Lipoprotein (a) promotes plasmin inhibition by α_2 -antiplasmin

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Plasmin inhibition by α_2 -antiplasmin (α_2 AP) is regulated by the vascular components fibrin(ogen) fragments, plasminogen and lipoprotein (a). Kinetic analysis demonstrates that CNBr-derived fibrinogen fragments completely protect plasmin from α_2 AP. Plasminogen and 6-aminohexanoic acid decrease the rate of inhibition by 5- and 10-fold respectively. These studies show that CNBr-derived fibrinogen fragments and 6-aminohexanoic acid bind plasmin kringle(s) with binding constants of 2 μ g/ml and 120 μ M respectively, and that plasminogen binds to α_2 AP with an affinity of 0.5 nM. The unmodulated inhibition is not effected by the presence of lipoprotein (a), but in the presence of protective CNBr-derived fibrinogen fragments the rate of inhibition is increased by the presence of the lipoprotein. The kinetics demonstrate that lipoprotein (a) binds to CNBr-derived fibrinogen fragments with an affinity of 4 nM, displacing plasmin from the protective surface. In addition, tissue-type plasminogen activator and trypsin inhibition by α_2 AP is not slowed by the presence of CNBr-derived fibrinogen fragments or plasminogen (Pg), respectively. These kinetics suggest that the initial reversible interaction between plasmin and α_2 AP is mediated by binding of the inhibitor to the kringle 1 domain of plasmin, with a reversible inhibition constant (K_i) of 5.0×10^{-10} M. Under conditions where this kringle-inhibitor interaction is blocked, the reversible inhibition still occurs between the plasmin and α_2 AP, but the initial K_i is increased to 5.0×10^{-9} M. These data suggest that, in the circulation, plasmin inhibition by α_2 AP may be down-regulated by fibrin, fibrin(ogen) fragments and Pg, but up-regulated by lipoprotein (a) in the presence of fibrin or fibrin(ogen) fragments. The lipoprotein (a)-mediated promotion of plasmin inhibition may provide an additional mechanism by which the lipoprotein impairs fibrinolysis and promotes atherosclerosis.

INTRODUCTION

Plasmin, the enzyme which degrades fibrin clots, is a serine proteinase containing a light and a heavy chain [for a brief review, see Castellino (1981)]. The light chain, containing the C-terminal catalytic domain, is similar to other serine proteinases, including trypsin and chymotrypsin. The heavy chain is composed of the N-terminal regulatory domains consisting of five different lysine-binding domains termed kringles 1–5. Fibrinolysis can be regulated either at the level of plasminogen (Pg) activation or by inhibition of plasmin activity. The regulation of Pg activation by components of the plasma and on the vascular surfaces has been extensively characterized [for a brief review, see Dano *et al.*, (1985)], whereas less effort has been focused on the regulation of plasmin inhibition by these components.

The activation of Pg is mediated primarily by tissue-type Pg activator (t-PA) [for a brief review, see Bachmann & Kruithof (1984)]. The rate of endogenous t-PA activation of Pg is extremely slow (Hoyalerts *et al.*, 1982) and is regulated by the presence of various vascular components. The rate of t-PA-mediated plasmin generation is enhanced by fibrinogen and its derivatives (Hoyalerts *et al.*, 1982; Ranby, 1982; Nieuwenhuizen *et al.*, 1983; de Serrano *et al.*, 1989; Harpel, *et al.*, 1985). The fibrinogen-enhanced Pg activation is in turn inhibited by lipoprotein (a), [Lp(a)] (Edelberg & Pizzo, 1990; Edelberg *et al.*, 1990; Loscalzo *et al.*, 1990).

In addition to regulating Pg activation, vascular components also govern the rate of plasmin inhibition. The primary inhibitor of plasmin in the circulation is the serine-proteinase inhibitor (serpin) α_2 -antiplasmin (α_2 AP) [for a brief review, see Lijnen &

Collen (1986)]. The inhibition of plasmin may be modelled as a two-step reaction involving a reversible complex-formation between α_2 AP and the enzyme, followed by a conformational change to form a functionally irreversible complex (Travis & Salvesen, 1983; Lijnen & Collen, 1986).

