The AH-site of plasminogen and two C-terminal fragments

A weak lysine-binding site preferring ligands not carrying a free carboxylate function

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Glu-plasminogen [native plasminogen (Glu-1–Asn-790)], Lys-plasminogen [plasmin-cleaved fragment of plasminogen (Lys-77–Asn-790)] and miniplasminogen [fragment of plasminogen (Val-440–Asn-790)] were all found to interact specifically with immobilized 6-aminohexyl ligands. The interactions apparently are mediated by a single weak lysine-binding site, termed the AH-site, as seen from the patterns of inhibition obtained from frontal-quantitative-affinity-chromatography experiments with 6-aminohexanoic acid and α -N-acetyl-L-lysine methyl ester as competing ligands. The AH-site, in contrast with the strong lysine-binding site of Glu-plasminogen and Lys-plasminogen, may prefer ligands not carrying a free carboxylate function and therefore may interact with lysine side chains of proteins. In Glu-plasminogen the AH-site is present, but is apparently only partially free to react. It is suggested that it participates in an intramolecular complex and that an equilibrium state between two Glu-plasminogen forms exists. It is further suggested that binding of the plasminogens to fibrin is mainly determined by the AH-site.

Plasmin (EC 3.4.21.7) is a serine proteinase that converts fibrin into soluble products in the last step of physiological fibrinolysis. Various ω -aminocarboxylic acid compounds analogous to L-lysine are well-known effectors of the fibrinolytic system (Alkjaersig, 1964; Muramutu *et al.*, 1965; Abiko *et al.*, 1969). Their effects apparently result from interactions with the so-called lysine-binding sites of plasminogen and plasmin, which are probably important regulatory sites of these proteins mediating several of the interactions in the main fibrinolytic reactions: conversion of plasminogen into plasmin, the actual-catalysed hydrolysis of fibrin and inhibition of plasmin by α_2 -antiplasmin (Ambrus *et al.*, 1968; Thorsen & Astrup, 1969; Landmann, 1973; Thorsen & Müllertz, 1974; Rickli & Otavsky, 1975; Christensen & Clemmensen, 1977, 1978; Wiman & Collen, 1978a,b; Christensen, 1978; Thorsen *et al.*, 1981). The presence of two or three classes of lysine-binding

sites of the plasminogens has been demonstrated (Markus *et al.*, 1978a,b), and much interest has been placed on one of these, the high-affinity or strong class of sites (Wiman & Collen, 1978a), whereas the possible importance of the other classes of lysine-binding sites has received little attention.

Native Glu-plasminogen, plasmin-cleaved Lys-plasminogen and two elastase-cleaved plasminogen fragments, Kringle 1+2+3 and Kringle 4 (Lys-77–Val-353 and Val-354–Ala-439), possess high-affinity lysine-binding sites, whereas miniplasminogen does not (Sottrup-Jensen *et al.*, 1977). But fibrin binds miniplasminogen and practically no Kringle 1+2+3 or Kringle 4. Furthermore the fibrin–miniplasminogen interaction is inhibited by ω -aminocarboxylic acids (Thorsen *et al.*, 1981; Suenson & Thorsen, 1981).

Clearly the presence of a weak(er) lysine-binding site in the miniplasminogen part of the plasminogen molecule, perhaps of great importance for the regulation of fibrinolysis, is indicated.

In the present paper I report some experiments designed to investigate that possibility. The ligand-specificity of the weak(er) lysine-binding site of the plasminogens was expected to differ from, but show some resemblance to, that of the strong

Abbreviations used: Glu-plasminogen, native-plasminogen (Glu-1–Asn-790); Lys-plasminogen, plasmin-cleaved fragment of plasminogen (Lys-77–Asn-790); miniplasminogen, fragment of plasminogen (Val-440–Asn-790); AH-Sepharose, 6-aminohexyl-substituted Sepharose 4B; f.q.a.c., frontal quantitative affinity chromatography.

lysine-binding site, as indeed indicated by the different affinities for ω -aminocarboxylic acids (Markus *et al.*, 1978a,b). Furthermore a normal amino acid side chain might fulfil the structural requirements and be a ligand, particular if the weak(er) lysine-binding site plays a role in protein-protein interactions. Because of its content of 6-aminoheptyl groups analogous to lysyl side chains, AH-Sepharose was considered a potential affinity adsorbent of the plasminogens, and the interactions of Glu-plasminogen, Lys-plasminogen and miniplasminogen with AH-Sepharose were investigated. It was found that the plasminogens all interact strongly with AH-Sepharose, but Lys-plasminogen and miniplasminogen more strongly than Glu-plasminogen. That is the same pattern as obtained in fibrin-binding studies (Thorsen *et al.*, 1981; Suenson & Thorsen, 1981).

