Conformation of Lys-Plasminogen and the Kringle 1–3 Fragment of Plasminogen Analyzed by Small-Angle Neutron Scattering†

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ABSTRACT: Native human Glu-plasminogen (Glu_1–Asn_{791}) was previously shown to have a radius of gyration of 39 Å and a shape best described by a prolate ellipsoid [Mangel, W. F., Lin, B., & Ramakrishnan, V. (1990) *Science 248*, 69–73]. Upon occupation of a weak lysine-binding site, the shape reversibly changes to that best described by a Debye random coil with a radius of gyration of 56 Å. Conversion from the closed to the open form is not accompanied by any change in secondary structure, hence the closed conformation is formed by interaction between domains, the five kringles and the protease domain, and this is abolished upon conversion to the open form. Here we analyzed by small-angle neutron scattering the conformations of human Lys-plasminogen (Lys_{78}–Asn_{791}) and the fragment K1–3 that contains the first three kringles of plasminogen (Tyr_{80}–Val_{338} or Tyr_{80}–Val_{354}). The shape of Lys-plasminogen was best described by a Debye random coil with a radius of gyration of 51 Å, and occupation of its lysine-binding sites by 6-aminohexanoic acid did not dramatically alter its conformation. Thus Lys-plasminogen was in the open form, similar to that of Glu-plasminogen with its lysine-binding sites occupied. The fragment K1–3 in the absence or presence of 6-aminohexanoic acid had a shape best described equally either by an elongated prolate ellipsoid or by a Debye random coil, with a radius of gyration of 29 Å. Our model for the two forms of plasminogen is that, in the closed form, domain interaction generates a compact, almost globular, structure. Upon ligand binding or removal of the NH_2-terminal peptide (Glu_1–Lys_{77}), domain interaction is abolished, but the domains remain intact. Flexibility in the polypeptide chains between the domains then gives rise to the extended and flexible structure that is characteristic of the open form. The structural differences between the closed and open forms of plasminogen are discussed in terms of functional differences relevant to the regulation of plasminogen activation.

Native human plasminogen, Glu-plasminogen,[1] is a single-chain polypeptide of 791 amino acids, including an NH_2-terminal glutamic acid (Forsgren et al., 1987). Plasmin, a serine protease, is formed by a plasminogen activator upon cleavage of the arginyl–valyl bond 561 amino acids from the NH_2 terminus of Glu-plasminogen (Robbins et al., 1967). The resulting two polypeptides, the 561 amino acid A chain and the 230 amino acid B chain, are held together by two disulfide bridges. There are six domains in plasminogen. At the NH_2 terminus are five kringles, triple-loop structures of about 80 amino acids constrained by three disulfide bridges (Sottrup-Jensen et al., 1978). The kringles have a high degree of sequence similarity and are autonomous structural and folding domains (Castellino et al., 1981; Ploplis et al., 1981; Novokhatny et al., 1984; Trexler & Patthy, 1983) that have evolved by exon shuffling (Patthy, 1985). The proteinase domain, which is homologous to chymotrypsin, is located at the C-terminal end. Plasmin can catalytically cleave Glu-plasminogen at the lysyl–lysyl bond at position 77–78, yielding a small NH_2-terminal peptide and the 714 amino acid variant Lys-plasminogen (Wallen & Wiman, 1972; Robbins et al., 1967).

Certain ω-amino acids bind to lysine-binding sites on Glu- and Lys-plasminogens. There are two classes of binding sites on Glu-plasminogen for 6-aminohexanoic acid, one strong site in kringle 1 (Lucas et al., 1983a,b) with a dissociation constant, K_d, of 9 μM and five weaker sites with a K_d of 5 mM (Markus et al., 1978a). With Lys-plasminogen there are also six binding sites, one with a K_d of 35 μM, one with a K_d of 0.26 mM, and four with a K_d of 10 mM (Markus et al., 1978b). Plasminogen is targeted by way of its lysine-binding sites, because it is through these sites that plasminogen binds to fibrin (Thorsen, 1975; Lucas et al., 1983a; Suenson & Thorsen, 1982; Bok & Mangel, 1985), to the extracellular matrix (Knudsen et al., 1986), and to the cell surface (Plow et al., 1986).

The presence of certain ω-amino acids alters the kinetics of activation of plasminogen. The rate of activation of Glu-plasminogen by urokinase, u-PA, increases 10-fold in the presence of concentrations of 6-aminohexanoic acid sufficient to saturate a weak lysine-binding site (Markus et al., 1978b; Claeys & Vermylen, 1974; Wallen & Wiman, 1975; Walther

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[1] Abbreviations: Glu-plasminogen, native plasminogen with an NH_2-terminal glutamic acid, Glu_1-Asn_{791}; HBS, Hepes-buffered saline; K1–3, a fragment of plasminogen containing the first three kringles, Tyr_{80}–Val_{338} or Tyr_{80}–Val_{354}; K4, a fragment of plasminogen containing the fourth kringle, Val_{355}–Ala_{440}; Lys-plasminogen, plasminogen with an NH_2-terminal lysine that is produced by plasmin cleavage of the lysyl–lysyl bond at position 77–78 of Glu-plasminogen, Lys_{78}–Asn_{791}; mini-plasminogen, a fragment of plasminogen containing the fifth kringle plus the protease domain, Val_{443}–Asn_{791}; NH_2-terminal peptide, the NH_2-terminal peptide of native plasminogen, Glu_1–Lys_{77}; R_g, radius of gyration; t-PA, tissue plasminogen activator; u-PA, urokinase.

