Location of the Intermediate and High Affinity \(\omega\)-Aminocarboxylic Acid-binding Sites in Human Plasminogen*

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Two elastase fragments of plasminogen, Tyr\(_2\)-Val\(_{127}\)/Tyr\(_{26}\)-Val\(_{127}\) and Val\(_{130}\)-Ala\(_{43}\) (kringle 1 + 2 + 3 and kringle 4), are known to bind to lysine-Sepharose. Elastase fragmentation of Glu- and Lys-plasminogen that lost affinity for lysine-Sepharose as a result of 1,2-cyclohexanedione modification yields kringle 1 + 2 + 3 that no longer displays affinity for lysine-Sepharose; the kringle 4 fragment, however, is bound normally to the affinity column. These results show that the binding site of kringle 4 is not functional in plasminogen and it is the kringle 1 + 2 + 3 region which ensures the affinity of the protein for lysine-Sepharose.

1,2-Cyclohexanedione treatment of isolated kringle 4 fragment under the same conditions as those used for plasminogen modification abolishes its affinity for lysine-Sepharose; therefore, lack of reaction of kringle 4 in plasminogen suggests that the residues involved in ligand binding by kringle 4 are protected from reaction in the intact protein.

Studies on kringle 4 + miniplasminogen revealed that the lysine-Sepharose binding site is already functional in this fragment; i.e. removal of kringle 1 + 2 + 3 renders kringle 4 accessible to external ligands.

The changes in the functional state of the kringle 4 binding site resemble the changes in the affinity of one of the weak \(\omega\)-aminocarboxylic acid-binding sites which upon proteolytic modification of Glu-plasminogen gives rise to the so-called intermediate binding site. On the basis of evidence presented in this paper, we propose that the intermediate site corresponds to the one present in kringle 4.

Our finding that the high affinity binding site of kringle 4 is not functional in plasminogen, by way of elimination also indicates that the single high affinity binding site of plasminogen must be the one carried by the first kringle of kringle 1 + 2 + 3.

Plasmin has a broad sequence specificity very similar to that of trypsin, yet its intravascular action is primarily directed toward a single protein, fibrin. Recently, a unified molecular model has been formulated that explains why the action of plasmin is normally restricted to fibrin (1). According to this model, regulation of fibrinolysis is achieved through the interaction of plasminogen with fibrin, plasminogen activators, and \(\alpha\)-antiplasmin. These interactions are apparently mediated by the \(\omega\)-aminocarboxylic acid-binding sites (lysine-binding sites) of plasminogen since these sites have been shown to coincide with the fibrin-binding sites of plasminogen (2-4) and with the sites essential for the rapid binding of plasmin by the fast acting plasmin inhibitor, \(\alpha\)-antiplasmin (5).

Native human plasminogen contains one strong \((K_d = 9 \mu M)\) and possibly four or five weak \((K_d = 5 \mu M)\) \(\epsilon\)-aminohexanoic-binding sites (5). In proteolytically modified plasminogen, the high affinity binding site is somewhat weaker \((K_d = 35 \mu M)\) and, at the expense of one of the weak sites, a new site appears which displays intermediate affinity for \(\epsilon\)-Ahx \((K_d = 260 \mu M)\); the remaining low affinity sites are essentially unchanged (6). The same pattern of high affinity, intermediate, and low affinity \(\omega\)-aminocarboxylic acid-binding sites was detected using tranexamic acid in the binding assay, the only difference being that each binding site shows an order of magnitude higher affinity for tranexamic acid than for \(\epsilon\)-Ahx (7). These studies thus demonstrated that plasminogen contains about five independent \(\omega\)-aminocarboxylic acid-binding sites of similar ligand specificity.

A striking feature of the structure of human plasminogen is the presence of five closely homologous triple loop structures, "kringles," in the heavy chain part of the molecule (8) (Fig. 1). Five-fold repetition of this unusual structure in plasmin led to the assumption (5) that each kringle might carry an \(\omega\)-aminocarboxylic acid-binding site. This view would be in accord with the observation that the binding site(s) responsible for the affinity of plasminogen for lysine-Sepharose resides in the heavy chain portion of plasmin (9) which is organized into the kringle structures. Moreover, fragments of plasminogen comprising kringle 1 + 2 + 3, kringle 4 (8), and kringle 1 (10) obtained by proteolysis of the molecule have been shown to display affinity for lysine-Sepharose. On the other hand, miniplasminogen, the plasminogen fragment that contains only the fifth kringle plus the light chain portion of plasmin is not adsorbed to lysine-Sepharose (8); therefore, kringle 5 is generally assumed to lack an \(\omega\)-aminocarboxylic acid-binding site.