The dissociation constants of the AH-Sepharose-plasminogen complexes obtained from f.q.a.c. experiments in the presence and in the absence of competing ligands, 6-aminohexanoic acid or α -N-acetyl-L-lysine methyl ester, showed that the interactions involve a weak lysine-binding site that binds 6-aminohexanoic acid with K_i approx. 0.3 mM, which I designate the aminohexyl or AH-site. It is apparently only partially free to react in Glu-plasminogen, which may possibly be due to an equilibrium state involving internal occupation of it. The results further show that lysine compounds not carrying a free carboxylate function are bound more tightly than 6-aminohexanoic acid at the AH-site. A new model of fibrinolysis that fits the results presented here and is compatible with other known features of the fibrinolytic system is presented. It is suggested that the AH-site, a weak lysine-binding site in the miniplasminogen part of the plasminogen molecule, is important for the initial steps of fibrinolysis, whereas the strong lysine-binding site may play a role later, perhaps facilitating the movement of plasmin on partially degraded fibrin, when the degradation has resulted in the formation of C-terminal lysine residues, which, as I see it, are the only likely ligands known to be present physiologically that answer exactly to the apparent ligand-structure requirements of the strong lysine-binding site.

Materials and methods

AH-Sepharose

AH-Sepharose was prepared by coupling of 1,6-diaminohexane to CNBr-activated Sepharose 4B. Only 25 mg of CNBr/ml of Sepharose 4B was used in the activation step, and coupling was performed at pH 9 at 10°C with 1 M-1,6-diaminohexane; this gave a rather low yield of coupled ligand but was done in order possibly to decrease the number of

ineffective ligands and also lower the probability of two-point attachments of the hexane (Parikh *et al.*, 1974). Estimates of the concentration of immobilized ligand were obtained from potentiometric titrations with HCl after addition of excess of NaOH, assuming that each immobilized ligand contained two basic groups, the isourea-coupling group and the 6-amino group. The approximate concentration of effective 6-aminoheptyl ligands thus obtained was 1.1 μ mol/ml of gel.

Proteins and other materials

Glu-plasminogen and Lys-plasminogen were prepared essentially as described by Deutsch & Mertz (1970), by the use of affinity chromatography on lysine-substituted Sepharose 4B. But, to obtain Glu-plasminogen, the eluate of the affinity column was passed through a Trasyol-substituted Sepharose 4B column, then gel-filtered on Sephadex G-25 to remove 6-aminohexanoic acid, and finally, after ultrafiltration to a suitable concentration, subjected to ion-exchange chromatography of DEAE-Sephadex A-50, whereas, to obtain Lys-plasminogen, plasmin was added to the eluate and that mixture left overnight at 4°C before the Trasyol-substituted Sepharose 4B column step. Miniplasminogen was prepared from Lys-plasminogen as described by Sottrup-Jensen *et al.* (1977).

The following materials were obtained from the indicated commercial sources: α -N-acetyl-L-lysine methyl ester (Sigma Chemical Co., St. Louis, MO, U.S.A.), 6-aminohexanoic acid (Fluka, Buchs, Switzerland), S-2251 (D-valyl-L-leucyl-L-lysine *p*-nitroanilide) (Kabi, Stockholm, Sweden), human serum albumin (Behringwerke, Marburg, Germany) and urokinase (Leo, Ballerup, Denmark).

F.q.a.c.

