Conformational Studies of Human Plasminogen and Plasminogen Fragments: Evidence for a Novel Third Conformation of Plasminogen†

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ABSTRACT: The conformations of Glu-plasminogen and defined proteolytic fragments, in the presence and absence of 6-aminohexanoic acid (6-AHA), *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (t-AMCHA), and benzamidine, were studied using three methods: size-exclusion high-performance liquid chromatography (SE-HPLC), small-angle X-ray scattering (SAXS), and dynamic laser light scattering (DLLS). The well-documented conformational change of Glu-plasminogen with 6-AHA or t-AMCHA was measured as a decrease in molecular elution time by SE-HPLC (8.93 ± 0.01 to 8.32 ± 0.01 min) and increases in radius of gyration (30.7 ± 0.1 to 49.8 ± 0.3 Å) and Stokes radius (40.6 ± 0.3 to 48.5 ± 0.3 Å) by SAXS and DLLS, respectively. The addition of benzamidine to Glu-plasminogen resulted in a conformation (radius of gyration 41.0 ± 0.4 Å and Stokes radius 46.6 ± 0.3 Å) distinct from that in the presence of 6-AHA. 6-AHA, but not benzamidine, induced significant conformational changes in Lys-plasminogen and kringles 1 + 2 + 3 + 4 + 5. We conclude that Glu-plasminogen adopts three distinct conformations involving two intramolecular interactions: one mediated by regions of the NH_2-terminal peptide and kringle 5, competed for by 6-AHA or benzamidine, and the other possibly between kringles 3 and 4, competed for by 6-AHA but not benzamidine.

The zymogen plasminogen is a multidomain glycoprotein ($M_r \simeq 90\,000$) which is converted to the serine protease plasmin by specific cleavage of the Arg_{561}–Val_{562} peptide bond (Robbins *et al.*, 1967) by plasminogen activators. Plasminogen is abundant in most body fluids, and its activation is involved in a variety of physiological and pathological processes requiring localized proteolysis, such as fibrinolysis (Wiman & Collen, 1978), inflammation (Brommer *et al.*, 1992), tissue remodeling (Ossowski *et al.*, 1979), ovulation (Beers, 1975), tumor cell invasion, and metastasis (Danø *et al.*, 1985).

Native Glu-plasminogen (with NH_2-terminal glutamic acid) is a multidomain protein, consisting of an amino terminal peptide (NTP),[1] five homologous kringle domains (K1–5), and a trypsin-like serine protease (SP) domain. Removal of the NTP by plasmin cleavage yields a plasminogen species named Lys-plasminogen which lacks NH_2-terminal residues Glu_1–Lys_{77}, Glu_1–Arg_{68} or Glu_1–Lys_{78}. Limited elastase digest of plasminogen results in kringle-containing fragments: K1–3, kringle 4, and K5-SP (Sottrup-Jensen *et al.*, 1978).

The high-resolution structure of plasminogen has not yet been determined. Electron micrographs suggest a compact 'spiral' structure for Glu-plasminogen (Tranqui *et al.*, 1979; Weisel *et al.*, 1994) which is supported by evidence from small-angle X-ray scattering and molecular modeling studies (Ponting *et al.*, 1992a). Further evidence of a compact structure has been obtained from the biochemical cross-linking studies of Bányai and Patthy (1984, 1985) which demonstrated the incorporation of a 3-Å cross-link between residues within the serine protease and kringle 2 domains [for a review of structure, see Ponting *et al.* (1992b)]. Electron microscopy of Lys-plasminogen and 6-AHA-liganded Glu-plasminogen has shown structures resembling a broad, shallow letter "u" (Weisel *et al.*, 1994). This implies that domains are ordered within these structures as they are within the amino acid sequence allowing for structural interactions only between domains adjacent in sequence.

The binding of plasminogen to fibrin (Lucas *et al.*, 1983; Wu *et al.*, 1990), cultured endothelial cells (Hajjar *et al.*, 1991; Wu *et al.*, 1992), histidine-rich glycoprotein (Lijnen *et al.*, 1980), thrombospondin (DePoli *et al.*, 1989), cellular receptors (Silverstein *et al.*, 1988; Miles *et al.*, 1991), components of the extracellular matrix (Knudsen *et al.*, 1986; Stack *et al.*, 1992), and the plasmin-inhibitor α_2-antiplasmin (Wiman *et al.*, 1979) is mediated via the so-called "lysine-binding sites" (LBS) located within the five kringle structures. Small ligands, structurally similar to lysine, have been used to characterize these binding sites (Petros *et al.*, 1989; Thewes *et al.*, 1990; Menhart *et al.*, 1991; Rejante *et al.*, 1991a) since they are thought to mimic the binding determinants of larger macromolecules. These ligands include ω-aminocarboxylic acids (ω-ACAs, e.g., 6-AHA and t-AMCHA) and others,

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[1] Abbreviations: ω-ACA, ω-aminocarboxylic acid; 6-AHA, 6-aminohexanoic acid (ε-amino-*n*-caproic acid); *t*-AMCHA, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid; AH, aminohexyl; SE-HPLC, size-exclusion high-performance liquid chromatography; DLLS, dynamic laser light scattering; SAXS, small-angle X-ray scattering; τ_E, molecular elution time; R_S, Stokes radius; R_G, radius of gyration; Glu-Plg, Glu-plasminogen, native plasminogen with NH_2-terminal glutamic acid (Glu_1–Asn_{791}); Lys-Plg, Lys-plasminogen, proteolytically modified form of plasminogen, predominantly residues Lys_{78}–Asn_{791} with NH_2-terminal lysine; K5-SP, kringle 5 and serine protease domain (miniplasminogen) of plasminogen (Val_{443}–Asn_{791}); K1–3, kringles 1 + 2 + 3 of plasminogen (Tyr_{80}–Val_{338} and Tyr_{80}–Val_{354}); K1–5, kringles 1 + 2 + 3 + 4 + 5 of plasminogen defined by Sottrup-Jensen *et al.* (1978); NTP, NH_2-terminal peptide of plasminogen, predominantly residues Glu_1–Lys_{78}; LBS, lysine-binding site; mAb, monoclonal antibody.