Studies on ligand binding by the plasminogen fragments have revealed that both kringle 1 and kringle 4 contain a single high affinity binding site for \(\omega\)-aminocarboxylic acids, kringle 1 + 2 + 3 carries a strong and a weak site, whereas no ligand binding could be demonstrated to miniplasminogen (10, 11). These sites thus account for three of the suggested five \(\omega\)-aminocarboxylic acid-binding sites of intact plasminogen. The discrepancy in the number of binding sites detectable in plasminogen and in the constituent fragments may be due to fragmentation itself; e.g. it may be the result of changes in the

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environment of these sites, some of which may become too weak to be detected.

Several lines of evidence support the suggestion that the environment of kringle sites does influence the affinity of the binding site for the ligand. When native Glu-Pg is converted to Lys-Pg leading to a much looser conformation and large changes in the relative position of entire domains (12), the high affinity binding site becomes weaker and one of the weak sites increases in affinity about 20-fold (5-7). Moreover, isolated kringle 1 and kringle 4 have been shown to bind e-Ahx with high affinity ($K_d = 16$ and $36 \mu M$, respectively; Ref. 10), comparable to that of the single high affinity binding site of Glu- and Lys-Pg. Obviously, one of these binding sites is weak in intact plasminogen and becomes a strong site only upon excision from plasminogen. Considering the fact that binding sites do experience such significant changes in affinity in different plasminogen derivatives, any coincidence of actual binding constants between isolated kringle and intact plasminogen seems to be fortuitous and cannot decide the question whether kringle 1 or kringle 4 carries the binding site that corresponds to the high affinity site of Glu- and Lys-Pg.

In this paper, we present evidence that kringle 4 does not function as a high affinity binding site in either Glu- or Lys-Pg, leaving kringle 1 as the only candidate for the high affinity binding site of plasminogen.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-Cyclohexanediene (Fluka) and e-aminoenohexanic acid (BDH) were commercial preparations. Porcine pancreatic elastase prepared according to a described procedure (13) was a generous gift of Dr. P. Tolnay (Institute of Drug Research, Budapest).

**Plasminogen and Plasminogen Fragments**—Human Glu-Pg was prepared from fresh citrated plasma by affinity chromatography on lysine-Sepharose as described by Deutsch and Mertz (14); the purification steps were performed in the presence of bovine pancreatic trypsin inhibitor (Trasylol, Bayer) to prevent conversion of Glu-Pg to Lys-Pg. Partially degraded plasminogen (Lys-Pg) was prepared from outdated plasma without the addition of trypsin inhibitor. The purity of the isolated plasminogen forms was checked by polyacrylamide gel electrophoresis in urea/acetic acid, pH 3.2, as described by Walther et al. (15). If necessary, the two forms of plasminogen were separated by DEAE-Sephadex chromatography (16). The concentration of plasminogen was measured spectrophotometrically according to $A_{280} = 16.8$ (17).

Kringle 1 + 2 + 3, kringle 4, and miniplasminogen were prepared by limited proteolysis of plasminogen with porcine pancreatic elastase followed by separation of the resulting fragments by gel filtration on Sephadex G-75 and affinity chromatography on lysine-Sepharose essentially as described by Sottrup-Jensen et al. (8). The purity of the isolated fragments was checked with 6-16% linear polyacrylamide gradient-DodSO$_4$, slab gel electrophoresis under both reducing and nonreducing conditions. The isolated fragments were quantitated by amino acid analysis on the basis of the known sequence of the fragments. Absorption coefficients were determined from amino acid analysis of the fragments and from their absorption spectra obtained on a Varian 635/D UV/VIS spectrophotometer. $e_{280}$ values determined for kringle 1 + 2 + 3 ($4.7 \times 10^3$ $M^{-1} \text{cm}^{-1}$), kringle 4 ($3.1 \times 10^3$ $M^{-1} \text{cm}^{-1}$), and miniplasminogen ($8.9 \times 10^2$ $M^{-1} \text{cm}^{-1}$) were routinely used for the determination of the concentration of the fragments. In the case of the kringle 1 + 2 + 3 cleavage variants, the amino acids present only in the sequence common to all variants (residues 79-97) but missing from the Ser$_{140}$-Val$_{150}$ region were used to determine the molar concentration by amino acid analysis. Since the Ser$_{140}$-Val$_{150}$ region is devoid of amino acids absorbing at 280 nm, the presence or absence of this region in kringle 1 + 2 + 3 preparations does not influence the molar absorption; therefore, the same absorption coefficient was used to determine the concentration of the various preparations. For the calculation of the concentration of the plasminogen fragments, the $M_r$ values determined by Sottrup-Jensen et al. (8) for kringle 4 (10,000), miniplasminogen (37,000), and kringle 1 + 2 + 3 variants (27,000, 33,000, 34,000, and 38,000) were used.