The method described by Kasai & Ishii (1978) was used. Affinity chromatography of Glu-plasminogen, Lys-plasminogen and miniplasminogen was performed with AH-Sepharose as affinity adsorbent and 6-aminohexanoic acid and N-acetyl-lysine methyl ester as competing ligands. A solution of the plasminogen in question (approx. 500 ml, 0.5 μ M) in buffer, where appropriate also containing the competing ligand at a desired concentration, was continuously added (flow rate 25 ml/h or 5 ml/h) to a column (0.9 cm $\times$ 10 cm, volume 6.4 ml) of AH-Sepharose. The column was kept at 22°C, whereas the bulk of material to be applied and the eluate was at 4°C. The buffer used was 0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.6, also containing 0.2 M-NaCl to avoid ion-exchange effects. The elution patterns were determined by measuring the concentration of plasminogen in each 0.5 ml or 0.25 ml fraction collected in an assay with S-2251 as substrate after addition of urokinase

and 6-aminohexanoic acid to a final concentration of 25 mM. None of the plasminogens were adsorbed on pure Sepharose 4B, and in the presence of 0.2 M-6-aminohexanoic acid they all showed an elution position similar to that of human serum albumin, which corresponded well to the bed volume of the AH-Sepharose column.

Calculations

The area corresponding to adsorbed plasminogen of each measured elution pattern was calculated as the sum of the plasminogen lacking in each of the eluted fractions compared with the amount of plasminogen in each eluted fraction in the presence of 0.2 M-6-aminohexanoic acid (area hatched in Fig. 1). A_0 is the area obtained in the absence of competing ligand and A_i areas are those obtained in the presence of a competing ligand at concentration i . K_i values were obtained assuming $A_0/A_i = 1 + i/K_i$ from a best fit of the data presented in Figs. 3 and 4.

Results

The f.q.a.c. method described by Kasai & Ishii (1978) for the determination of dissociation constants of protein-ligand complexes was used. The principle of that method is as follows. A solution of protein is continuously added to an affinity column. The protein is bound to the immobilized ligand, and thus the protein content of the eluate is less than that of the solution added until equilibrium prevails within the column. From the amount of protein bound in various conditions, it is possible to determine, for example, the dissociation constants of protein-soluble ligand complexes as outlined in Fig. 1.

The elution patterns of Glu-plasminogen, Lys-plasminogen and miniplasminogen obtained from a column of AH-Sepharose in the presence and in the absence of 6-aminohexanoic acid and α -N-acetyl-L-lysine methyl ester with the use of f.q.a.c. are shown in Fig. 2. It is seen that Glu-plasminogen, Lys-plasminogen and miniplasminogen all interact strongly with AH-Sepharose and that both 6-aminohexanoic acid and N-acetyl-lysine methyl ester inhibit these interactions. At the experimental conditions used (0.1 M-NaH₂PO₄/Na₂HPO₄, pH 7.6, containing 0.2 M-NaCl) none of the plasminogens was adsorbed on normal cationic ion-exchangers. It therefore seems justified to assume that only specific binding to AH-Sepharose occurs.

Plots of the ratio of the molar amounts of protein immobilized at equilibrium in the absence (A_0) and in the presence (A_i) of inhibitor, A_0/A_i , versus the concentration of the inhibitor, i , are shown in Figs. 3 and 4. N-Acetyl-lysine methyl ester is seen

Fig. 1. Principle of the f.q.a.c. method for the determination of dissociation constants, K_i , of protein-soluble ligand complexes

To an affinity column containing an immobilized ligand, L (molar amount, l), is continuously added a protein solution (concentration, p_0) also containing a soluble competing ligand (or inhibitor), I (concentration, i). Until equilibrium prevails more and more protein is being bound to the column and the protein concentration of the eluate is less than p_0 . At equilibrium no more protein is being bound and the concentration of unbound protein equals p_0 in the column and in the eluate. The molar amount of bound protein at equilibrium is determined from that lacking in the eluate. It is equal to the area A_i hatched in the Figure. The curve (—) is obtained in the presence of I at a concentration low enough to only partially compete for the immobilization of the protein (e.g. $i = 0$) and the curve (----) is one obtained in the presence of I at a concentration sufficiently high to inhibit totally (99–99.9%) the immobilization of the protein, so that the elution pattern (----) equals that of a protein with no affinity to the column. An area A_i (volume times concentration) is thus the molar amount of protein bound at equilibrium in the presence of inhibitor at the concentration i . In particular A_0 is the amount bound in the absence of inhibitor, $i = 0$. The dissociation constant of the protein-immobilized ligand complex is:

$$K_i = \frac{p_0(l - A_i)}{\left(1 + \frac{i}{K_i}\right)A_i}$$

where K_i is the dissociation constant of the protein-soluble ligand (inhibitor) complex. If A_0 and A_i ($< A_0$) are negligibly small compared with l , $A_0/A_i = (l - A_0)(1 + i/K_i)/(l - A_i) = (1 + i/k_i)$, so that K_i may be determined from a number of areas (molar amounts of bound protein) A_0 and A_i obtained at various i values.

to be a simple competitive inhibitor of all of the AH-Sepharose-plasminogen interactions. Linear competitive patterns are obtained also of 6-aminohexanoic acid as the inhibitor of Lys-plasminogen and miniplasminogen binding, whereas two effects of 6-aminohexanoic acid can be distinguished on the Glu-plasminogen-AH-Sepharose interaction.

Fig. 2. Elution patterns of miniplasminogen, Lys-plasminogen and Glu-plasminogen obtained in six series of *f.q.a.c.* experiments on *AH-Sepharose*

(a) Mixtures of miniplasminogen (m-Pg) with 6-aminohexanoic acid ($H_2NC_5H_{10}CO_2H$): (1) $i = 0$; (2) $i = 0.167$ mM; (3) $i = 0.333$ mM; (4) $i = 0.6$ mM; (5) $i = 1.8$ mM; (6) $i = 200$ mM. (b) Mixtures of m-Pg with *N*-acetyl-lysine methyl ester (Ac-Lys-OMe): (1) $i = 0$; (2) $i = 0.02$ mM; (3) $i = 0.07$ mM; (4) $i = 0.28$ mM; (5) $i (H_2NC_5H_{10}CO_2H) = 200$ mM. (c) Mixtures of Lys-plasminogen (Lys-Pg) with $H_2NC_5H_{10}CO_2H$: (1) $i = 0$; (2) $i = 0.2$ mM; (3) $i = 0.4$ mM; (4) $i = 0.8$ mM; (5) $i = 2.4$ mM; (6) $i = 200$ mM. (d) Mixtures of Lys-Pg with Ac-Lys-OMe: (1) $i = 0$; (2) $i = 0.0167$ mM; (3) $i = 0.05$ mM; (4) $i = 0.02$ mM; (5) $i (H_2NC_5H_{10}CO_2H) = 200$ mM. (e) Mixtures of Glu-plasminogen (Glu-Pg) with $H_2NC_5H_{10}CO_2H$: (1) $i = 0$; (2) $i = 0.003$ mM; (3) $i = 0.03$ mM; (4) $i = 1.8$ mM; (5) $i = 5.4$ mM; (6) $i = 18$ mM; (7) $i = 200$ mM. (f) Mixtures of Glu-Pg with Ac-Lys-OMe: (1) $i = 0$; (2) $i = 0.09$ mM; (3) $i = 0.24$ mM; (4) $i = 0.72$ mM; (5) $i (H_2NC_5H_{10}CO_2H) = 200$ mM. Experiments in the presence of inhibitor at a concentration sufficiently high to inhibit totally the immobilization of the proteins were performed only with 6-aminohexanoic acid ($i = 200$ mM). The principle of the experiments is explained in Fig. 1.

The K_i values determined are summarized in Table 1. Lys-plasminogen and miniplasminogen interact equally well with the AH-Sepharose column, both using a site, the AH-site, that binds *N*-acetyl-lysine methyl ester K_i approx. $30\text{ }\mu\text{M}$ and 6-amino-hexanoic acid with K_i approx. $300\text{ }\mu\text{M}$ (Table 1). The AH-site thus is a low-affinity lysine-binding site. No effects of 6-aminohexanoic acid binding to the high-affinity lysine-binding site of Lys-plasminogen are seen (Fig. 3) ($K_i = 35\text{ }\mu\text{M}$, Markus *et al.*, 1978a). It is noteworthy that the AH-site binds the two compounds approx. 10 times more strongly than it does 6-aminohexanoic acid. In contrast with the high-affinity lysine-binding site, the AH-

site may prefer lysine-analogous compounds that do not carry a free carboxylate function.