Several solution-phase studies suggest that the α_2 AP inhibition of plasmin is initiated by an interaction between a C-terminal region of the inhibitor and the kringle domains of the proteinase, and that blocking this interaction slows the inhibition (Wiman & Collen, 1978; Lijnen & Collen, 1986). For example, studies by Anonick & Gonias (1991) show that the binding of plasmin kringles to fibrin decreases the rate of plasmin inhibition. Furthermore, studies with the zymogen Pg demonstrate that the inhibitor binds to Pg (Christensen & Clemmensen, 1978) and that the interaction is localized to one of the kringles 1–3 domains of the zymogen (Wiman *et al.*, 1979). More recent studies demonstrate that a 26-residue peptide from the C-terminus of α_2 AP confers this kringle-binding capacity to the inhibitor (Hortin *et al.*, 1989) and that absence of this peptide decreases the rate of plasmin inhibition (Clemmensen *et al.*, 1981). α_2 AP inhibition of miniplasmin, which lacks the first four kringle domains, is over 50 times slower than the inhibition of the intact enzyme (Wiman *et al.*, 1979). However, kringle domains are neither necessary nor sufficient for the inhibitory activity of α_2 AP. The inhibitor rapidly inactivates trypsin and chymotrypsin, serine proteinases which lack kringle domains (Wiman & Collen, 1978; Wiman, 1981). In addition, t-PA, which contains N-terminal domains homologous with the kringles 1 and 2 of Pg, is inhibited slowly by the inhibitor (Korninger & Collen, 1981).

The importance of the kringle- α_2 AP interaction suggests that other kringle-containing proteins may modulate the interaction

Abbreviations used: α_2 AP, α_2 -antiplasmin; 6-AHA, 6-aminohexanoic acid; bzI-IDGA-pNA·2HCl, N-benzyl-Ile-Glu-Gly-Arg p-nitroanilide dihydrochloride; Lp(a), lipoprotein (a); Pg, plasminogen; t-PA, tissue-type plasminogen activator; VLK-amc, Val-Leu-Lys-7-amino-4-methylcoumarin; VLK-pNA·2HCl, Val-Leu-Lys p-nitroanilide dihydrochloride; serpin, serine-proteinase-inhibitor.

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of plasmin and α_2 AP. Lp(a) is a low-density lipoprotein possessing an apoprotein (a) domain which shows considerable sequence similarity to Pg (Eaton *et al.*, 1987; McLean *et al.*, 1987). Sequence data demonstrate that the apoprotein (a) contains up to 37 copies of a kringle similar to kringle 4 of Pg, as well as a single copy of a kringle-5-like domain. Through its kringle-4 domains Lp(a) can compete with Pg for binding to various surfaces, including cell surfaces (Gonzalez-Gronow *et al.*, 1989; Hajjar *et al.*, 1989; Miles *et al.*, 1989) and fibrin and CNBr-derived fibrinogen fragments (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990; Edelberg *et al.*, 1990).

Clinically elevated levels of Lp(a) are associated with a depression of fibrinolytic activity (Hamsten *et al.*, 1987). Elevated levels of Lp(a) are also associated with an increased risk of both the development of atherosclerotic lesions [for a review, see Edelberg & Pizzo (1991)] and the restenosis of saphenous-vein by-pass grafts (Hoff *et al.*, 1988). The pathological effects of Lp(a) may be due to a decrease in the rate of plasmin generation (for a review, see Edelberg & Pizzo (1991)), but the lipoprotein may also have a role in the inhibition of plasmin. However, the potential role of Lp(a) in the regulation of plasmin inhibition has not been examined.

MATERIALS AND METHODS

Reagents

The fluorometric plasmin substrate Val-Leu-Lys-7-amino-4-methylcoumarin (VLK-amc) was purchased from Enzyme System Products, Livermore, CA, U.S.A. The chromogenic plasmin substrate Val-Leu-Lys *p*-nitroanilide dihydrochloride (VLK-pNA·2HCl) and the thrombin substrate *N*-benzyl-Ile-Glu-Gly-Arg *p*-nitroanilide dihydrochloride (bzI-IDGA-pNA·2HCl) were purchased from Helena Laboratories, Beaumont, TX, U.S.A. All other reagents were of the best grade commercially available.