Partial digestion of Glu-Pg with porcine pancreatic elastase was carried out under the conditions described for limit digestion with elastase but the amount of elastase was 10 times lower; therefore, intermediate fragments were still prevalent in the digest at the time when the proteolysis was arrested. Intermediates were separated from remaining plasminogen as well as from kringle 4 by gel filtration on Sephadex G-150 columns (1.4 x 70 cm) equilibrated with 0.3 M ammonium bicarbonate. The fractions containing the intermediates were pooled and were further fractionated by lysine-Sepharose chromatography.

$1,2$-Cyclohexanediene Modification—Modification of Glu-Pg, Lys-Pg, and kringle 4 (0.1 mM with the arginine-specific reagent: cHxO$_2$ was performed in 50 mM sodium borate, 500 mM NaCl, pH 8.0, at 37°C according to a described procedure (18). Aliquots were taken during reaction and were gel-filtered on Sephadex G-25 columns equilibrated with 0.1 M sodium borate buffer, pH 8.0, to remove reagents. Affinity of modified Glu-Pg, Lys-Pg, and kringle 4 for lysine-Sepharose was assayed at 25°C by column chromatography as described previously (19).

In preparative experiments, Glu-Pg and Lys-Pg were incubated with cHxO$_2$ under the above conditions until about half of the molecules lost affinity for lysine-Sepharose. Plasminogen that lost affinity for lysine-Sepharose was subjected to elastase fragmentation according to the procedure of Sottrup-Jensen et al. (8) with the minor modification that both the digestion and gel filtration and affinity chromatography steps were performed in 0.1 M borate buffer, pH 8.0. The digest was first chromatographed on lysine-Sepharose; the adsorbed material was eluted with 0.1 M e-Ahx. Both the adsorbed and nonadsorbed materials were further fractionated on Sephadex G-75 columns. At each stage of fractionation, the composition of the samples was also checked by 6-16% linear polyacrylamide gradient-DodSO$_4$, slab gel electrophoresis.

**Amino Acid Analysis**—Protein samples were hydrolyzed for 24 h in 6 N HCl at 110°C in the presence of mercaptoethanol. The composition of the hydrolysates was determined on a JEOL JLC-5AH amino acid analyzer and the extent of arginine modification of cHxO$_2$-treated samples was determined on the basis of the loss of arginine residues and appearance of modified arginine in the elution profile (18).

**RESULTS**

In a previous paper (19), we have shown that chemical modification of carboxyl and guanidino groups of Glu-plasminogen abolishes the affinity of the protein for lysine-Sepharose, but presence of $\omega$-aminocarboxylic acids in the reaction mixture protects against loss of affinity. On the basis of our results, we concluded that the essential carboxyl and guanidino groups are responsible for binding the amino and carboxyl functions of $\omega$-aminocarboxylic acids, respectively. In the present study, modification of arginyl residues of Lys-plasminogen with cHxO$_2$ was similarly found to cause the rapid loss of affinity of the protein for lysine-Sepharose (Fig. 2). The presence of e-Ahx in the reaction mixture prevents this loss of affinity, indicating that reaction of arginine residues...
in the \(\omega\)-amino carboxylic acid binding sites is responsible for the abolition of affinity for the lysine-Sepharose. Both Glu-Pg and Lys-Pg contain a total of five independent \(\omega\)-amino carboxylic acid-binding sites (5-7), all of which, at least in principle, could mediate the binding of the protein to lysine-Sepharose. Assuming that this is the case, modification of all five lysine-binding sites would be a precondition of loss of affinity.

Lysine is bound to the Sepharose matrix via its \(\alpha\)-amino group; therefore, the ligand of lysine-Sepharose functionally corresponds to \(\epsilon\)-Ahx. We may then assume that the affinity of the matrix-bound ligand to the different binding sites is properly represented by the affinity of the sites for \(\epsilon\)-Ahx. According to theoretical considerations and practical experience (20), \(K_a\) values larger than \(10^{-3}\) M are usually useless in affinity chromatography, and the above considerations argue against the involvement of the weak binding sites in the binding of plasminogen to lysine-Sepharose.\(^7\) The latter assumption then implies that the binding of plasminogen to lysine-Sepharose is mediated only by the high affinity site; therefore, abolition of the affinity for lysine-Sepharose by chemical modification reflects only the reaction of this site, whereas the weak binding sites may well remain intact in the protein that lost affinity for lysine-Sepharose.