The interaction of Glu-plasminogen with AH-Sepharose is less strong than those of Lys-plasminogen and miniplasminogen (Fig. 2). In this respect AH-Sepharose binding mimics the fibrin binding of the plasminogens reported by Suenson & Thorsen (1981). In contrast with the plasminogen-AH-Sepharose interaction, that of Glu-plas-

Fig. 3. Ratio of molar amounts of miniplasminogen (□ and ○) and Lys-plasminogen (■ and ●) immobilized on the AH-Sepharose column in the absence (A_0) and in the presence (A_i) of inhibitor, A_0/A_i , plotted against the concentration of inhibitor, i

A_0 and A_i values were obtained from the data presented in Fig. 2. Theory predicts that $A_0/A_i = 1 + i/K_i$. Inhibitor: □ and ■, *N*-acetyl-lysine methyl ester; ○ and ●, 6-aminohexanoic acid.

Fig. 4. Ratio of the molar amounts of Glu-plasminogen immobilized on the AH-Sepharose column in the absence (A_0) and in the presence (A_i) of inhibitor, A_0/A_i , plotted against the concentration of inhibitor, i

The plots are similar to those of Fig. 3, but show the data for Glu-plasminogen. Inhibitor: △, *N*-acetyl-lysine methyl ester; ▲, 6-aminohexanoic acid. In order to illustrate the two effects of 6-aminohexanoic acid the data are shown twice with different scales on the abscissa.

Table 1. Dissociation constants of various plasminogen-ligand complexes determined by f.q.a.c. on AH-Sepharose
For experimental details see the text.

	K_i for AH-Sepharose (μM)	K_i for <i>N</i> -acetyl-lysine methyl ester (μM)	K_i for 6-aminohexanoic acid (μM)
Glu-plasminogen	210*	240†	$\sim 10^\ddagger$ 5600†
Lys-plasminogen	23*	25†	295†
Miniplasminogen	24*	26†	290†

* From $K_i = P_0/I/A_0$.
† Competitive inhibition of the plasminogen-AH-Sepharose interaction.
‡ Non-competitive inhibition of the Glu-plasminogen-AH-Sepharose interaction.

minogen is effected by 6-aminohexanoic acid binding to the high-affinity lysine-binding site (Figs. 3 and 4).

The strong lysine-binding site of Lys-plasminogen does not interact with the AH-Sepharose column, so it seems very unlikely if that of Glu-plasminogen should do so, particularly since a less strong AH-Sepharose binding results. Furthermore, *N*-acetyl-lysine methyl ester inhibits the AH-Sepharose binding of the Glu-plasminogen in a simple linear competitive way, indicating that only one site (namely the AH-site) is involved. It seems reasonable therefore to assume that no direct interaction exists between the strong lysine-binding site of Glu-plasminogen and the AH-Sepharose column and that it is necessary to seek another explanation of its weak binding and of the two effects of 6-aminohexanoic acid (Fig. 4 and Table 1).

An f.q.a.c. experiment with an equimolar mixture of Lys-plasminogen and the *N*-terminal peptide Glu-1-Glu-75 of Glu-plasminogen (1.0 μ M) resulted in 10% (approx.) inhibition of the AH-Sepharose binding of Lys-plasminogen (Fig. 5). The mixture was obtained from Glu-plasminogen by plasminic cleavage followed by removal at the Lys-76 residue by carboxypeptidase B to avoid the presence of a C-terminal lysine residue.

This inhibition leads to an estimated K_i approx. 8 μ M of the apparent Lys-plasminogen-peptide (AH-site) complex. Since the free peptide apparently interacts rather strongly with the AH-site of Lys-plasminogen, it may also participate in an intramolecular Glu-plasminogen AH-site interaction. It is well known that Glu-plasminogen exists in different conformational forms and that lysine-analogous ligands stabilize a Glu-plasminogen