Proteins

Human Pg glycoforms 1 and 2 was isolated by affinity chromatography as previously described by Deutsch & Mertz (1970) and modified by Brockway & Castellino (1972) and Gonzalez-Gronow & Robbins (1984). Plasmin glycoform 2 was generated by incubation of Pg glycoform 2 with urokinase-Sepharose as previously described (Stack *et al.*, 1990). t-PA was generously given by Dr. Henry Berger, Wellcome Research Laboratories, Research Park, NC, U.S.A. Trypsin was purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). This preparation was 90% active by active-site titration (Chase & Shaw, 1967). α_2 AP was generously given by Dr. Steven Gonias, University of Virginia, Charlottesville, VA, U.S.A., and was purified as previously described (Wiman, 1981). Human fibrinogen was obtained from Kabivitrum, Stockholm, Sweden. CNBr-derived fibrinogen fragments were prepared as previously described (Nieuwenhuizen *et al.*, 1983). Lp(a) was purified from plasma as previously described (Edelberg *et al.*, 1989).

Plasmin inhibition by α_2 AP

The rate of plasmin inhibition by α_2 AP was determined by adding equimolar amounts of plasmin and α_2 AP to a reaction solution and continuously measuring the activity of uninhibited plasmin. Inhibition reaction samples contained plasmin (1.0 nM), α_2 AP (1.0 nM) and VLK-amc (40 μ M), in 1 ml of 50 mM-Tris/HCl/100 mM-NaCl/0.01% Tween 80, pH 7.4. The reaction mixtures were incubated at 37 °C, and the hydrolysis of the VLK-amc was measured continuously by a Shimadzu (Columbia, MD, U.S.A.) RF-540 spectrofluorophotometer using an excitation

wavelength of 340 nm and an emission wavelength of 450 nm. Control samples contained no α_2 AP. All kinetic analyses were performed at least in triplicate. The relative concentration of active plasmin was determined by dividing the initial velocity of VLK-amc hydrolysis in the inhibition reaction samples by the velocities of the control samples. The inhibition rate constant (k_2) was determined from the equation:

$$1/[E]_t - 1/[E]_0 = k_2 t$$

where $[E]_0$ and $[E]_t$ are the concentrations of active enzyme at the initial and measured times of the reaction respectively (Fersht, 1984).

Effect of CNBr-derived fibrinogen fragments, 6-aminohexanoic acid (6-AHA), Pg and Lp(a) on plasmin inhibition by α_2 AP

The rate of plasmin inhibition by α_2 AP was determined in the presence of CNBr-derived fibrinogen fragments, 6-AHA, Pg and Lp(a). The inhibition reactions were performed as described above with the addition of 0–12 μ g of CNBr-derived fibrinogen fragments/ml, 0–1.2 mM-6-AHA, 0–8 nM-Pg or 0–20 nM-Lp(a). The inhibition rate constant (k_2) of each reaction was calculated as described above, and all kinetic analyses were performed at least in triplicate. The maximum fold change in the k_2 (α) induced by the presence of a modulator molecule and the dissociation constant of the modulator (K_{Mo}) are calculated from the equation:

$$\beta = (K_A/[Mo] + 1)/(\alpha - 1),$$

where α is the value of $k_a/(k_2 - k_a)$ (Edelberg & Pizzo, 1991b). A plot of β , the Modulation Factor, versus the inverse of the modulator concentration, yielded the values for α and K_{Mo} .

