Inhibition of plasmin by fibrinogen

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The kinetics of inhibition of the amidolytic activity of plasmin on D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) by fibrinogen and fibrin were determined. Reciprocal ($1/v$ versus $1/[S]$) plots of plasmin inhibition by $0.50\ \mu\text{M}$ -fibrinogen showed a non-linear downward curve. The Hill coefficient (h) was 0.68, suggesting negative co-operativity. By contrast, fibrin produced a simple competitive inhibition of plasmin ($K_i = 12\ \mu\text{g/ml}$). Addition of $0.1\ \text{mM}$ -6-aminohexanoic acid shifted the non-linear curve obtained in the presence of fibrinogen to a straight line as for controls, indicating that 6-aminohexanoic acid abolishes the fibrinogen-induced inhibition. Transient exposure of the enzyme to pH 1.0 abrogates the ability of fibrinogen to inhibit plasmin activity. Acidification had no effect on the V_{max} , but increased the K_m of plasmin. The present evidence for modulation of plasmin reveals a novel mechanism for control of fibrinolysis by fibrinogen, a component of the coagulation system and the precursor of the physiological substrate of plasmin.

INTRODUCTION

The fibrinolytic process, mediated through the plasminogen activation cascade, is under stringent metabolic control. Accordingly, extracellular plasminogen-activator activity is regulated at various stages, including the level of biosynthesis, secretion from producer cells, activation of inactive proenzyme forms, and interaction of different enzyme forms with activators and inhibitors [1]. The plasminogen activation cascade culminates in formation of plasmin. The non-specific and extremely potent serine protease plasmin (EC 3.4.21.7) fulfils pivotal functions in thrombolysis and fibrinolysis [2,3]. In addition to its major function as a fibrin-cleaving enzyme, plasmin participates in a variety of specific and limited proteolytic reactions involved in the processing of several intermediates of the fibrinolytic mechanism. For example, plasmin converts Glu-plasminogen into Lys-plasminogen, which is a preferable substrate for plasminogen activators that transform plasminogen to plasmin [3–5]. Plasmin activates pro-urokinase to active urokinase [6–8] and converts high-molecular-mass urokinase into low-molecular-mass urokinase [5,9]. It also re-activates complexes of plasminogen activators formed with their specific inhibitors, such as plasminogen-activator inhibitor type-1 [10]. Plasmin activity is subject to regulation through inhibition by several circulating inhibitors, and in particular the irreversible inhibitor α_2 -antiplasmin [11–13]. The interaction between plasmin and α_2 -antiplasmin is impaired by fibrinogen and certain degradation products of fibrinogen [11].

The plasmin molecule consists of two polypeptide chains; the heavy chain (A) contains five homologous triple-loop structures (kringles) in which lysine-binding sites (LBS) are located [5,11,14]. These LBS mediate the interaction of plasmin with fibrin, α_2 -antiplasmin, fibrinogen and synthetic amino acids such as 6-aminohexanoic acid (AHA) and tranexamic acid [3,11,14,15]. The second polypeptide is a 25 kDa light chain (B) enclosing the catalytic site of the enzyme. The amino acid sequence of the B chain closely resembles that of other serine proteases, e.g. thrombin, trypsin, chymotrypsin and elastase [16].

It is well known that binding of ligands to LBS in plasmin or in its parent molecule plasminogen induces conformational changes in these proteins [15,17–22]. Until recently the functional significance of the conformational changes in plasmin, e.g. their

potential effect on the active site of the enzyme, was unknown. We have recently reported that interaction of ligands such as ampicillin with LBS in plasmin affects the function of the catalytic site [23]. Similarly, Scully *et al.* [24] found changes in plasmin activity as a consequence of interaction of pro-urokinase with LBS in the plasmin molecule.

In the present study we show that plasmin activity is regulated by fibrinogen via a regulatory site involving LBS.

MATERIALS AND METHODS

Materials

The chromogenic substrate D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) and plasmin were obtained from Kabi Diagnostics, Stockholm, Sweden. The commercial Kabi plasmin was produced by activation of human Glu-plasminogen in the presence of AHA. By polyacrylamide-gel electrophoresis we confirmed that this preparation consists of a single protein of $\sim 80\ \text{kDa}$, corresponding to Lys-plasmin. Fibrinogen (plasminogen-free) from bovine blood was obtained from Miles Scientific, Naperville, IL, U.S.A. Washed fibrin from bovine blood and AHA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The fibrinogen and fibrin were dissolved by layering the protein powders on phosphate-buffered saline, pH 7.4. After an overnight incubation at room temperature without agitation, the mixture was centrifuged at $3000\ g$ for 10 min. Protein concentration in the clear supernatant solution was determined, and this solution was taken for the experiments. Electrophoresis of the fibrinogen on a 5%-polyacrylamide gel without SDS gave the expected single protein band, indicating the purity of the protein solution. To rule out possible contamination by small molecules, the experiments were also repeated with fibrinogen and fibrin dialysed against phosphate-buffered saline in dialysis bags permitting diffusion of molecules of $< 10\ \text{kDa}$. The undialysed and dialysed proteins had the same effects in all experiments.