form that is similar to Lys-plasminogen (Abiko *et al.*, 1969; Brockway & Castellino, 1972; Castellino *et al.*, 1973; Markus *et al.*, 1979). I propose that essentially two forms of Glu-plasminogen exist, an A-form with an internally occupied AH-site and a B-form with a free AH-site, where the equilibrium state is given by an internal dissociation constant, $K_{int.} = [A]/[B]$ (dimensionless). After all, the differences between Glu-plasminogen and Lys-plasminogen stem from the presence or absence of the *N*-terminal residues Glu-1-Lys-76. If we assume that the strength of *N*-acetyl-lysine methyl ester and AH-Sepharose binding of Glu-plasminogen in the hypothetical absence of intramolecular inhibition (no A-form present) would equal those of Lys-plasminogen, then $K_{i(Glu)} = K_{i(Lys)}(1 + K_{int.})$, and that leads to $K_{int.} = 9.5$ in both cases, corresponding to the presence of approx. 10% of the B-form at equilibrium in the absence of external AH-site ligands.

The effect of 6-aminohexanoic acid binding to the strong lysine-binding site of Glu-plasminogen apparently leads to a shift of the equilibrium between A- and B-forms of Glu-plasminogen ($K_{int.} = 20$). I propose that this is due to destruction of a B'-form that can exist only when the strong lysine-binding site is free and that differs from other B-form by having the *N*-terminal part of the molecule weakly bound intramolecularly to the low-affinity lysine-binding site of Kringle 1 reported to interact with the plasminogen peptide Glu-1-Met-57 by Lerch *et al.* (1980) (Scheme 1). Such a shift of equilibrium between the A- and B-forms of Glu-plasminogen also may explain the inhibition of plasminogen activation at low concentrations of antifibrinolytic amino acids noted by Thorsen & Astrup (1974) and Markus *et al.* (1979).

Discussion

Glu-plasminogen, Lys-plasminogen and miniplasminogen were found to interact specifically with immobilized 6-aminohexyl ligands at a site designated the AH-site, which is apparently identical with a weak lysine-binding site that binds 6-aminohexanoic acid with $K_i = 0.3$ mM in Lys-plasminogen but is not found (free) in Glu-plasminogen (Markus *et al.*, 1978a,b). The AH-site is found in miniplasminogen, and apparently no other lysine-binding sites of the plasminogens interact with the Sepharose AH-column. The AH-site interacts approx. 10 times more strongly with lysyl compounds than with 6-aminohexanoic acid. It may, in contrast with the strong lysine-binding site of Glu-plasminogen and Lys-plasminogen, prefer ligands that do not carry a free carboxylate function, and thus normal lysyl side chains of proteins are potential ligands of it. This suggests,

Fig. 5. Elution patterns of Lys-plasminogen in the absence (1) and in the presence of an equimolar amount of *N*-terminal peptide Glu-1-Glu-75 of Glu-plasminogen (2) in f.q.a.c. experiments on AH-Sepharose with $p_0 = 1 \mu$ M. In the presence of 6-aminohexanoic acid ($i = 200$ mM) the same elution pattern (3) corresponding to total inhibition of the immobilization of the Lys-plasminogen was obtained with both preparations. The principle of the experiments is explained in Fig. 1.

Scheme 1. Model illustrating the A-, B- and B'-forms of Glu-plasminogen

B- and B'-forms are able to bind lysyl ligands (▲) to the free AH-site (<), A-form is not. The A/B equilibrium is shifted when 6-aminohexanoic acid (■) binds to the strong lysine-binding site, since such molecules are prevented from forming the B'-form, which differs from the other B-form by having the N-terminal part of the molecule weakly bound intramolecularly to the low-affinity lysine-binding site of Kringle 1 (▷). The strong lysine-binding site is illustrated only with bound 6-aminohexanoic acid.

in my opinion, that the AH-site may be a primary protein-binding site of the plasminogens, whereas the strong lysine-binding site may interact particularly with plasmin-cleaved protein fragments carrying C-terminal lysine residues. In particular, the AH-site is probably responsible for the binding of the plasminogens to undegraded fibrin. The results presented here further lead me to propose the existence of two forms of Glu-plasminogen differing mainly with respect to the status of the AH-site (Scheme 1): the A-form, which is dominating in the absence of external AH ligands, and which has an internally occupied AH-site, where the intramolecular bond keeps the molecule in a 'closed' conformation, inhibits the access of plasminogen activators and the fibrin-binding, and the B-form, of which the AH-site is free and the conformation is more open and the access of plasminogen activators is not inhibited.