\(^7\) Note that the affinity of \(\omega\)-amino carboxylic acid-binding sites for lysine is about two orders of magnitude lower than that for \(\epsilon\)-Ahx (5, 21). Therefore, if binding of lysine were a more valid model of the binding to lysine-Sepharose, the weak binding sites would be even less likely to contribute to affinity of plasminogen to lysine-Sepharose.

![Fig. 2. Loss of affinity of human Lys-Pg and isolated kringle 4 fragment for lysine-Sepharose under 1,2-cyclohexanedione modification. The reaction mixture contained 0.1 mm protein and 50 mM \(\text{CH}_3\text{O}\) in 50 mM sodium borate, 500 mM NaCl buffer, pH 8.0. Reaction of Lys-Pg with \(\text{CH}_3\text{O}\) in the absence (c) and presence (b) of \(\epsilon\)-Ahx (10 mm). Reaction of kringle 4 with \(\text{CH}_3\text{O}\) in the absence (a) and presence (c) of \(\epsilon\)-Ahx (10 mm). Loss of affinity of Glu-Pg as a result of \(\text{CH}_3\text{O}\) modification under the same conditions is also included for comparison (x; Ref. 19).](http://www.jbc.org/)

![Fig. 3. Elastase fragments of 1,2-cyclohexanedione-modified plasminogen. Glu-Pg was subjected to modification with \(\text{CH}_3\text{O}\); the form that had lost affinity for lysine-Sepharose was isolated as the fraction which had not been held up by the column and was subjected to elastase fragmentation as described under "Experimental Procedures." The digest was again chromatographed on lysine-Sepharose and both the adsorbing (A) and nonadsorbing (B) fractions were gel filtered on a Sephadex G-75 column (1.4 x 62 cm). The polyacrylamide gradient-DDodSO\(_4\) gel electrophoresis pattern of the reduced samples indicates that kringle 4 is present in the fraction adsorbed to lysine-Sepharose (A); the other fragments appear in the nonadsorbing fraction (B).](http://www.jbc.org/)

\(\omega\)-Aminocarboxylic Acid-binding Sites of Human Plasminogen

**TABLE 1**

| Elastase fragments | Native Glu-Pg* | \(\text{CH}_3\text{O}\)-modified Glu-Pg* |
|--------------------|---------------|--------------------------------------|
| Bound to lysine-Sepharose |                |                                      |
| Pool 1*            | 50.7 ± 1.3    | 6.6 ± 3                              |
| Pool 2             | 10.7 ± 0.3    | 10.9 ± 1                             |
| Not bound to lysine-Sepharose |            |                                      |
| Pool 1             | 38.2 ± 1.5    | 82.1 ± 6                             |
| Pool 2             | 0.5           | 0.5                                  |

* The values presented are the average of data obtained in six independent experiments.

\(\omega\)-plasminogen was modified with \(\text{CH}_3\text{O}\) until about half of the plasminogen lost affinity for lysine-Sepharose. In these experiments, only the plasminogen form no longer bound to lysine-Sepharose was used. The values presented are the average of data obtained in four independent experiments.

* Pools of Sephadex G-75 chromatography are defined as shown in Fig. 3. Pool 1 contains fragments of \(M_r > 20,000\); pool 2 contains kringle 4 (\(M_r = 10,000\) ).
hand, appear in the fractions lacking affinity for lysine-Sepharose, together with miniplasminogen.

These data indicate that the \(\omega\)-aminocarboxylic acid-binding site of kringle 4 is not functional in Glu-Pg as a lysine-Sepharose-binding site. The parallelism between the loss of affinity of plasminogen and loss of affinity of kringle 1 + 2 + 3 confirms that the lysine-Sepharose-binding site of plasminogen resides in the kringle 1 + 2 + 3 fragment.

**Shielding of the \(\omega\)-Aminocarboxylic Acid-binding Site of Kringle 4 in Plasminogen**—The finding that \(\text{CH}_{2}O\) modification of Glu-plasminogen did not destroy the affinity of the kringle 4 fragment for lysine-Sepharose contrasts sharply with data obtained on isolated kringle 4. As shown in Fig. 2, \(\text{CH}_{2}O\) modification of an isolated kringle 4 fragment under the same conditions as those used for modification of plasminogen leads to loss of affinity at a rate similar to that found for plasminogen.