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et al., 1975). The rate of activation of Lys-plasminogen by u-PA is 10-fold higher than that of Glu-plasminogen and is not affected by the presence of 6-aminohexanoic acid. Occupation of a weak lysine-binding site in Glu-plasminogen lowers the K_m for activation with u-PA by 10-fold (Peltz et al., 1982; Lijnen et al., 1984; Banyai & Patthy, 1985; Urano et al., 1987a,b) down to the physiological concentration of plasminogen (Sherry, 1968; Rabiner et al., 1969).

The binding of certain ω-amino acids alters some of the physical properties of Glu-plasminogen but not of Lys-plasminogen. The presence of 6-aminohexanoic acid causes a reversible decrease in the sedimentation coefficient (Alkjaersig, 1964) from 5.75 to 4.85 S (Brockway & Castellino, 1972; Violand et al., 1978), an increase of 7% in the intrinsic fluorescence, and a decrease in the rotational relaxation time from 262 to 158 ns (Castellino et al., 1973). The concentration of 6-aminohexanoic acid required to produce half the change in the sedimentation coefficient or intrinsic fluorescence is about 3 mM, which implies that occupation of a weak lysine-binding site is involved (Markus et al., 1978a). This conformational change is similar to that observed upon conversion of Glu-plasminogen to Lys-plasminogen or to Lys-plasmin. In both cases, the sedimentation coefficient is decreased, the Stokes radius is increased, and the circular-dichroism pattern in the near UV is changed (Wallen & Wiman, 1972; Violand et al., 1978; Sjoholm et al., 1973).

Although a change in the conformation of plasminogen was known to occur upon occupation of a weak lysine-binding site, the nature of this change and the precise physical dimensions involved were unknown. Recently we characterized the shape and secondary structure of Glu-plasminogen in the presence and absence of 0.05 M 6-aminohexanoic acid (Mangel et al., 1990). Here we characterize the shapes of Lys-plasminogen and the K1–3 fragment that contains the first three kringles (Tyr_{80}–Val_{338} or Tyr_{80}–Val_{354}). Lys-plasminogen is a physiologically important molecule, as it is formed during several of the early stages of fibrinolysis, and its formation contributes to an acceleration of plasminogen activation (Suenson & Thorsen, 1988). The shape of K1–3 was characterized to see if its conformation is sensitive to occupation of its lysine-binding sites. Small-angle scattering was employed, because it can be used to study directly and precisely conformational changes occurring in proteins in solution. Analysis of the low-angle part of the scattered intensity yields the radius of gyration and the molecular weight without the use of any assumptions, while the data at higher angles can be used to estimate the approximate shape of the molecule (Glatter & Kratky, 1982).

Materials and Methods

Human Glu- and Lys-plasminogens were purified and characterized as described in Varadi and Patthy (1983) and Bok and Mangel (1985). The fragment of plasminogen containing the first three kringles, K1–3 (Tyr_{80}–Val_{338}, Tyr_{80}–Val_{354}), was prepared by limited proteolysis of plasminogen with porcine pancreatic elastase (Sottrup-Jensen et al., 1978) as described in Varadi and Patthy (1981). All small-angle scattering experiments were performed in Hepes-buffered saline (HBS), which contained 0.01 M Hepes, pH 7.2, 0.137 M NaCl, 2.68 mM KCl, 0.91 mM $CaCl_2$, and 0.49 mM $MgCl_2$. In some cases 16.9 μM bovine pancreatic trypsin inhibitor or 1 μM dansylglutamylglycylarginyl chloromethyl ketone (Higgins & Lamb, 1986) was present. Prior to each experiment the plasminogens were fractionated on a Sephacryl S-200 column (Pharmacia) in HBS to remove possible aggregates and then concentrated by filtration under nitrogen pressure with an Amicon PM-10 ultrafiltration membrane. Concentrated protein solutions were dialyzed for 15 h, with one change of buffer, against a 50-fold volume of HBS made up in D_2O. After each experiment, the plasminogens were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970), which indicated that they remained intact (data not shown).

Small-angle neutron-scattering measurements were done on the H9B spectrometer (Schneider & Schoenborn, 1984) in the High-Flux Beam Reactor at Brookhaven National Laboratory. The mean incident wavelength varied, depending upon the experiment, from 4.5 to 7.5 Å. The protein concentration in samples varied from 1 to 5 mg/mL for Lys-plasminogen and K1–3 and was around 15 mg/mL for Glu-plasminogen. The path lengths for the samples were 1 mm in H_2O for Glu-plasminogen, 3 mm in D_2O for Glu- and Lys-plasminogens, and 4 mm in D_2O for K1–3. The transmissions were 0.77–0.78 in 3 mm of D_2O and 0.72 in 4 mm of D_2O. All measurements were performed at 4 °C.