Methods

The amidolytic activity of plasmin was assayed by measuring the cleavage of S-2251 to form the chromogen *p*-nitroaniline. In the standard assay, 0.006 casein unit [25] of plasmin was incubated in $225\ \mu\text{l}$ of phosphate-buffered saline, pH 7.4, con-

Abbreviations used: LBS, lysine-binding site; AHA, 6-aminohexanoic acid.

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taining different concentrations of the substrate as indicated, and 25 μ l of polyethylene glycol (5 g/l; average $\sim$ 6 kDa). The rate of change of A_{405} was monitored with a Diagnostics Pasteur LP-200 plate reader. The reaction was followed for up to 10 min, and the rates were calculated for the linear portion of the activity curves. Fibrinogen or fibrin was added to the reaction mixture at concentrations as indicated in the appropriate legends.

Human plasmin solution was prepared by dissolving the enzyme in 50% (v/v) glycerol in 2.0 mM-HCl containing 5 g of polyethylene glycol (average $\sim$ 6 kDa)/l, as recommended by the manufacturer. Acidification of the enzyme was accomplished by increasing the HCl concentration of the dissolving solution to 194 mM to give pH 1.0. After 60 min at room temperature, the acidified enzyme was added to the assay mixture for determination of its activity. The buffering capacity in the reaction mixture was sufficient to revert the pH to 7.4.

RESULTS

In the present study the chromogenic substrate S-2251 was utilized to measure plasmin activity. The assay was performed with a plasmin standard of 0.025 casein unit/ml and at a S-2251 concentration equal to the K_m , i.e. 0.147 mM, unless specified otherwise. Under these conditions the reaction rate is 30.5 nmol/min. The effect of addition of increasing concentrations of fibrinogen on this activity was studied in the experiment shown in Fig. 1. Fibrinogen produced a concentration-dependent inhibition of plasmin activity. Half-maximal inhibition of the amidolytic activity of the enzyme was achieved at a fibrinogen concentration of 0.232 mg/ml (0.58 μ M). Fibrin similarly inhibited plasmin, with a half-maximal inhibition at 0.100 mg of fibrin/ml.

The amino acid analogue AHA interacts with LBS in several proteins such as plasmin and plasminogen [3,11,12,26]. In a previous study we have noted that AHA impedes the inhibition of plasmin by the β -lactam antibiotic ampicillin [23]. To establish whether the fibrinogen-induced inhibition of the enzyme involves interaction with LBS, the effect of AHA on plasmin inhibition by fibrinogen was determined. Plasmin activity was measured at $[S] = K_m$, with increasing concentrations of AHA and in the presence of a fibrinogen concentration known to produce 50% inhibition of the activity. As seen in Fig. 2, the inhibition of the

Fig. 1. Effect of fibrinogen and fibrin on the amidolytic activity of plasmin

Increasing concentrations of fibrinogen ($\circ$) and fibrin (Δ) were added to the standard assay of plasmin activity with substrate S-2251. Substrate concentration was 0.147 mM, equal to the K_m value. Activity is given as a percentage, taking 30.5 nmol/min as 100%. Means $\pm$ S.E.M. of triplicate determinations are shown.

Fig. 2. Effect of AHA on plasmin activity

Increasing concentrations of AHA were added to a standard assay of plasmin-mediated amidolysis in the absence ($\circ$) or presence (Δ) of 0.58 μ M-fibrinogen. Substrate concentration was 0.147 mM, equal to the K_m . Activity (means $\pm$ S.E.M.) is given as a percentage, taking 30.5 nmol/min as 100%.

amidolytic activity by fibrinogen was relieved by increasing the concentration of AHA in the assay mixture. The inhibitory effect of fibrinogen was completely abolished at approx. 0.06 mM-AHA. In the absence of fibrinogen, AHA had a small stimulatory effect, causing a 10% increase in the amidolytic activity of plasmin. A similar stimulation of plasmin activity in the absence of fibrinogen and a similar ability to prevent the fibrinogen effect was also noted with lysine in the same concentration range (results not shown). In contrast, glutamic acid, even up to 2 mM, did not affect the inhibition of plasmin by fibrinogen. Thus the effect of fibrinogen is highly likely to involve interaction with the same LBSs that bind AHA or lysine but do not interact with glutamic acid.