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such as aminohexyl (AH) ligands lacking the carboxylate group.

Kringle 1 possesses a high-affinity binding site for 6-AHA and AH-type ligands (Lerch *et al.*, 1980; Matsuka *et al.*, 1990) as well as for α_2-antiplasmin (Wiman *et al.*, 1979) and histidine-rich glycoprotein (Lijnen *et al.*, 1980). Isolated kringle 4 has been shown to bind 6-AHA with moderate affinity, although it appears to possess little or no affinity for AH-type ligands (Petros *et al.*, 1989; Rejante *et al.*, 1991a). The kringle 4 LBS, however, may bind internal lysine and/or arginine residues within polypeptides, as evidenced by intermolecular interactions in crystals of kringle 4 (Mulichak *et al.*, 1991) and by its affinity for tetranectin, which contains no C-terminal lysine or arginine residues (Clemmensen *et al.*, 1986). Kringle 5 binds both 6-AHA and AH-type ligands with relatively low affinity (Novokhatny *et al.*, 1989; Thewes *et al.*, 1990) and is the only plasminogen kringle that possesses an additional affinity for benzamidine (Váradi & Patthy, 1981; Thewes *et al.*, 1990).

One or more of the kringle 1, 4, and 5 LBSs have been considered to mediate interactions of plasminogen with a variety of cell types (Miles *et al.*, 1991; Gonzalez-Gronow *et al.*, 1989; Wu *et al.*, 1992; Burge *et al.*, 1992) and with fibrin (Suenson & Thorsen, 1981; Lucas *et al.*, 1983; Wu *et al.*, 1990). Lucas *et al.* (1983) concluded that kringle 1 possesses the highest affinity for intact fibrin. In contrast, others (Thorsen *et al.*, 1981; Wu *et al.*, 1992, 1990) have shown that the site possessing the highest affinity for both cultured endothelial cells and intact fibrin resides within kringle 5 and that the lower affinity site resides within kringle 1. Kringle 4 has been shown to possess little or no affinity for intact fibrin (Thorsen *et al.*, 1981; Lucas *et al.*, 1983; Wu *et al.*, 1990), although it may bind to partially degraded fibrin(ogen) (Rejante *et al.*, 1991b).

The binding of Glu-plasminogen to fibrin induces a large-scale conformational change which facilitates its activation to plasmin (Walther *et al.*, 1975; Peltz *et al.*, 1982). A similar conformational change is thought to occur upon the binding of 6-AHA and t-AMCHA to one or more of the plasminogen LBS as observed by sedimentation analysis (Alkjaersig, 1964; Violand *et al.*, 1978; Markus *et al.*, 1978), fluorescence polarization (Castellino *et al.*, 1973), stopped-flow fluorescence spectroscopy (Christensen & Mølgaard, 1992), small-angle neutron scattering (Mangel *et al.*, 1990), and size-exclusion chromatography (Wallén & Wiman, 1972). The 6-AHA-bound Glu-plasminogen structure resembles that of Lys-plasminogen (Sjöholm *et al.*, 1973; Weisel *et al.*, 1994).

The binding of benzamidine has been shown to induce a conformational change within Glu-plasminogen (Holleman *et al.*, 1975). This has led us (Ponting *et al.*, 1992a) to concur with Christensen (1984) that this change is due to the displacement from the kringle 5 LBS [also known as the 'AH-site' (Christensen, 1984)] of a lysine-type ligand within the NTP. However, this benzamidine-induced conformational change appears to be smaller in magnitude to that induced by 6-AHA (cf. Markus *et al.*, 1978). This indicates that benzamidine- and 6-AHA-liganded Glu-plasminogen adopt different conformations. Moreover, it appears that plasminogen fragments lacking the NTP (namely, Lys-plasminogen and kringles 1–5) may also undergo ligand-induced conformational changes (Markus *et al.*, 1979; Rickli & Otavsky, 1975): it is apparent that disruption of a K5–NTP intramolecular interaction cannot account for these changes.

We have investigated this apparent paradox by studying the conformations of plasminogen and plasminogen fragments in the presence and absence of both ω-ACAs (6-AHA and t-AMCHA) and benzamidine using three different techniques. It is concluded that the structure of benzamidine-bound Glu-plasminogen differs from those of native Glu-plasminogen and ω-ACA-bound Glu-plasminogen and that, therefore, Glu-plasminogen may adopt three distinct conformations *in vitro*. Preliminary results of these studies have been presented at the Eleventh International Congress on Fibrinolysis, Copenhagen, Denmark, June 29–July 3, 1992.

EXPERIMENTAL PROCEDURES

All chemicals were analytical grade and obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Glu- and Lys-Plasminogen. Human plasminogen samples were purchased from the Plasma Fractionation Laboratory (PFL), Churchill Hospital (Oxford, U.K.), BioPool (Umeå, Sweden), and Kabi Pharmaceuticals (Stockholm, Sweden). Plasminogen was also isolated from plasma Cohn fraction III (Plasma Fractionation Plant, Elstree, U.K.) on lysine–Sepharose essentially as described by Deutsch and Mertz (1970). With the exception of BioPool Glu-plasminogen, all other plasminogen samples were gel filtered on Sephacryl S-200 to remove trace (<5% by absorbance at 280 nm) high-molecular-mass contaminating proteins. These contaminants were antigenically identified as including immunoglobulin-G and fibronectin.

The purity of plasminogen preparations and the absence of plasmin were confirmed by gradient SDS-PAGE (Laemmli, 1970) (Figure 1) and by SE-HPLC (Figure 2—other data not shown). Glu- and Lys-plasminogen status was determined by acetic acid/urea polyacrylamide gel electrophoresis (acid/urea-PAGE) adapted from the method of Panyim and Chalkley (1969). Furthermore, the NH_2-terminal residue of the PFL Glu-plasminogen preparation was determined as glutamic acid by NH_2-terminal sequencing. Protein concentrations were determined spectrophotometrically at 280 nm using $A_{280}^{1\%}$ values of 16.8 and 17.4 for Glu-plasminogen and Lys-plasminogen, respectively (Wallén & Wiman, 1972; Christensen, 1988).

