PAI an apparent k_1 value of $5.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ was obtained in the absence and $6.1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the presence of 300 nM-fibrin. For the reaction of LM_r uPA with PAI the apparent k_1 values were 7.7×10^6 and $8.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively.

Furthermore, a CNBr digest of fibrinogen (33 mg/l) and fibrinogen itself (0.3–3.0 mM) did not influence the reaction rate between PAI and sc-tPA.

DISCUSSION

In this study we have used a direct kinetic approach to get information on the reaction rates, expressed as second-order rate constants, for the reactions between different plasminogen activators and re-activated PAI. With this system it was possible to evaluate the influence on the reactions of different low- M_r and high- M_r ligands and explore the possibility of 'second-site' interactions between activators and PAI. It has previously been demonstrated that PAI reacts about equally fast with tPA and urokinase, and the rate constants have been estimated to be about $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [2–4]. The exact mechanism of the reaction is not known, but since PAI has a primary structure typical for members of the serpin superfamily of proteins [5,6], and since it forms an enzymically inactive very stable 1:1 stoichiometric complex with the enzymes [3,4,7], the reaction probably follows the overall scheme for such inhibitor–enzyme reaction, that is an initial rapid formation of a reversible proteinase–inhibitor complex followed by a slower transition into a more stable complex.

From the technical point of view, the direct kinetic systems employed in this study were quite difficult to perform. The sensitivity of the activator-specific substrates used necessitated the use of about 10 nM of the reactants. Under these conditions the reactions were virtually completed within 30 s and during this time the total change of absorbance at 405 nm was less than 0.01. Therefore we have not been able to extend the study to very low substrate concentrations (below K_m). This is especially true when the k_{cat} value is small, as is the case with D-Ile-Pro-Arg-NH-Np and sc-tPA.

The apparent rate constants obtained for the different PAI–plasminogen activator reactions were inversely dependent on the concentration of activator substrate. However, by use of eqn. (3) (see the Materials and methods section) very similar rate constants were obtained in spite of different substrate concentrations. The data indeed suggest that a free active site in the enzyme is a prerequisite for the rapid formation of complexes with PAI. The reaction rates obtained for all systems were very high and similar (1.6×10^7 – $3.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$). This is comparable with the results obtained with the plasmin– α_2 -antiplasmin reaction [17]. It is well known that a so-called 'second-site' interaction, between a lysine-binding site in the plasmin A-chain and a complementary site in α_2 -antiplasmin, is of importance for the high reaction rate between these compounds [18]. The studies with different kinds of ligands in the present investigation were carried out in order to demonstrate eventual second-site interactions also in the reaction

between PAI and plasminogen activators. No influence was observed with any of the antifibrinolytic amino acids, guanidinium chloride or heparin. However, DFP-inactivated tPA inhibited the reaction between all the different plasminogen activators and PAI quite efficiently: a decrease in $k_{1(\text{app.})}$ to 50% was obtained at about 180 nM with DFP-inactivated sc-tPA, with the corresponding concentration with the two-chain derivative being about 1.3 μM . These data clearly show that an interaction other than that mediated by the active site in tPA is of importance for the high reaction rate.

Furthermore, since the reactions between PAI and all the plasminogen activators studied were equally affected by DFP-inactivated tPA, these enzymes must have another structure than the active site in common. It is possible that this structure also resides in the B-chain portions of the molecules, since LM_r uPA only has a small piece, 21 amino acid residues, left of the A-chain [19]. It might even be speculated that this site is situated in the vicinity of the peptide bond that becomes cleaved during 'activation' of the activators. This could explain the difference in potency between DFP-inactivated sc-tPA and tc-tPA in inhibiting the PAI-plasminogen activator reactions.

Addition of fibrin to the reaction mixture of sc-tPA, PAI and D-Ile-Pro-Arg-NH-Np caused a decrease of the apparent rate constant for inactivation of the enzyme. A decrease in $k_{1(\text{app.})}$ to 50% was obtained at about 30 nM soluble fibrin. This effect was not observed with any of the other enzymes. In agreement with a previous report [20], fibrin also stimulated the amidolytic activity of sc-tPA. This phenomenon depended on a decrease of K_m in the presence of fibrin. Indeed, in calculating the k_1 by eqn. (3), no effect was observed on the rate constant for tPA inactivation by PAI. Nevertheless, this may still be a very relevant physiological mechanism, since the K_m of the tPA-catalysed plasminogen activation is strongly decreased by fibrin [16,21]. Hence tPA physiologically active in the fibrinolytic process would according to these mechanisms be protected against inactivation by PAI. This would indeed be a plausible explanation for some previously published data suggesting that inactivation of tPA by PAI in whole plasma or blood was delayed in the presence of fibrin [3,22].

This kinetic study of the inactivation of plasminogen activators by PAI was performed with PAI that had become inactivated during purification and subsequently re-activated by treatment with guanidinium chloride. It is not certain that this type of PAI behaves in exactly the same way as the 'native' molecules. However, in agreement with the present results it has been reported earlier that sc-tPA is inactivated by PAI in non-fractionated plasma with a rate constant of about $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [2,3]. Furthermore, it is not likely that the 'second-site' phenomenon described in this work is

something that occurred by chance as a consequence of the guanidinium chloride treatment. Nevertheless, once we have learned to purify PAI in a way that preserves its native physiological properties, the kinetic data have to be confirmed with such material.

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