Effect of Lp(a) on CNBr-derived fibrinogen-fragment-modulated plasmin inhibition by α_2 AP

The rate of CNBr-derived fibrinogen-fragment-modulated plasmin inhibition by α_2 AP was determined in the presence of Lp(a). The inhibition reactions were performed as described above in the presence of CNBr-derived fibrinogen fragments at 2.0 μ g/ml, the calculated value of K_{Mo} for CNBr-derived fibrinogen fragments. Additionally, increasing concentrations of Lp(a) (0–20 nM) were added to the reactions. The k_2 of each reaction was calculated as described above, and all kinetic analyses were performed at least in triplicate. The inhibition-enhancing effects of Lp(a) on the modulated reaction were analysed by an equation which describes the suppression of a modulated inhibition reaction, where the suppressor competes for the modulator, as previously described (Edelberg & Pizzo, 1991b). In this equation

$$k_2 = \frac{k_a \{1 + \alpha([Mo]/K_{Mo})/(1 + [Su]/K_{Su})\}}{1 + ([Mo]/K_{Mo})/(1 + [Su]/K_{Su})}$$

the suppressor of the modulator (Su) is Lp(a) and K_{Su} is the dissociation constant of Lp(a) for the CNBr-derived fibrinogen fragments. The solution to this equation is solved empirically.

Effect of CNBr-derived fibrinogen fragments on t-PA inhibition by α_2 AP

The rate of α_2 AP inhibition of t-PA was determined by incubating the enzyme and inhibitor and measuring the activity of free enzyme by the addition of Pg. t-PA (20 nM) and α_2 AP (200 nM) were incubated in 100 μ l of 50 mM-Tris/HCl/100 mM-NaCl/0.01% Tween 80, pH 7.4. At various times 10 μ l of the reaction mixture was removed and added to a 90 μ l sample of Pg (200 nM), VLK-pNA·2HCl (300 nM) and CNBr-derived fibrinogen fragments (50 μ g/ml). All kinetic analyses were performed at least in triplicate. The rate of Pg activation was

determined indirectly by the measurement of the rate of VLK-pNA·2HCl hydrolysis previously described (Edelberg *et al.*, 1990). The rate of t-PA inhibition by α_2 AP was then determined employing the equation:

$$\ln [E] = k_2 [I]_0 t$$

where $[I] \gg [E]$, and the concentration of the inhibitor is taken as a constant (Fersht, 1984). The rate of the inhibition was also determined in the presence of CNBr-derived fibrinogen fragments (0–50 $\mu\text{g/ml}$).

Effect of Pg on trypsin inhibition by α_2 AP

The rate of trypsin inhibition by α_2 AP was determined by continuous monitoring as described above for plasmin inhibition. Equimolar concentrations of active-site-titrated trypsin (1.0 nM) (Chase & Shaw, 1967) and α_2 AP (1.0 nM) were incubated in the presence of bzI-IDGA-pNA·2HCl (2 mM), and the rate of substrate hydrolysis was determined by a plot of A_{405} versus time. All kinetic analyses were performed at least in triplicate. The concentration of free trypsin was then determined as described above for plasmin inhibition and the k_2 was calculated. The rate of the inhibition was also determined in the presence of Pg (20 nM).

RESULTS

Plasmin inhibition by α_2 AP

The rate of plasmin inhibition by α_2 AP was determined as described above. The second-order inhibition constant was $1.4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is in good agreement with previously published values (Wiman & Collen, 1978; Anonick & Gonias, 1991).

Effect of CNBr-derived fibrinogen fragments, 6-AHA, Pg and Lp(a) on plasmin inhibition by α_2 AP

The rate of plasmin inhibition by α_2 AP in the presence of various concentrations of CNBr-derived fibrinogen fragments, 6-AHA, Pg and Lp(a) was determined as described above. Figs. 1(a)–3(a) show that the rate of inhibition is decreased by the addition of CNBr-derived fibrinogen fragments, 6-AHA or Pg respectively. The replots of these data in Fig. 1(b)–3(b) demonstrate that CNBr-derived fibrinogen fragments completely abolish inhibition, whereas 6-AHA and Pg decrease the rate of the reaction by 10- and 5-fold respectively. The modulation constants, α and K_{mo} , for these modulators are listed in Table 1. The addition of Lp(a) alone did not effect the rate of the inhibitors (results not shown).