To assess further the possibility that fibrinogen inhibits plasmin through interaction at a site different from the catalytic site, plasmin activity was assayed at increasing concentrations of the chromogenic substrate. Fig. 3 is a double-reciprocal plot of the experimental data, showing that a straight line is obtained for plasmin activity in a control experiment conducted in the absence

Fig. 3. Lineweaver-Burk plot of inhibition of plasmin by fibrinogen

Plasmin activity on substrate S-2251 was assayed under the following conditions: $\circ$, no additions; $\bullet$, fibrinogen (0.58 μ M); $\square$, AHA (0.1 mM); ∇ , fibrinogen and AHA at the above concentrations. The experiment was repeated three times, and results of a representative experiment are given.

Fig. 4. Lineweaver-Burk plot of inhibition of plasmin by fibrin

Plasmin activity on substrate S-2251 was assayed in the absence (○) or presence (Δ) of fibrin (0.14 mg/ml). The experiment was repeated three times, and results of a representative experiment are given.

of fibrinogen. However, when $0.58 \mu\text{M}$ -fibrinogen was added, a downward curvature of the Lineweaver-Burk plot was obtained. The non-linear curve suggests existence of negative co-operativity. Calculation of the Hill coefficient from the experiment shown in Fig. 3 gave a value of $h = 0.68$. Fig. 3 also demonstrates that, on addition of 0.1 mM -AHA, the effect of fibrinogen was abolished and the double-reciprocal plot approached that obtained for the fibrinogen-free control. The level of fibrinogen used in this experiment was below that needed for competitive inhibition of the chromogenic activity, since the K_i for inhibition by fibrinogen under similar conditions has been shown to be $5.4 \mu\text{M}$ [11]. When AHA was added in the absence of fibrinogen, the line that was obtained was superimposable on the control line. Thus, although AHA alone had no significant effect on plasmin activity, it prevented the inhibition of the enzyme by fibrinogen.

Fibrin, the physiological substrate of plasmin, resembles fibrinogen in its ability to bind to the active site of the enzyme as

Fig. 5. Effect of fibrinogen and fibrin on plasmin after treatment of the enzyme at pH 1.0

Plasmin was transiently exposed to pH 1.0 as described in the Materials and methods section. Increasing concentrations of fibrinogen (○) or fibrin (Δ) were added to the standard assay of plasmin activity with substrate S-2251. Substrate concentration was 0.147 mM . Activity is given as a percentage, taking 15.0 nmol/min as 100%. Means $\pm$ S.E.M. of triplicate determinations are given.

Fig. 6. Lineweaver-Burk plot of plasmin after treatment of the enzyme at pH 1.0

Plasmin was transiently exposed to pH 1.0 as described in the Materials and methods section. Activity on S-2251 was assayed in the absence (○) or presence (Δ) of $0.58 \mu\text{M}$ -fibrinogen. The experiment was repeated three times, and values from a representative experiment are given.

well as to LBS [14]. Fig. 4 depicts a Lineweaver-Burk plot showing the inhibitory effect of fibrin on the chromogenic activity of plasmin. In the presence of fibrin a straight line is obtained, intersecting with the $1/v$ axis at about the same V_{max} value as for the control in the absence of fibrin. A K_i value of $12 \mu\text{g/ml}$ was obtained for the inhibition by fibrin. Hence fibrin is a simple competitive inhibitor of plasmin, and does not seem to affect the enzyme at additional site(s).

The present experimental evidence as well as previous studies [23,24] suggest the existence of separate catalytic and regulatory sites on plasmin. It might therefore be possible to affect these distinct sites in a differential manner. Towards this end, we tested the effect of transient acidification of the enzyme on its catalytic function and on its amenability to regulation. Exposure of plasmin to pH 1.0 for 1 h before its assay at pH 7.4 results in a 50% decrease in enzyme activity as compared with the non-acid-treated enzyme control: the acid-treated enzyme displayed activity of 15.0 nmol/min , whereas that of non-acid-treated control enzyme was 30.5 nmol/min . This inhibition was observed at a substrate concentration equal to the K_m value of intact plasmin. As seen in Fig. 5, acid treatment notably changed the response of plasmin to fibrinogen, since no inhibition of enzyme activity was observed by fibrinogen at levels of up to 0.5 mg/ml ($1.25 \mu\text{M}$). Rather, a small stimulatory effect of fibrinogen was noted on the acid-treated enzyme. Similar concentrations of fibrinogen were inhibitory to untreated plasmin (cf. Fig. 1). Thus acidification eliminated the fibrinogen-induced inhibition of plasmin. By contrast, acidification did not impair the inhibitory effect of fibrin, indicating that inhibition by fibrin involves a different mechanism.