\(\text{CH}_{2}O\) modification of kringle 4 has been performed and both the form that lost and the form that retained affinity for lysine-Sepharose have been analyzed for loss of arginine. The results show that whereas untreated kringle 4 has 3.9 arginines (the theoretical number based on sequence is 4) the bound form has 3 arginine residues, and the form not bound to lysine-Sepharose has only 2 arginine residues (Table IIA). Data obtained in several similar experiments showed that the extent of \(\text{CH}_{2}O\) modification of kringle 4 practically does not influence the arginine content of the two forms, only alters the relative amounts of the bound and not bound forms. This indicates that about one arginine is rapidly modified without affecting the affinity of kringle 4 for lysine-Sepharose; reaction of a second arginine residue, however, abolishes its affinity. Apparently the remaining two arginines resist \(\text{CH}_{2}O\) modification. The presence of \(\epsilon\)-Ahx in the reaction mixture protects kringle 4 from loss of affinity, suggesting that the essential arginine is involved in ligand binding, probably by interaction with the carboxyl group of the \(\omega\)-aminocarboxylic acids.

**Table II**

| Experiment | Krigle 4 bound to lysine-Sepharose | mol arginine/mol kringle 4 | Bound to lysine-Sepharose | Not bound to lysine-Sepharose | Amol arginine |
|------------|-----------------------------------|--------------------------|---------------------------|-------------------------------|--------------|
| 1          | 70                                | 3.1                      | 2.1                       | 1.0                           |              |
| 2          | 60                                | 3.1                      | 2.1                       | 1.0                           |              |
| 3          | 55                                | 2.9                      | 2.0                       | 0.9                           |              |
| 4          | 35                                | 2.8                      | 1.9                       | 0.9                           |              |

**B. Effect of \(\text{CH}_{2}O\) modification of Glu-Pg on the arginine content of Glu-Pg and of the corresponding kringle 4 fragments**

Glu-Pg was subjected to \(\text{CH}_{2}O\) modification until 65% of the molecules lost affinity for lysine-Sepharose. The two forms of Glu-Pg were separated by affinity chromatography on lysine-Sepharose and kringle 4 fragments were prepared from both plasminogen forms by elastase fragmentation. The arginine content of the samples was determined following acid hydrolysis. The values in parentheses are the theoretical numbers of arginine residues based on the sequence of plasminogen and kringle 4.

As shown in Table IIB, kringle 4 in plasminogen is essentially unaltered by \(\text{CH}_{2}O\) modification of Glu-Pg under conditions which in the case of \(\text{CH}_{2}O\) modification of isolated kringle 4 (Experiment 4 in Table IIA) led to extensive modification of the two reactive arginines of kringle 4. The fact that the same insignificant modification occurred in the fragment derived from both the plasminogen form that lost and the form that retained affinity for lysine-Sepharose also emphasizes that the slight degree of modification does not have any role in influencing whether plasminogen is bound or not to lysine-Sepharose.

The fact that both arginine residues susceptible to chemical modification in isolated kringle 4 resist modification in Glu-Pg suggests that these residues might be involved in interactions that prevent their reaction. The fact that not only the single arginine indispensable for affinity for lysine-Sepharose is protected from reaction in plasminogen indicates that not only the binding site but also other parts of kringle 4 are involved in interactions with the rest of the plasminogen molecule.

Modification experiments on Lys-Pg showed that the rate of reaction with \(\text{CH}_{2}O\) is similar to that of Glu-Pg (Fig. 2). Elastase fragmentation of the form of Lys-Pg that lost affinity led to results similar to those obtained for Glu-Pg, i.e., the protein yielded functionally intact kringle 4 fragments. This result indicates that in Lys-Pg the binding site of kringle 4 is not capable of functioning as a lysine-Sepharose binding site. The data also suggest that the groups masking the kringle 4 binding site are, at least in part, provided by structures of plasminogen other than those present in the NH\(_2\)-terminal activation peptide. To answer the question which regions of plasminogen may be responsible for masking the kringle 4 binding site, we subjected Glu-Pg to partial elastase fragmentation employing 10 times lower elastase to plasminogen ratios than in the case of limit digestion. The partial digest was fractionated by gel filtration on Sephadex G-150. As shown in Fig. 4 the digest contained a substantial amount of undigested plasminogen as well as large amounts of intermediary fragments.