Effect of Lp(a) on fibrinogen-fragment-modulated plasmin inhibition of α_2 AP

The rate of plasmin inhibition by α_2 AP in the presence of CNBr-derived fibrinogen fragments and various concentrations of Lp(a) was determined as described above. Fig. 4 demonstrates that whereas the rate of plasmin inhibition is slowed in the presence of CNBr-derived fibrinogen fragments, it is increased significantly by the addition of increasing concentrations of Lp(a). Lp(a) increases the rate of the inhibition towards the unmodulated rate. The dissociation constant, K_{su} , of Lp(a) in this reaction was calculated as described above and is listed in Table 1.

Effect of CNBr-derived fibrinogen fragments on t-PA inhibition by α_2 AP

The rate of t-PA inhibition by α_2 AP was determined as

Fig. 1. Kinetic analysis of the effects of CNBr-derived fibrinogen fragments on the rate of plasmin inhibition by α_2 AP

(a) The second-order inhibition rate constant, k_2 , of plasmin inhibition by α_2 AP measured in the presence of increasing concentrations of CNBr-derived fibrinogen fragments. The values for k_2 were calculated as described in the Materials and methods section. (b) A replot of the modulation factor (β) versus reciprocal concentration of CNBr-derived fibrinogen fragments. The experiments were performed, and the values of β were calculated, as described in the Materials and methods section.

described above. The second-order inhibition rate constant of this reaction was $3.3 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is in good agreement with previously published values (Korninger & Collen, 1981). The addition of CNBr-derived fibrinogen fragments do not alter the rate of the inhibition (results not shown).

Effect of Pg on trypsin inhibition by α_2 AP

The rate of trypsin inhibition by α_2 AP was determined as described above. The second-order inhibition rate constant of this reaction was $3.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is in good agreement with previously published values (Wiman & Collen, 1978). The addition of Pg did not alter the rate of the inhibition (results not shown).

DISCUSSION

α_2 AP is the primary inhibitor of plasmin formed in the plasma (for a review, see Lijnen & Collen, 1986). The inhibition is rapid and involves an interaction with both the catalytic domain of plasmin, and the N-terminal regulatory kringle domains as well. Modulation of the initial interaction between these kringles and the inhibitor may be important in the regulation of fibrinolytic

Fig. 2. Kinetic analysis of the effects of 6-AHA on the rate of plasmin inhibition by α_2AP

(a) The second-order inhibition rate constant (k_2) of plasmin inhibition by α_2AP measured in the presence of increasing concentrations of 6-AHA. The values for k_2 were calculated as described in the Materials and methods section. (b) A replot of the modulation factor (β) versus reciprocal concentration of 6-AHA. The experiments were performed and the values of β were calculated as described in the Materials and methods section.

Fig. 3. Kinetic analysis of the effects of plasminogen on the rate of plasmin inhibition by α_2AP

(a) The second-order inhibition rate constant (k_2) of plasmin inhibition by α_2AP measured in the presence of increasing concentrations of Pg. The values for k_2 were calculated as described in the Materials and methods section. (b) A replot of the modulation factor (β) versus reciprocal concentration of Pg. The experiments were performed and the values of β were calculated as described in the Materials and methods section.

activity in the vasculature by the presence of fibrin(ogen) degradation fragments, Pg and Lp(a).

Several previous studies have demonstrated that modulators which block the interaction between the kringle and α_2AP will decrease the rate of the inhibition (Wiman & Collen, 1978; Wiman *et al.*, 1978, 1979; Christensen & Clemmensen, 1978; Anonick & Gonias, 1991). The present study demonstrates that the rate of plasmin inhibition by α_2AP is decreased by CNBr-derived fibrinogen fragments, 6-AHA and Pg. But unlike the previous studies the present study employs new kinetic analyses which allow the determination of the absolute rate of the modulated inhibition. Kinetic analysis shows that CNBr-derived fibrinogen fragments can competitively prevent the inhibition, whereas 6-AHA and Pg only decrease the rate of inhibition. The observed decreases in the rate of inhibition may result from the prevention of a kringle-inhibitor interaction. Kinetic analysis of the effects of various vascular components on the rate of the inhibition allows not only the evaluation of the kinetic contribution to this interaction to the rate of the inhibition, but also allows the formulation of a model of plasmin inhibition in the vasculature.