Preparation of Plasminogen Fragments K1–3 and K5-SP. Plasminogen fragments K1–3 and K5-SP were prepared by elastase (porcine pancreas) digestion (Sottrup-Jensen *et al.*, 1978) of 1 g of purified plasminogen (1:30 molar ratio elastase to plasminogen) for 1 h 40 min at 37 °C; the activity of elastase was inhibited by addition of 1 mM (final concentration) diisopropyl fluorophosphate, and the digest passed through a lysine–Sepharose column (4.4 × 12.5 cm). The nonbinding material (K5-SP) was desalted on Sephadex G-25 (4.4 × 21 cm) into 0.1 M NH_4HCO_3 and lyophilized. The lysine-binding material (K1–3 and kringle 4) was eluted with 50 mM 6-AHA and gel filtered on Sephadex G-75 (5 × 91 cm) in 0.1 M NH_4HCO_3 to separate the K1–3 fragment from kringle 4.

Preparation of Plasminogen Fragment K1–5. Kringle 1–5 fragment was prepared by autodigestion of 300 mg of plasminogen (10 mg cm^{-3} in 0.05 M sodium phosphate buffer, pH 7.5) for 24 h at 37 °C initiated by streptokinase activation (1:100 molar ratio streptokinase to plasminogen). Similar procedures for the generation of K1–5 have previously been described (Reddy & Wagner, 1980; Wu *et al.*, 1990). The opalescent digest was centrifuged and the supernatant applied to a lysine–Sepharose column (2.5 × 10.5 cm) equilibrated and washed with 0.15 M NaCl/0.05 M Tris buffer, pH 7.5. Bound material was eluted with 0.05 M 6-AHA in wash buffer and subsequently gel filtered on a calibrated Ultragel AcA44

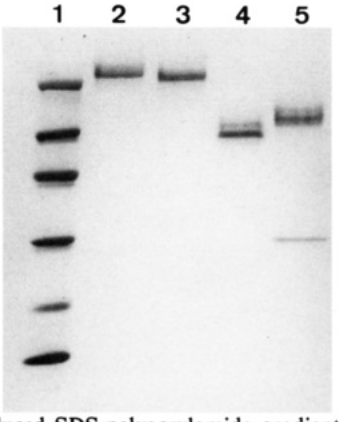

FIGURE 1: Reduced SDS-polyacrylamide gradient (10–20%) gel electrophoresis of plasminogen, plasminogen fragment K1–5, and plasmin. Samples: lane 1, Pharmacia low-molecular-mass standards (phosphorylase b, 92 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 400); lane 2, Glu-plasminogen; lane 3, Lys-plasminogen; lane 4, Kl–5; lane 5, plasmin.

column (4.4 × 82 cm; LKB, Bromma, Sweden) equilibrated in 0.1 M NH_4HCO_3. The molecular mass of the major peak (66% by absorbance at 280 nm) was estimated as 60 000 by gel filtration and 64 000 by reduced SDS-PAGE analysis (Figure 1).

The identity of the fragment was confirmed using NH_2-terminal sequence analysis, immunological characterization, affinity for benzamidine–agarose, and an enzymatic assay. The NH_2-terminal sequence of the fragment was found to be either Lys_{78} (residues 78–97 sequenced) or Val_{79} (residues 79–93 sequenced) using an Applied Biosystems 470A/120A protein sequencer. Using a dot blot-type assay, the fragment cross-reacted with monoclonal antibodies (mAbs) (American Diagnostica Inc., #3642 and #3647, respectively) directed against the K1–3 and K4 fragments of plasminogen. No cross-reaction with mAbs (American Diagnostica Inc., #3641 and #3644, respectively) directed against the NTP or the K5-SP fragment was observed. The fragment bound benzamidine–agarose (Affinity Chromatography Ltd., U.K.) indicating the presence of a functional kringle 5 domain. Finally, the fragment was shown to possess no amidolytic activity, using the chromogenic substrate D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide (S-2251, Kabi Diagnostica, U.K.) following the addition of urokinase-type plasminogen activator. It is concluded that this fragment contains the five kringle domains of plasminogen, although the presence of some residues of the COOH-terminal serine protease domain cannot be discounted. Protein concentrations were determined spectrophotometrically at 280 nm using $A_{280}^{1\%}$ values of 15.4, 17.0, and 18.5 for K5-SP, K1-5, and K1-3, respectively (Christensen, 1988; Summaria *et al.*, 1979; Matsuka *et al.*, 1990).

Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). SE-HPLC experiments were performed using a Hewlett-Packard 1090 HPLC system with diode array detector and fitted with a Tosa Haas TSK G3000SWx1 column (0.78 × 30 cm). Samples (25 μL containing 25–50 μg of protein) were applied to the column equilibrated in 0.1 M sodium phosphate buffer, pH 6.8, with and without 50 mM 6-AHA as indicated, at ambient temperature, with a flow rate of 1 cm^3 min^{-1}.

The SE-HPLC eluant was monitored at a wavelength of 205 nm or at 210 nm in the presence of 6-AHA. The measured elution time (τ_E) was estimated by the HPLC Hewlett-Packard integrator. Ligand-induced conformational changes were detected by changes in τ_E-values. Elution times were calculated as the mean of the results from 10 or more repeat experiments. Plasminogen framgent K1–3 is known to be at least 4-fold heterogeneous [two C-terminal elastase-cleavage sites and two glycoforms thereof (Sottrup-Jensen *et al.*, 1978)] and was found to be partially resolved into two peaks by SE-HPLC. The results shown here for K1–3 are the averaged τ_E-values for the two peaks.