The fractions containing intermediates, miniplasminogen, and kringle 1 + 2 + 3 variants but not containing plasminogen or kringle 4 (fractions 4-6 in Fig. 4) were used for further experiments. The pooled fractions (Fig. 5A) were chromatographed on lysine-Sepharose to remove miniplasminogen present in the sample (Fig. 5B). All intermediates and variants of kringle 1 + 2 + 3 were bound to the lysine-Sepharose column (Fig. 5C). (No further attempt was made in the present study to separate the different intermediates from kringle 1 + 2 + 3 or from each other.) Sample C (Fig. 5) was then subjected to a second limit digestion with elastase to determine the composition of the intermediates. Affinity chromatography on lysine-Sepharose showed that redigestion resulted in the reappearance of miniplasminogen (Fig. 5D). The fraction adsorbed to lysine-Sepharose contained kringle 4 in addition to the variants of kringle 1 + 2 + 3 (Fig. 5E). The reappearance of miniplasminogen at the expense of the disappearance of the intermediates proves that there is a fragment among the intermediates that corresponds to kringle 4 + miniplasminogen.\(^3\) Quantitative evaluation of the data showed that the molar amount of kringle 4 formed in the second digest is about three times higher than that of miniplasminogen. Obviously, the remaining amount of kringle 4 comes from variants of kringle 1 + 2 + 3 + 4 (Table III).

\(^3\) Unpublished data of J. Powell and F. J. Castellino, quoted in Ref. 22, also led these authors to conclude that a fragment, corresponding to kringle 4 + miniplasminogen is formed during the initial stages of elastase digestion of human plasminogen.
The molar quantity of each component has been determined spectro-photometrically. The quantity of undigested plasminogen, or kringle 1 digest of sample was chromatographed on a Sephadex G-150 column (1.4 × 70 cm). The polyacrylamide gradient-DodSO4 gel electrophoresis pattern of reduced samples taken from some of the fractions is also included. Fraction 1 contains undigested plasminogen; fractions 8 and 9 contain predominantly kringle 1 + 2 + 3 variants and miniplasminogen. Fractions 4-6, rich in intermediates but devoid of plasminogen, were used for further studies on the composition of the intermediates.

The fact that kringle 4 + miniplasminogen is bound to lysine-Sepharose shows that kringle 4 is functional in this fragment. 

**DISCUSSION**

**Binding of Plasminogen to Lysine-Sepharose Is Not Mediated by All ω-Aminocarboxylic Acid-binding Sites**—It is tacitly assumed by most authors that the terms ω-aminocarboxylic acid-binding site and lysine-Sepharose binding-site are synonymous and can be used interchangeably. This opinion has not been questioned even in the recent literature, although it was shown by Markus and co-workers (5, 6) that not a single, but about five different ω-aminocarboxylic acid-binding sites are found in plasminogen, only one of which has high affinity for ω-aminocarboxylic acids. Our finding that the affinity of Glu-Pg to lysine-Sepharose can be eliminated by chemical modification, although at least one of the ω-aminocarboxylic acid-binding sites (the one on kringle 4) remains functionally intact, proves that lysine-Sepharose binding is not mediated by all ω-aminocarboxylic acid-binding sites.

The results presented in this paper suggest that only the complex formed between high affinity binding sites and lysine-Sepharose has a suitably low $K_d$ value to mediate detectable binding to the lysine-substituted carrier. All presently available data are consistent with this interpretation since all plasminogen forms and fragments which bind to lysine-Sepharose (Glu-Pg, Lys-Pg, kringle 1, kringle 1 + 2 + 3, kringle 4) harbor a high affinity ω-aminocarboxylic acid-binding site (5-8, 10, 11, 23). The fact that there is only one strong binding site in plasminogen but two independent lysine-Sepharose binding fragments does not contradict this conclusion, since the binding sites of both nonoverlapping fragments display high affinity for ligands (10, 11). The observation that kringle 4 does not function as a lysine-Sepharose-binding site in plasminogen, whereas it binds to the affinity column as an isolated fragment, assigns kringle 4 to the binding site that increases in affinity by almost three orders of magnitude upon excision.