The effect of fibrinogen on the acid-treated enzyme is further shown in the double-reciprocal plot depicted in Fig. 6. Regardless of the absence or presence of fibrinogen, the acid-treated enzyme presented a V_{max} value close to that obtained with the untreated enzyme (cf. Fig. 3). Thus fibrinogen fails to inhibit plasmin that was transiently exposed to pH 1.0. In other experiments we noted that it is also possible to interfere with the catalytic activity without impairment of the regulatory function. For example, when plasmin activity was assayed at pH 6.3, the catalytic activity was decreased by 50% (at $[S] = K_m$), fibrinogen was inhibitory to the enzyme, and AHA was still able to abolish this inhibition (results not shown).

DISCUSSION

The observations in the present study indicate that plasmin activity is negatively controlled by fibrinogen (Figs. 2 and 3). As further shown in Fig. 2, the fibrinogen-induced inhibition can be totally abolished by AHA, suggesting that fibrinogen mediates its effect through binding to LBS. These sites are located in the heavy chain of plasmin, whereas the catalytic site is in the light chain of the enzyme [14,16]. The present findings are in accord with our previous report that certain ligands such as the β -lactam antibiotic ampicillin interact with plasmin at a regulatory site which contains a lysine-binding moiety and is clearly distinguished from the active site [23].

The downward curvature of the Lineweaver-Burk plot in the presence of fibrinogen (Fig. 3) suggests negative co-operativity. Co-operative phenomena were also recently reported in kinetic analysis of plasmin activity [23,24].

The effects of AHA suggest interaction between different sites of plasmin: AHA completely abolishes the fibrinogen-mediated inhibition throughout the whole range of substrate concentrations tested. It also reverts the curved Lineweaver-Burk plot in the presence of fibrinogen to a straight line overlapping the control line. AHA alone has no significant effect on plasmin activity.

Plasmin loses its susceptibility to inhibition by fibrinogen as a consequence of a transient acidification at pH 1.0 (Fig. 5). Kinetic analysis of plasmin activity after this acidification revealed that the catalytic site of the enzyme continued to function with an approximately similar $V_{\max}$, albeit with an increased K_m (compare Fig. 6 with Fig. 3). The ability to obliterate the regulatory function with only minimal interference with the catalytic activity is in line with the localization of these domains to distinct and distant sites. The two domains exhibit different characteristics and can be modified independently. Although treatment at pH 1.0 abolished regulation, assay of activity at pH 6.3 mainly impaired catalysis, with minimal effect on the regulatory function.

A plasmin oligomer, with each monomer having a regulatory and a catalytic site, could produce the results observed here. However, the existence of plasmin in forms exhibiting higher molecular masses was never documented. An alternative potential mechanism could be based on mutual interactions between a regulatory and a catalytic site in the known plasmin molecule. Such interactions would explain both the negative and the positive co-operativity phenomena that were observed with plasmin: both phenomena have in common binding of a regulator to the regulatory site resulting in suppression of the catalytic activity. For negative co-operativity, such as with fibrinogen (Fig. 3) or pro-urokinase [24], binding of the substrate to the catalytic site further enhances the inhibitory action of the regulator. For positive co-operativity, such as with ampicillin polymer [23], the binding of the substrate to the catalytic site decreases the inhibitory effect exerted by the ligand at the regulatory site. Therefore, increasing substrate levels relieve the inhibition.

Regulation of plasmin by fibrinogen is likely to have important physiological implications. A potentially active reservoir of the enzyme may be continuously maintained in the blood. The suppressed enzyme will be quickly activated as soon as circulating fibrinogen is converted into fibrin. Thus clot formation relieves the inhibition of plasmin to promote fibrinolysis. This possibility is supported by the clinical presentation of a patient with α_2 -antiplasmin deficiency, haemorrhagic diathesis and a normal level of plasma fibrinogen [27]. Existence of this entity suggests that, in spite of the absence of α_2 -antiplasmin, fibrinogen is

protected from cleavage by plasmin *in vivo* by the mechanism outlined above. Furthermore, this phenomenon tacitly implies that the physiological role of α_2 -antiplasmin is to inhibit plasmin-induced clot lysis, whereas its systemic effect upon fibrinogen is limited.