Small-Angle X-ray Scattering (SAXS). SAXS data were acquired on station 2.1 of the synchrotron radiation source of the Daresbury Laboratory (Daresbury, U.K.) (Towns-Andrews *et al.*, 1989) using camera lengths of 5 m (data sets A, B, C, and D) and 2 m (data sets E and F). Prior to X-ray illumination, each Glu-plasminogen sample was shown, using the techniques of SE-HPLC, DLLS, and acid/urea-PAGE, to contain a single molecular mass species characterized by a single conformation (acid/urea-PAGE data not shown). The sources for the Glu-plasminogen samples used in these experiments were BioPool and PFL. Protein samples were held within thin mica walls in a target cell cooled to 4 °C.

Scattered X-rays were detected as a function of angle using a quadrant detector system (Lewis *et al.*, 1988). Spectra were calibrated in terms of the scattering vector **Q** using the well-known diffraction pattern of wet rat tail collagen ($Q = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle and the wavelength $\lambda = 1.54$ Å). Data were acquired for values of ($Q > 0.012$ $Å^{-1}$ (data sets A, B, C, and D) and $Q > 0.016$ $Å^{-1}$ (data sets E and F). As discussed previously (Ponting *et al.*, 1992a), data-smearing effects were not significant for this experimental configuration and, also, significantly accurate in-beam molecular mass determination was not possible.

Time-dependent scattering spectra were acquired for both buffer and protein-plus-buffer samples. Subsequently, these were normalized to account for fluctuations in beam intensity and X-ray absorption within samples. Difference intensity spectra, $I(Q)$, of the scattering from the protein over that from the buffer were calculated by subtracting the buffer spectrum from the protein-plus-buffer spectrum. Variations in these spectra were monitored as a function of time in order to determine the onset of radiation damage: typically, molecular aggregation occurred after approximately 30 s of illumination.

Analysis of data was performed using the Guinier approximation (Guinier, 1939) for the intensity spectra at small Q:

$$I(Q) \simeq I(0) \exp(-R_G^2 Q^2/3)$$

Molecular radii of gyration (R_G) were calculated from the gradient of the linear portions of Guinier plots ($\log_e I(Q)$ vs Q^2) using a least-squares fitting procedure. Calculations of distance distributions from scattering data were not possible due to an insufficiently large Q range.

Dynamic Laser Light Scattering (DLLS). All DLLS studies were performed using a Protein Solutions Ltd. (Hertford, U.K.) Model 801 molecular size detector. This exploits the technique of dynamic laser light scattering to determine the translational diffusion coefficient (D_T) using photon correlation spectroscopy (Claes *et al.*, 1992). The Stokes radius (R_S) is calculated from D_T using the Stokes–Einstein equation:

$$D_T = \frac{k_B T}{6\pi\eta R_S}$$

where k_B is Boltzmann's constant, T is the absolute temperature, and η is the solution viscosity.

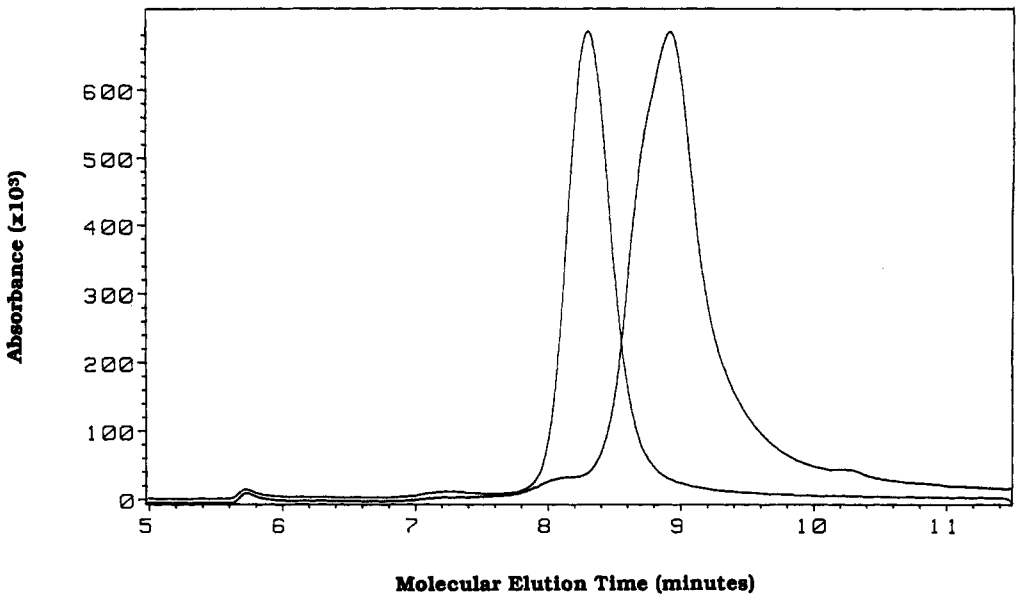

FIGURE 2: Size-exclusion HPLC elution profiles of Glu-plasminogen in the absence (right peak, absorbance 205 nm) and presence (left peak, absorbance 210 nm) of 50 mM 6-aminohexanoic acid.

Lyophilized protein samples were dissolved in 0.2 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, centrifuged for 10 min at 4000*g*, and filtered through a 0.1-μm filter (Anotop, U.K.) upon injection into the molecular size detector sample cell. Typically, concentrations in the range 1–3 mg cm^{-3} were used.

No significant change in R_S-values was observed over a range of concentrations (0.5–6.0 mg cm^{-3}) for Glu-plasminogen and a control, bovine α-chymotrypsinogen A (Sigma Chemical Co.). Data therefore were not required to be extrapolated to infinite dilution. Approximately 50 individual R_S-values were acquired for each sample and statistically averaged. An individual measurement was discounted if its discrete autocorrelation function did not correspond to that of a monodisperse suspension of particles. The DLLS results described herein do not include analysis of K5-SP, since samples were shown to contain contaminating aggregates.