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**Table III**

Composition of the intermediate elastase fragments of human plasminogen

| Elastase fragments | (a) Partial elastase digest | (b) Complete elastase digest of α |
|--------------------|-----------------------------|--------------------------------|
| Miniplasminogen    | 0                           | 18 nmol                        |
| Kringle 4          | 0                           | 55 nmol                        |
| Kringle 1 + 2 + 3  | (47 nmol)                   | 84 nmol                        |
| Kringle 4 + miniplasminogen | (18 nmol)       | 0                              |
| Kringle 1 + 2 + 3 + 4 | (37 nmol)                 | 0                              |

4 The observation that all kringle 1 + 2 + 3 + 4 variants bind to lysine-Sepharose is not surprising and does not say anything about whether kringle 4 of these intermediates is functional or not since the kringle 1 + 2 + 3 portion alone may well suffice for binding.
from plasminogen. This assignment leaves the high affinity binding site of kringle 1 + 2 + 3, located in the kringle 1 portion by Lerch et al. (10), as the only candidate for the single strong ω-aminocarboxylic acid-binding site of plasminogen.

Role of the ω-Aminocarboxylic Acid-binding Site of Kringle 4 in Controlling the Conformation of Plasminogen—Plasminogen appears to exist in two conformational states and the position of the equilibrium of the two conformers is controlled by the functional state of the ω-aminocarboxylic acid-binding sites (6, 7). Saturation of these sites with ligands shifts the equilibrium position as detected by numerous physicochemical techniques. The conformational change is best described as a general loosening of the plasminogen molecule (manifested in decreasing the sedimentation coefficient from 5.51 to 4.15, increase in molecular weight determined by gel filtration, increase in intrinsic fluorescence; Refs. 7, 16, and 24).

The conformational equilibrium may also be shifted in favor of the looser conformer when Glu-Pg is converted to Lys-Pg, i.e. when approximately 70 residues are proteolytically removed from the NH2-terminus of plasminogen (6, 7). Lys-Pg was found to bind specifically the NH2-terminal peptide. This interaction was prevented by ω-aminocarboxylic acids (25), leading to the hypothesis that the ligands and the peptide region compete for the same binding site in plasminogen. According to this hypothesis, the specific interaction of the NH2-terminal peptide region with an ω-aminocarboxylic acid-binding site is responsible for the maintenance of the compact conformation of Glu-Pg; both removal of the NH2-terminal activation peptide or saturation of this binding site with ligands eliminates this interaction and leads to the looser conformer of plasminogen.

The removal of the NH2-terminal peptide from native plasminogen was indeed found to uncover a binding site in Lys-Pg (6, 7). This binding site is one of the weak sites in Glu-Pg. Removal of the activation peptide increases its affinity about 20-fold (parallel with the transconformation of plasminogen), giving rise to the so-called intermediate ω-aminocarboxylic acid-binding site.

In this study, we have demonstrated that kringle 4 carries the ω-aminocarboxylic acid-binding site that experiences a vast increase in affinity upon proteolysis of plasminogen. We suggest that the binding site of kringle 4 corresponds to the site that has been shown to undergo an increase in affinity upon proteolytic conversion of Glu- to Lys-Pg.

On the basis of the data obtained in the present investigation, we propose the following model for the role of kringle 4 in controlling the conformation of plasminogen.

In intact Glu-plasminogen, kringle 4 is involved in interactions with certain regions of plasminogen; these regions probably involve the activation peptide and, directly or indirectly, some segments of kringle 1 + 2 + 3. The lysine-binding site and other portions of kringle 4 participate in these interactions. The intramolecular interactions compete for the binding site; therefore, it appears as one of the weak sites for small ligands and the interactions protect the binding site from chemical modification. The shielded site cannot mediate the binding of the molecule to lysine-Sepharose.

When the activation peptide is lost during conversion of Glu- to Lys-Pg, some of the interactions involving the binding site are eliminated and the molecule undergoes a large conformational change since these interactions are responsible for maintaining the compact conformation of Glu-Pg. Partial elimination of the interactions competing for the binding site is reflected in an increase in the affinity of the site for small ligands: the site displays intermediate affinity for ω-aminocarboxylic acids. In Lys-Pg, however, the kringle 1 + 2 + 3 region still interferes with binding to lysine-Sepharose; removal of the kringle 1 + 2 + 3 segment uncovers the kringle 4 site which may then already serve as a lysine-Sepharose binding site, e.g. in kringle 4+ in plasminogen or in the isolated kringle 4 fragment. The affinity of the isolated kringle 4-binding site is no longer decreased by competing interactions; therefore, its affinity for ligands is comparable to that of the high affinity binding sites of plasminogen or kringle 1.