The kinetic contribution of this plasmin kringle- α_2AP interaction to the rate of the inhibition is calculated from the modulation constants of 6-AHA and Pg, which bind to the

Table 1. Modulation constants of plasmin inhibition by α_2AP

α is the change in the k_2 when the modulator is bound to either the enzyme or the inhibitor; K_{Mo} is the dissociation constant of the modulator from either the enzyme or the inhibitor; K_{Su} is the dissociation constant of the suppressor from the modulator.

Modulator suppressor	α	K_{Mo}	K_{Su}
CNBr-derived fibrinogen fragments	0 ± 0.002	$2.0 \pm 0.1 \mu g/ml$	—
6-AHA	0.1 ± 0.002	$120 \pm 6 \mu M$	—
Plasminogen	0.2 ± 0.01	$0.5 \pm 0.03 nM$	—
Lp(a)*	—	—	$4.0 \pm 1.0 nM$

* In the presence of CNBr-derived fibrinogen fragments.

kringle domains of plasmin and the kringle-binding site of α_2AP respectively. The initial interaction in plasmin inhibition involves a reversible binding between the enzyme and the inhibitor and thereby contributes to the kinetics of the initial reversible inhibition step (K_i). The results of the 6-AHA- and Pg-mediated prevention of this binding decrease the inhibition rate by approx.

Fig. 4. Kinetic analysis of the effects of Lp(a) on the rate of CNBr-derived fibrinogen-fragment-moulded plasmin inhibition by α_2 AP

The second-order inhibition rate constant (k_2) of plasmin inhibition by α_2 AP measured in the presence of a constant concentration of CNBr-derived fibrinogen fragments (2.0 μ g/ml) and increasing concentrations of Lp(a) in nM. The experiments were performed and the values for k_2 were calculated as described in the Materials and methods section.

10-fold, therefore decreasing the initial affinity of the inhibitor for the enzyme by 10-fold. The decrease in affinity of α_2 AP for plasmin increases the initial K_i of the reaction.

The affinity of α_2 AP for the kringles of plasmin is reflected by the unmodulated K_i of the inhibition. The binding constant of this interaction between the kringles and the inhibitor can be calculated from the kinetics of the reaction in the presence of Pg. Pg decreases the rate of plasmin inhibition with a modulation constant (equivalent to a binding constant to α_2 AP) of 5.0×10^{-10} M, representing the strength of the kringles-inhibitor interaction. Using these values of the reversible binding constants, the rate of the second, irreversible, step is calculated as 7.0×10^{-3} s⁻¹. These values for the unmodulated reaction are in good agreement with those of previous kinetic studies (Wiman & Collen, 1978; Longstaff & Gaffney, 1991), which further demonstrates that the interaction between the plasmin kringles and the α_2 AP kringles binding site accounts for the affinity of the reversible phase of the inhibition reaction.

In the absence of the kringles-inhibitor interaction, the reversible binding constant of the inhibition is decreased approx. 10-fold to 5.0×10^{-9} M. This initial lower-affinity interaction between the enzyme and the inhibitor most likely involves the binding of the inhibitor to the catalytic domain of plasmin. Such binding must be involved in the rapid α_2 AP-mediated inhibition of trypsin, which contains only a catalytic domain. Moreover, the rate of trypsin inhibition is almost five times lower than that of plasmin inhibition, and trypsin inhibition is unaffected by the presence of Pg. This further suggests that the kringles interaction is the initial reversible step in the inhibition of plasmin, but that, in the absence of this interaction, the inhibitor initially reversibly binds to the catalytic domain of the enzyme.

Kringles interactions are probably also involved in the fibrinogen-fragment-mediated decrease in the rate of plasmin inhibition. But unlike the effects of 6-AHA and Pg, CNBr-derived fibrinogen fragments completely inhibit the reaction. As previously determined in the presence of Pg and 6-AHA, the binding of plasmin kringles domains to CNBr-derived fibrinogen fragments decreases the rate of inhibition by 5–10-fold. However, since the inhibition is totally blocked, there must be an additional mechanism for modulation by CNBr-derived fibrinogen fragments. This mechanism is probably a steric effect of the CNBr-derived fibrinogen fragments on the plasmin. CNBr-

derived fibrinogen fragments may block the bound enzyme from interacting with the inhibitor through non-kringle-dependent binding sites.