RESULTS

The techniques of size-exclusion HPLC, small-angle X-ray scattering, and dynamic laser light scattering were used to characterize the conformations of plasminogen and plasminogen fragments in the presence and absence of 6-AHA, t-AMCHA, and benzamidine. SE-HPLC experiments investigated the conformations of native Glu-plasminogen (Glu-Plg) and plasminogen fragments (Lys-Plg, K1–5, K1–3, and K5-SP) in the presence and absence of 6-AHA (50 mM) but not benzamidine due to its strong absorbance of ultra-violet light (190–280 nm). SAXS experiments characterized the conformations of Glu-Plg in the presence of 50 mM 6-AHA, t-AMCHA, and benzamidine. DLLS experiments studied the effects of 6-AHA (100 mM) and benzamidine (50 mM) on the conformations of Glu-Plg and all fragments except K5-SP.

Size-Exclusion HPLC. Figure 3 shows the molecular elution times of Glu- and Lys-Plg, K5-SP, and kringle-containing fragments K1–3 and K1–5, both with and without 50 mM 6-AHA. Addition of 6-AHA to Glu-Plg caused a significant decrease in τ_E-values from 8.93 ± 0.01 to 8.32 ± 0.01 min, representing a structural alteration from a "smaller" to "larger" molecular shape. This large-scale conformational change is fully consistent with that observed by other

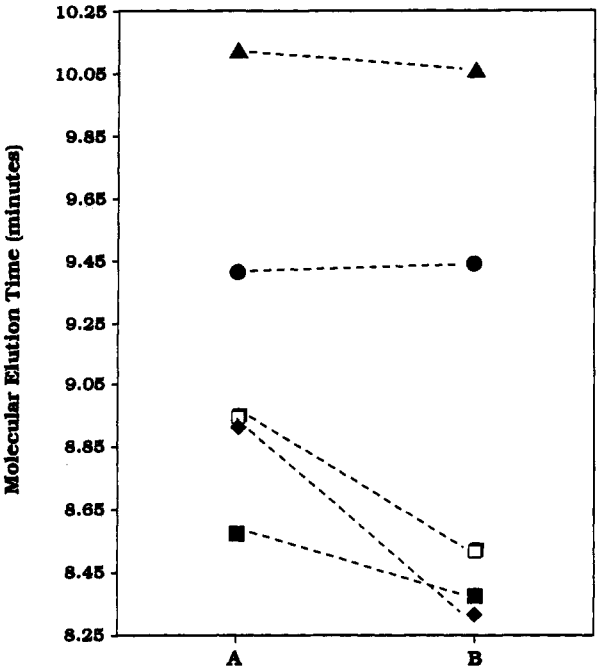

FIGURE 3: Molecular elution times measured by SE-HPLC in the absence (A) or presence (B) of 50 mM 6-aminohexanoic acid for Glu-plasminogen (◆), Lys-plasminogen (■), K1–5 (□), K1–3 (●), and K5-SP (▲). Error bars showing the standard deviation of 10 replicates are contained within the symbols.

techniques, for example, sedimentation analysis (Markus *et al.*, 1978). Data were equivalent for four different Glu-Plg preparations. No further changes in τ_E-values occurred when the 6-AHA concentration was increased to 100 mM (data not shown). No changes in τ_E-values were observed for six nonallosteric protein standards (α_2-antiplasmin, ovalbumin, bovine serum albumin, cytochrome *c*, soybean trypsin inhibitor, and aldolase, data not shown), demonstrating that changes in τ_E-values are due solely to changes in protein conformation.

Smaller changes in elution time were also noted for both Lys-Plg and K1–5 but not for fragments K5-SP and K1–3 (Figure 3). These results are statistically significant and indicative of conformational changes occurring within both