The markedly different K_m values of urokinase-catalysed conversion of the plasminogens into plasmin obtained by Wohl *et al.* (1978, 1980) and those obtained by us previously (Christensen & Müllertz, 1977; Christensen, 1977) apparently are due to their use of a lysyl substrate and our use of an arginyl substrate to detect plasmin in the course of the reaction; the lysyl substrate apparently interacts with the AH-site of the plasminogens and changes the equilibrium between the Glu-plasminogen forms. Apparently urokinase converts only the B-form of Glu-plasminogen and is unable to bind the A-forms (U. Christensen & H. Ipsen, unpublished work). I have previously shown that the activity of (Lys-)plasmin is increased by allosteric binding of lysine-analogous compounds, and according to the K_i values obtained the allosteric site may be identical with the AH-site (Christensen, 1978).

Scheme 2. Model of fibrinolysis

Symbols not explained in Scheme 1 are: $\blacktriangle$, fibrin monomer with its lysyl ligand; $\blacksquare$, fibrin degradation product with C-terminal lysine residue; AP, α_2 -antiplasmin.

The complex-formation between plasmin and α_2 -antiplasmin is apparently dependent, not on the AH-site, but on a lysine-binding site in the Kringle 1 part of plasmin (Christensen & Clemmensen, 1977, 1978; Christensen *et al.*, 1979; Wiman & Collen, 1978a,b; Wiman, 1980), so that a B'-form of Glu-plasminogen may yield some protection from interaction with α_2 -antiplasmin.

Below I present a hypothesis on the regulation of fibrinolysis that takes the AH-site into account and may explain the efficient and specific hydrolysis of fibrin catalysed by plasmin (Scheme 2).

1. In normal plasma only Glu-plasminogen and no Lys-plasminogen or miniplasminogen is present. Glu-plasminogen exists mainly in the A-form, which is not converted into plasmin by activator enzymes that convert plasminogen into plasmin.

2. In the presence of fibrin (native), Glu-plasminogen binds and fibrin-bound Glu-plasminogen exists only in an activatable form. Since the uptake of Glu-plasminogen probably does not change the plasma concentration of Glu-plasminogen significantly, fibrin may well be saturated with Glu-plasminogen. The conditions *in vivo* may be very different from experimental conditions *in vitro* in this respect.

3. Activator-catalysed conversion of bound Glu-plasminogen results in Glu-plasminogen bound to fibrin at the AH-site. Internal Glu-

plasmin-Kringle 1 (weak lysine-binding site) interaction partially protects it from α_2 -antiplasmin.

4. The AH-site interaction brings a (number of) particular fibrin peptide bonds in close contact with the catalytic site of plasmin, so that cleavages occur only at specific points of the fibrin molecule. During fibrinolysis a number of C-terminal lysine residues are formed, since plasmin preferentially cleaves Lys-Xaa bonds of fibrin, and such residues bind to the strong lysine-binding site of plasmin. Plasmin gets from one cleavage point to another by alternating strong lysine-binding site and AH-site-interactions and is not released until the fibrin network is dissolved.

5. Termination of fibrinolysis involves several mechanisms that protect the organism from damaging cleavages catalysed by plasmin. Binding of internal lysyl ligand to the AH-site in the miniplasmin part of the molecule converts most of the free Glu-plasmin into an inactive A-form, the interaction with α_2 -antiplasmin is no longer inhibited by internal Glu-plasmin-Kringle 1 interaction, and autocatalytic cleavage of the primary Glu-plasmin- α_2 -antiplasmin complex to a similar Lys-plasmin complex further facilitates the final formation of α_2 -antiplasmin-plasmin complex, which irreversibly inhibits plasmin. Further, since AH-site interactions apparently are stronger than active-site interactions of plasmin, the binding of proteins may preferentially occur at the AH-site

and diminish plasmin-catalysed protein cleavage greatly, because only a limited part of the protein, perhaps containing no Lys-Xaa or Arg-Xaa bonds, is accessible to the active site. The main features of this hypothesis are shown in Scheme 2.

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