The present study does not imply that fibrinogen affects the plasmin-mediated fibrinolysis under physiological conditions *in vivo*. Fibrinogen and fibrin compete on binding to the same LBS, and consequently the high local concentration of fibrin in the clot precludes an inhibitory effect of fibrinogen on clot lysis by plasmin.

Another interesting possibility is that binding of ligands to the regulatory site could protect plasmin against irreversible inactivation by inhibitors such as α_2 -antiplasmin. Indeed, fibrin as well as AHA were reported to impede irreversible inhibition of plasmin by α -antiplasmin [14]. Fibrinogen had a similar protecting effect [11]. The apparently paradoxical protecting effect of fibrinogen can now be resolved on the basis of its suppressive effect on the catalytic site.

In previous studies we have shown that certain ligands can induce non-linear kinetics of plasmin activity as a function of substrate concentration, suggesting positive co-operativity [23]. Taken together with the present results and the work of Scully *et al.* [24], these findings imply that plasmin fulfils a function of a regulatory enzyme. Enzymes possessing this property often catalyse unidirectional reactions, and plasmin-catalysed reactions are all irreversible.

REFERENCES

- Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S. & Skriver, L. (1985) *Adv. Cancer Res.* **44**, 139–266
- Wiman, B. & Collen, D. (1978) *Nature (London)* **272**, 549–550
- Collen, D. (1979) *Thromb. Haemostasis* **41**, 77–89
- Violand, B. D. & Castellino, F. J. (1976) *J. Biol. Chem.* **251**, 3906–3912
- Saksela, O. (1985) *Biochim. Biophys. Acta* **823**, 35–65
- Kasai, S., Arimura, H., Nishida, M. & Suyama, T. (1985) *J. Biol. Chem.* **260**, 12377–12381
- Pannell, R. & Gurewich, V. (1987) *Blood* **69**, 22–26
- Petersen, L. C., Lund, L. R., Nielsen, L. S., Dano, K. & Skriver, L. (1988) *J. Biol. Chem.* **263**, 11189–11195
- Barlow, G. H., Francis, C. W. & Marder, V. J. (1981) *Thromb. Res.* **23**, 541–547
- Sprengers, E. D. & Kluft, C. (1987) *Blood* **69**, 381–387
- Wiman, B., Lijnen, H. R. & Collen, D. (1979) *Biochim. Biophys. Acta* **579**, 142–154
- Wiman, B. & Collen, D. (1978) *Eur. J. Biochem.* **84**, 573–578
- Juhan-Vague, I., Aillaud, M. F. & Serradimigni, A. (1986) *Haemostasis* **16**, Suppl. 3, 16–20
- Verstraete, M. & Collen, D. (1986) *Blood* **67**, 1529–1541
- Rickli, E. E. & Otavsky, R. I. (1975) *Eur. J. Biochem.* **59**, 441–447
- Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis* **3**, 191–209
- Alkjaersig, N. (1964) *Biochem. J.* **93**, 171–183
- Brockway, W. J. & Castellino, F. J. (1971) *J. Biol. Chem.* **246**, 4641–4647
- Violand, B., Byrne, R. & Castellino, F. J. (1978) *J. Biol. Chem.* **253**, 5395–5401
- Markus, G., DePasquale, J. L. & Wissler, F. C. (1978) *J. Biol. Chem.* **253**, 727–732
- Collen, D. & De Cock, F. (1974) *Thromb. Res.* **5**, 777–779
- Collen, D. & De Cock, F. (1975) *Thromb. Res.* **7**, 235–238
- Higazi, A. & Mayer, M. (1989) *Biochem. J.* **260**, 609–612
- Scully, M. F., Ellis, V., Watahiki, Y. & Kakkar, V. V. (1989) *Arch. Biochem. Biophys.* **268**, 438–446
- Sgouris, J. T., Inman, J. K., McCall, K. B., Hyndman, L. A. & Andersen, H. D. (1960) *Vox Sang.* **5**, 357–376
- Francis, C. W. & Marder, V. J. (1986) *Annu. Rev. Med.* **37**, 187–204
- Koie, K., Kamiya, T., Ogata, K. & Takamatsu, J. (1978) *Lancet* **ii**, 1334–1336