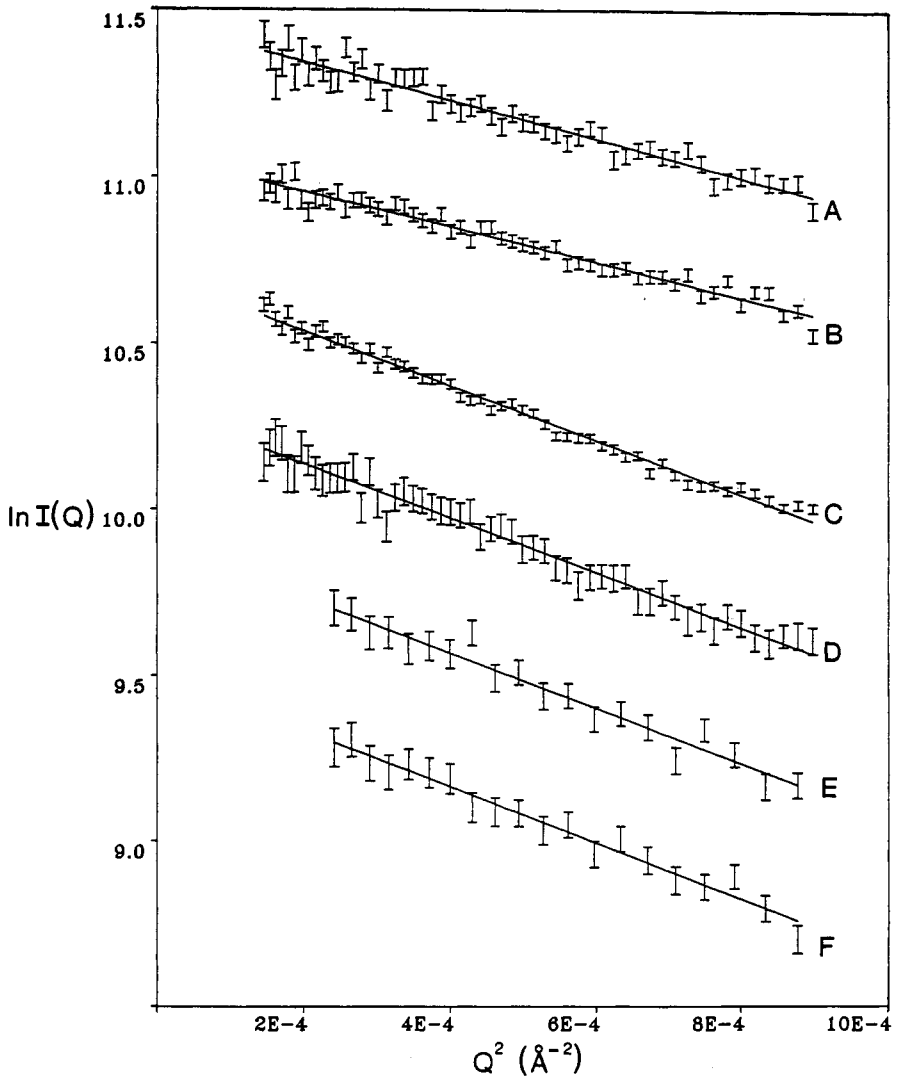

FIGURE 4: Guinier plots ($\log_e I(Q)$ vs Q^2) for Glu-plasminogen at protein concentrations of 10 mg cm^{-3} (data sets A, C, and E) and 5 mg cm^{-3} (B, D, and F). Data sets A and B, C and D, and E and F correspond to Glu-plasminogen plus 50 mM benzamidine, plus 50 mM t-AMCHA, and plus 50 mM 6-AHA, respectively. Data have been scaled vertically. Error bars represent the error in counting statistics only. Values for the molecular radius of gyration were calculated from the linear least-squares fits to the data (see text).

Lys-Plg and K1–5 fragments. Furthermore, they are consistent with the results of DLLS experiments (Figure 5).

Concentrations of 6-AHA up to 100 mM were shown by SE-HPLC not to cause molecular aggregation, in agreement with the results of Barlow *et al.* (1984). Structural parameters obtained by DLLS and SAXS therefore correspond to the monomeric species only.

Small-Angle X-ray Scattering. The Guinier plots of human Glu-Plg samples containing 50 mM benzamidine (data sets A and B), 50 mM t-AMCHA (data sets C and D), and 50 mM 6-AHA (data sets E and F) are shown in Figure 4. Molecular radii of gyration, calculated using Guinier analyses of the linear portions of these data ($0.012 \leq Q \leq 0.030$ Å^{-1}), are given in Table 1 together with the value for the R_G of unliganded Glu-Plg, which we reported previously (Ponting *et al.*, 1992a). The values of the reduced χ^2 for the linear least-squares fit to the data were all approximately 1, as required. Scattering curves and radii of gyration for each set of conditions showed no protein concentration dependence; hence, no extrapolation of data to zero concentration was required.

The accuracy of Guinier analysis is ensured if data points are acquired above a minimum value of Q: $Q_{min} < \pi/D$ where D is the maximum intramolecular distance (Glatter & Kratky, 1982). For these experiments, this criterion is satisfied as $Q_{min} = 0.012$ Å^{-1}, and hence, $D < 260$ Å, which is larger than the published maximum distance for liganded Glu-Plg (Mangel *et al.*, 1990). The error in R_G caused by the use of the Guinier approximation over the range ($0.012 \leq Q \leq 0.030$ Å^{-1}) is estimated as approximately 2–4% (Glatter & Kratky, 1982).

Table 1: Radius of Gyration of Glu-Plasminogen with Different Ligands, Determined Using the Method of Guinier (1939)

data set	ligand (50 mM)	prot. conc. (mg cm^{-3})	radius of gyration (R_G, Å)[a]	Q range (Å^{-1})
	no ligand[b]	10.0	30.7 ± 0.1	$0.022 \leq Q \leq 0.060$
	no ligand[b]	6.5	30.5 ± 0.2	$0.022 \leq Q \leq 0.060$
A	benzamidine	10.0	42.1 ± 0.6	$0.012 \leq Q \leq 0.030$
B	benzamidine	5.0	40.2 ± 0.5	$0.012 \leq Q \leq 0.030$
C	t-AMCHA	10.0	49.8 ± 0.3	$0.012 \leq Q \leq 0.030$
D	t-AMCHA	5.0	49.7 ± 0.8	$0.012 \leq Q \leq 0.030$
E	6-AHA	10.0	50.1 ± 1.5	$0.016 \leq Q \leq 0.030$
F	6-AHA	5.0	50.4 ± 1.6	$0.016 \leq Q \leq 0.030$

[a] The errors quoted for individual measurements are due to counting statistics only. Errors due to the use of the Guinier approximation were estimated as ≈2–4%, using a rodlike approximation for the plasminogen structure (Glatter & Kratky, 1982). [b] Values for nonliganded plasminogen are taken from Ponting *et al.* (1992a).

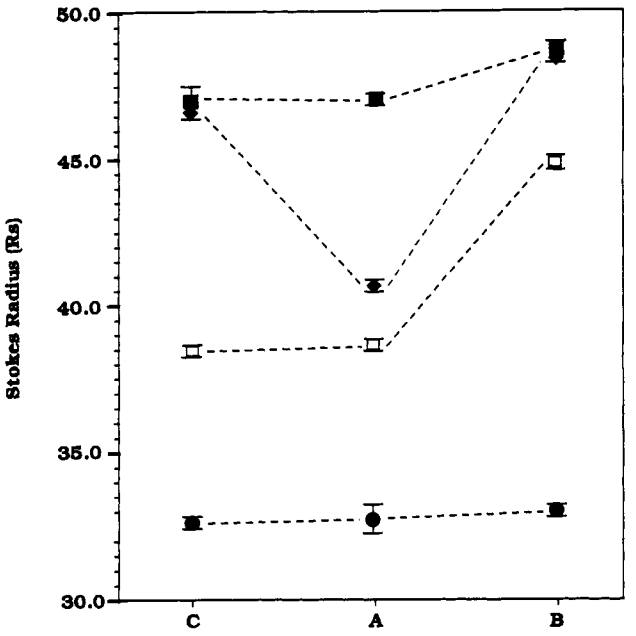

FIGURE 5: Stokes radii measured by DLLS in the absence of ligand (A) or the presence of 100 mM 6-aminohexanoic acid (B) or 50 mM benzamidine (C) for Glu-plasminogen (◆), Lys-plasminogen (■), K1–5 (□), and K1–3 (●).