The initial interaction between α_2 AP and the kringles of plasmin may involve one of the first three lysine-binding sites of the enzyme. Previous studies demonstrated that α_2 AP binds the kringles of Pg and that the domains 1–3 are preferentially involved (Christensen & Clemmensen, 1978; Wiman *et al.*, 1979; Sugiyama *et al.*, 1988). More recent studies by Sugiyama *et al.* (1988) demonstrate that tryptic fragments of the C-terminus of α_2 AP have a higher affinity for the Pg kringles 1–3 domains than for the kringles 4 domain. In addition, the 6-AHA kinetic modulation experiments further suggest that the lysine-binding site of the first kringles of plasmin is involved in the initial reaction with α_2 AP. 6-AHA binds to plasmin with a dissociation constant of 120 μ M, a binding constant consistent with 6-AHA binding to the Pg kringles 1 (Markus *et al.*, 1978). Furthermore, Lp(a) contains only kringles 4 and 5 and does not on its own affect the rate of the inhibition. From these data we suggest that the reaction between plasmin and α_2 AP is mediated by an initial interaction between the kringles-1 domain of the enzyme and the kringles-binding site of the inhibitor.

This model of the initial interaction of the α_2 AP with plasmin is consistent with the results of the t-PA-inhibition experiment. t-PA contains N-terminal domains that show a high degree of sequence identity with the first two kringles of Pg. The sequence of the t-PA kringles suggests that both should confer fibrin specificity, but only the second domain contains an intact lysine-binding site (Van Zonneveld *et al.*, 1986a,b; Verheijen *et al.*, 1986). A possible result of the absence of a functionally homologous first kringles is the low rate of t-PA inhibition by α_2 AP. Furthermore, experiments with CNBr-derived fibrinogen fragments demonstrate that the intact second kringles is not involved in the inhibition reaction. The binding of t-PA kringles 2 to CNBr-derived fibrinogen fragments does not further decrease the rate of the slow inhibition. These experiments also suggest that, unlike plasmin, the t-PA catalytic domain does not interact with the CNBr-derived fibrinogen fragments and therefore is available for inhibition by α_2 AP as well as activation of Pg.

The present kinetic findings demonstrate that the regulation of the inhibitor-kringles 1 interaction in the circulation may be important in the control of fibrinolysis. The concentrations of Pg and α_2 AP in the plasma are 2 μ M and 1 μ M respectively (Lijnen & Collen, 1986). On the basis of the dissociation constant of Pg for α_2 AP, most of the inhibitor in the plasma would circulate in complex with Pg. This interaction would decrease the rate of plasmin inhibition by almost 10-fold. However, when Pg binds to fibrin and is converted into plasmin by t-PA, α_2 AP would be released from the Pg kringles, freeing it to be a 'faster' inhibitor of plasmin. When α_2 AP circulates with Pg, the inhibitor is not as effective and the zymogen is not active. The regulation of plasmin activity is tightly controlled by the kringles-inhibitor interaction. Plasmin lyses the fibrin and is protected from inhibition while bound to the clot, but if dissociated from the clot the enzyme is rapidly inhibited by the free inhibitor.

The role of Lp(a) in the regulation of plasmin inhibition is equally interesting. Lp(a) has no direct activity on the rate of plasmin inhibition, but in the presence of CNBr-derived fibrinogen fragments Lp(a) increases the rate of the inhibition. The kinetics demonstrate that Lp(a) acts as a competitive suppressor of fibrinogen-fragment-modulated inhibition. Lp(a) competes with plasmin for binding sites on the CNBr-derived fibrinogen fragments, resulting in plasmin dissociation from the fragments followed by inhibition by α_2 AP. These results suggest that, in the vasculature, Lp(a) may promote the premature inhibition of fibrinolysis by promoting plasmin inhibition.

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