Interactions of the kringle 4 binding site both with the activation peptide and with the kringle 1 + 2 + 3 segment are assumed by the model to play a significant role in maintaining the compact conformation of Glu-Pg. Accordingly, elimination of both types of interactions by saturating the kringle 4-binding site with ligand in Glu-plasminogen allows the large conformational change to occur. Previously, removal of the activation peptide was found to lead to changes in physicochemical parameters similar to those induced by ω-aminocarboxylic acids (24); therefore, it was suggested that interaction with the activation peptide was responsible entirely for maintaining the more compact conformation (26).

In this study, we have demonstrated that kringle 4 carries the ω-aminocarboxylic acid-binding site that experiences a vast increase in affinity upon proteolysis of plasminogen. We suggest that the binding site of kringle 4 corresponds to the site that has been shown to undergo an increase in affinity upon proteolytic conversion of Glu- to Lys-Pg.

In this study, we have demonstrated that kringle 4 carries the ω-aminocarboxylic acid-binding site that experiences a vast increase in affinity upon proteolysis of plasminogen. We suggest that the binding site of kringle 4 corresponds to the site that has been shown to undergo an increase in affinity upon proteolytic conversion of Glu- to Lys-Pg. This means that the ω-aminocarboxylic acid-binding site that controls the conformation of the plasminogen molecule participates in intramolecular interactions with regions other than the activation peptide. This conclusion is in agreement with our observation that removal of the activation peptide is not sufficient to eliminate all interactions interfering with the kringle 4-binding site; the kringle 1 + 2 + 3 region also interacts with the binding site.

Autonomy of the Kringle Structures—The model assumes that the binding constellation of amino acid side chains of kringle 4 is fully formed even in Glu-Pg and the changes in its affinity on going from Glu- to Lys-Pg and to isolated kringle 4 simply reflect changes in its accessibility rather than stepwise evolution of the binding site. This view is based on the following considerations.

When the efficiency of different ligands to induce the transconformation of Glu-Pg is studied, the saturation of the weak binding site controlling conformation (6) (i.e. kringle 4 in its inactive state) is investigated. Studies on the influence of the same series of ω-aminocarboxylic acids have revealed that this binding site has a very strict structural requirement for ligands, the optimum distance between the amino and carboxyl group of the ligand being 0.68 nm (24). The affinity of the binding site for either shorter or longer ligands decreases precipitously, suggesting that the side chains involved in binding the amino and carboxyl groups tolerate little deviation from the optimal length. The sharp structural requirement indicates a rigid binding site since in the case of a more motile binding site a much less sharp distance requirement is expected.

Studies on the influence of the same series of ω-aminocarboxylic acids on the binding of isolated kringle 4 to lysine-Sepharose (23) have revealed that the binding site (i.e. the kringle 4-binding site in its high affinity state) has exactly the same structural requirement as in Glu-Pg: optimal distance between the amino and carboxyl group is also 0.68 nm and affinity decreases on both sides of this length.

The insensitivity of the crucial geometry of the kringle 4-binding site to whether it is present in plasminogen or isolated indicates that the binding site is fully formed in Glu-Pg. The simplest explanation for changes of three orders of magnitude in affinity without changes in the very strict ligand specificity is that the accessibility of the ligand binding site is the only
feature which changes during stepwise degradation of the protein. The fact that a fragment comprising little more than 16% of the total plasminogen molecule preserves the lysine-binding site with unimpaired binding specificity emphasizes the remarkable autonomy of this structure.

A similar insensitivity of the binding site geometry to proteolytic degradation of plasminogen can be observed also in the case of the high affinity binding site carried by the kringle 1 + 2 + 3 fragment. This binding site, as shown in the present paper, corresponds to the single high affinity site of both Glu- and Lys-Pg. Winn et al. (23), using an assay system based on the binding of the proteins to lysine-Sepharose, which therefore detects only high affinity binding sites, showed that kringle 1 + 2 + 3, Glu-Pg, and Lys-Pg all display an identical ligand specificity spectrum. Neither the optimal distance (0.68 nm) nor the sharpness of the distance requirement differs in the different plasminogen forms and fragments, although the environment of the kringles changes enormously on going from Glu- to Lys-Pg and to kringle 1 + 2 + 3. These data were interpreted to mean that the primary factor that decides the susceptibility of these regions to these different proteases is the flexibility of these probably random peptide structures. It seems therefore likely that the peptide segments connecting kringle 3 to kringle 4 and kringle 4 to kringle 5 serve as hinges for the large movements of the kringles relative to each other during the conformational changes of plasminogen, but that little change occurs within the kringles.

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