From these results, it is apparent that the conformations of Glu-Plg in the presence of either 50 mM 6-AHA or 50 mM t-AMCHA are likely to be similar. However, it is also apparent that Glu-Plg in the presence of 50 mM benzamidine ($R_G \simeq 40$ Å) differs from both native Glu-Plg ($R_G \simeq 30$ Å) and 6-AHA- or t-AMCHA-liganded Glu-Plg ($R_G \simeq 50$ Å). As for native Glu-Plg (Ponting *et al.*, 1992a), our measured R_G for t-AMCHA-liganded Glu-Plg is less than that found by Mangel *et al.* (1990). The reasons for these differences remain unknown.

Dynamic Laser Light Scattering. The Stokes radii of Glu-Plg, Lys-Plg, and kringle-containing fragments K1–3 and K1–5 were measured in the absence and presence of 50 mM benzamidine or 100 mM 6-AHA (Figure 5). Titration experiments showed no significant difference between R_S-values for 6-AHA ligand concentrations of 10 and 100 mM; hence, results obtained by SE-HPLC, DLLS, and SAXS using different ligand concentrations are comparable.

Data derived by DLLS for all samples plus and minus 6-AHA correspond to SE-HPLC results (Figure 3): Glu-Plg undergoes a large conformational change (R_S = 40.6 ± 0.3 to 48.5 ± 0.3 Å) upon the addition of 100 mM 6-AHA and Lys-Plg undergoes a smaller change (R_S = 47.0 ± 0.3 to 48.6 ± 0.4 Å) as does the fragment K1–5 (R_S = 38.6 ± 0.3 to 44.9 ± 0.3 Å). Fragment K1–3 does not appear to undergo a measurable conformational change in the presence of 6-AHA.

Stokes radius measurements obtained using 50 mM benzamidine differ substantially from values obtained using 100 mM 6-AHA. This concentration of benzamidine does not appear to induce conformational changes in Lys-Plg, K1–3, and K1–5 samples. The benzamidine-induced conformational change in Glu-Plg (R_S = 40.6 ± 0.3 to 46.6 ± 0.3 Å) is significantly smaller than that induced by 100 mM 6-AHA. This is not due to an insufficient concentration of benzamidine, since concentrations greater than 50 mM produced no further conformational effect (data not shown) which is in agreement with the results of Holleman *et al.* (1975).

Addition of 6-AHA (>0.01 mM) to Glu-Plg samples containing 50 mM benzamidine produced an increase in Stokes radius from 46.6 ± 0.3 to 48.9 ± 0.7 Å. These results demonstrate that addition of 50 mM benzamidine to Glu-Plg induces the adoption of a second, partially extended conformation, which is dissimilar from a third, fully extended conformation, adopted when in the presence of 6-AHA (± benzamidine).

DISCUSSION

The conformational change of Glu-Plg upon the addition of >1 mM 6-AHA or t-AMCHA has been previously studied using a variety of techniques (Alkjaersig, 1964; Castellino *et al.*, 1973; Christensen & Mølgaard, 1992). We have investigated this change using size-exclusion HPLC, small-angle X-ray scattering, and dynamic laser light scattering. The increase in Stokes radius by DLLS upon the addition of 10 mM 6-AHA was measured as 40.6 ± 0.3 to 48.5 ± 0.3 Å, which is consistent with previous values obtained using sedimentation analysis (Bányai & Patthy, 1984; Barlow *et al.*, 1984). The equivalent change in radius of gyration measured by SAXS for 50 mM 6-AHA or t-AMCHA was found to be 30.7 ± 0.1 to 49.8 ± 0.3 Å.

Spiral-type Glu-Plg structures, imaged by electron microscopy (Tranqui *et al.*, 1979; Weisel *et al.*, 1994), have been found to be consistent with results of SAXS and molecular modeling studies (Ponting *et al.*, 1992a) and biochemical intramolecular cross-linking studies (Bányai & Patthy, 1984, 1985). A spiral model of Glu-Plg offers a structural explanation for the large 6-AHA-induced conformational change [for a detailed discussion, see Ponting *et al.* (1992b)]. A spiral domain arrangement within Glu-Plg implies proximity of the NH_2-terminal peptide to the C-terminal domains, allowing for a postulated intramolecular and lysine-dependent interaction between the NTP and the AH-site of kringle 5 (Christensen, 1984). The NTP–K5 interaction is disrupted upon the addition of those ligands which bind kringle 5, thereby initiating a large change in conformation.

This proposed mechanism additionally accounts for the conformational change induced by 50 mM benzamidine (Figure 5), also observed by Holleman *et al.* (1975). Benzamidine binds the LBS of kringle 5 (Thewes *et al.*, 1990) but not the LBS of kringles 1–4 (Váradi & Patthy, 1981; Rejante *et al.*, 1991a). The benzamidine-induced conformation of Glu-Plg, however, is not equivalent to that induced by 6-AHA (Figure 5); addition of 6-AHA to benzamidine-liganded Glu-Plg is required to induce the fully extended conformation. It is apparent that Glu-Plg may adopt at least three conformations. We propose to define these conformations as: Glu-plasminogen (α) (in the absence of ligands), Glu-plasminogen (β) (in the presence of 50 mM benzamidine), and Glu-plasminogen (γ) (in the presence of ≥10 mM 6-AHA or t-AMCHA (± benzamidine)) (Table 2).

Demonstration of three conformations of Glu-Plg allows interpretation of the 6-AHA-induced conformational changes within Lys-Plg and K1–5 observed by SE-HPLC and DLLS (Figures 3 and 5) and sedimentation analysis (Markus *et al.*, 1979; Rickli & Otavsky, 1975). Similar values for the Stokes radius of Lys-Plg (R_S = 47.0 ± 0.3 Å) and Glu-Plg with benzamidine (R_S = 46.6 ± 0.3 Å) suggest that these molecules both adopt β-conformations, differing only by the absence of the NTP in Lys-Plg. The absence of a conformational change within Lys-Plg upon the addition of benzamidine (Figure 5) supports this interpretation. Addition of 6-AHA to Lys-Plg therefore effects a β- to γ-conformational change. An

Table 2: Changes in Conformation of Glu- and Lys-Plasminogen and Kringles 1–5 Measured by SE-HPLC (τ_E), DLLS (R_S), SAXS (R_G)

	Glu-plasminogen	Lys-plasminogen	kringles 1–5
α-conformation	no ligand τ_E = 8.93 ± 0.01 min R_S = 40.6 ± 0.3 Å R_G = 30.7 ± 0.2 Å		
β-conformation	+ benzamidine[a] τ_E = ND[c] R_S = 46.6 ± 0.3 Å R_G = 42.1 ± 0.6 Å	no ligand τ_E = 8.58 ± 0.01 min R_S = 47.0 ± 0.3 Å R_G = ND[c]	no ligand τ_E = 8.94 ± 0.01 min R_S = 38.6 ± 0.3 Å R_G = ND[c]
γ-conformation	+ 6-AHA[b] τ_E = 8.32 ± 0.01 min R_S = 48.5 ± 0.3 Å R_G = 50.1 ± 0.6 Å	+ 6-AHA[b] τ_E = 8.38 ± 0.01 min R_S = 48.6 ± 0.3 Å R_G = ND[c]	+ 6-AHA[b] τ_E = 8.54 ± 0.01 min R_S = 44.9 ± 0.3 Å R_G = ND[c]

[a] 50 mM benzamidine. [b] 50 mM (SE-HPLC and SAXS) or 100 mM (DLLS) 6-AHA. [c] ND-not determined.

equivalent mechanism is suggested for a β- to γ-conformational change in K1–5.

Our data establish the existence of at least three plasminogen conformations differing by the occupation or otherwise of two LBS by two intramolecular binding ligands. The similarity of Lys-Plg and benzamidine-liganded Glu-Plg conformations and the dissimilarity of these with the unliganded Glu-Plg conformation establish the AH-site of kringle 5 as the first of these intramolecular binding sites and the NTP as possessing its binding ligand.

The conformational change of K1–5 (this work; Rickli & Otavsky, 1975), but not of K1–3 (this work; Ramakrishnan *et al.*, 1991), with 6-AHA establishes the K3–5 region as possessing both the second intramolecular binding site and its ligand. This leads directly to the assignment of the kringle 4 LBS as the second binding site since the kringle 3 LBS possesses a very low 6-AHA affinity (Matsuka *et al.*, 1990) and since the kringle 5 AH-site has already been assigned an independent function. This assignment is substantiated by the results of others that access to the kringle 4 LBS is sterically hindered in plasminogen, but not in the isolated kringle (Plow & Collen, 1981; Vâli & Patthy, 1982), and that binding of a mAb to the kringle 4 LBS results in acceleration of Glu-Plg activation by two-chain urokinase-type plasminogen activator (Cummings & Castellino, 1985).

A region of kringle 3 (-Cys_{256}-Leu-Lys-Gly-Thr-) is tentatively proposed as the ligand for the second intramolecular binding site, since it is identical to a region of tetranectin (-Cys_{20}-Leu-Lys-Gly-Thr-); tetranectin possesses substantial affinity for plasminogen via the kringle 4 LBS (Clemmensen *et al.*, 1986). Molecular modeling of kringle 3 from the crystallographic structure of kringle 4 (Mulichak *et al.*, 1991) has indicated that this ligand possesses the required stereochemistry for a kringle 4 binding ligand (results not shown).

DLLS experiments showed that >0.01 mM 6-AHA is required to induce the β- to γ-conformational change in benzamidine-liganded Glu-Plg. This concentration of 6-AHA does not induce the α- to β-change. This demonstrates that the two binding sites, which regulate the two large-scale structural changes, are positively cooperative. The changes are ordered, sequential ($\alpha \Leftrightarrow \beta \Leftrightarrow \gamma$), and preclude a fourth conformation, in which only the NTP–K5 intramolecular interaction is maintained. A similar conclusion has been reached independently by Christensen and Mølgaard (1992).

The finding that Glu-Plg may adopt three conformations *in vitro* may serve to illuminate the specificity of plasminogen activation *in vivo*. The binding of Glu-Plg to intact fibrin accelerates its activation to plasmin (Walther *et al.*, 1975; Peltz *et al.*, 1982) due to a large conformational change and a concomitant increase in the accessibility of its activation peptide bond to plasminogen activators. This conformational change is likely to be of the α- to β-type, with fibrin competing with the NTP ligand for the kringle 5 AH-site. This is argued since kringle 5 possesses the highest affinity of plasminogen kringles for intact fibrin (Thorsen *et al.*, 1981; Wu *et al.*, 1990) and the NTP is considered to be bound internally to kringle 5 within Glu-Plg (α) (see above).

Fibrin-induced acceleration of plasminogen activation is known to initiate plasmin degradation of fibrin and concomitant generation of C-terminal lysine residues, which act as further plasmin(ogen)-binding sites (Christensen, 1985; Fleury & Anglés-Cano, 1991). We postulate that binding of Glu-plasmin(ogen) (β) to these C-terminal lysines via the kringle 4 LBS induces a β- to γ-conformational change due to displacement of its intramolecular binding ligand. Although the significance of the β- to γ-structural change to fibrinolysis is not readily apparent, it may be important for those plasmin-dependent processes which involve molecules possessing affinity for the kringle 4 LBS, which include tetranectin (Clemmensen *et al.*, 1986), collagen type IV (Stack *et al.*, 1992), and endothelial cell surface receptors (Gonzalez-Gronow *et al.*, 1989).

These postulated mechanisms of conformational change make no mention of the kringle 1 LBS. It is suggested that this site participates not by regulating conformation but by forming complexes with α_2-antiplasmin and histidine-rich glycoprotein. The binding of α_2-antiplasmin to kringle 1 does not interfere with the binding and activation of plasminogen on the fibrin surface but instead decreases the generation of C-terminal lysine-binding sites by the inhibition of plasmin (Anglés-Cano *et al.*, 1992).

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