In each experiment, the scattering was measured from the sample, a cell containing the buffer that had a transmission nearly identical to that of the sample, an empty cell, and a blocked beam. Scattered neutrons were measured on an He^3 area detector. To obtain the scattered intensity from the macromolecule, we radially averaged the scattering and corrected for background and buffer scattering. The intensity was then normalized for beam intensity (which was monitored with a low-efficiency fission detector during the measurement), thickness, transmission, and protein concentration.

The radius of gyration and forward scatter were estimated from a least-squares fit to the linear region of Guinier plots of the data [ln $[I(k)]$ vs k^2] by using the assumption that at low angles $I(k) = I(0) \exp(-k^2R_g^2/3)$. The range for the linear region was k = 0.014–0.024 $Å^{-1}$ for Glu- and Lys-plasminogens and was 0.017–0.030 $Å^{-1}$ for K1–3.

Shape analysis was performed by fitting the entire scattering curve to model functions for spheres, ellipsoids of revolution, and cylinders (Kratky, 1963) by using the nonlinear least-squares estimation algorithm of Marquardt (1963). A fit was also done to an unconstrained Gaussian (Debye coil), whose scattering function is given by

$$I(k) = 2I(0)\frac{(e^{-x} + x - 1)}{x^2}$$

where $x = \langle R^2 \rangle k^2$ and $\langle R^2 \rangle$ is the square of the radius of gyration (Debye, 1947; Kirste & Oberthur, 1982). In each case, a constant term was added as a parameter to the function to be fit, to account for any residual incoherent scattering. The radius of gyration and the forward scatter were also allowed to float during the modeling.

Results

For small-angle neutron scattering, the intensity at very small angles obeys the Guinier relationship (Guinier & Fournet, 1955). The radius of gyration, R_g, is most accurately measured in D_2O from the slope of a Guinier plot. Guinier plots of the scattering data obtained in D_2O with Lys-plasminogen in the absence and presence of 0.05 M 6-aminohexanoic acid are shown in Figure 1. The radius of gyration of Lys-plasminogen was 51 Å (Table I). In the presence of 0.05 M 6-aminohexanoic acid, the radius of gyration was 52 Å. Similar experiments with K1–3 shown in Figure 2 yielded an R_g in D_2O of 31 Å and 28 Å in the presence of 0.05 M 6-aminohexanoic acid (Table I).

The shape of a molecule and its internal structure are reflected in the higher angle scattering intensity (Glatter &

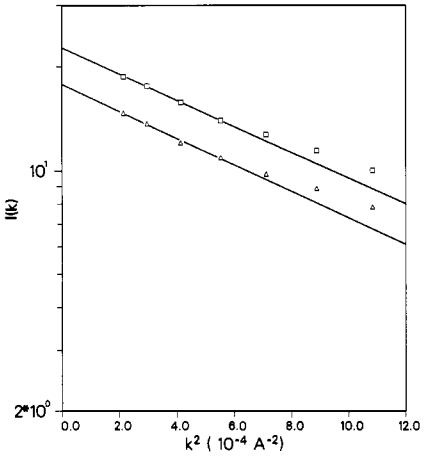

FIGURE 1: Guinier plots [ln $[I(k)]$ vs k^2] of the low-angle scattering from Lys-plasminogen in D_2O without (□) and with (△) 0.05 M 6-aminohexanoic acid. $I(k)$ is the scattered intensity as a function of $k = 4\pi \sin \theta/\lambda$, where 2θ is the scattering angle and λ is the mean wavelength.

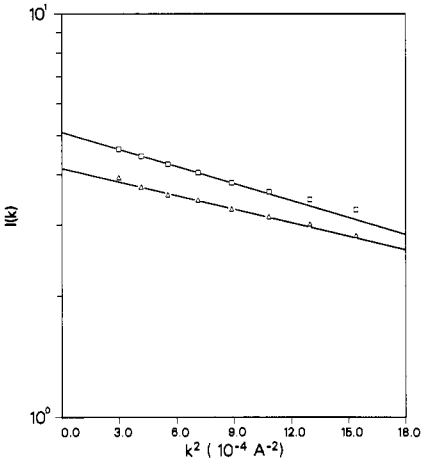

FIGURE 2: Guinier plots of the low-angle scattering from K1-3 in D_2O with (□) and without (△) 0.05 M 6-aminohexanoic acid.

Table I: Physical Parameters Obtained from Low-Angle Neutron Scattering

	radii of gyration (Å)		
parameter	Glu-pmg[a]	Lys-pmg	K1-3
(-) 6-AHA[b]	39.0 ± 0.6	51.0 ± 2.0	31.2 ± 1.0
(+) 6-AHA	56.1 ± 1.1	51.7 ± 2.0	27.8 ± 1.0

[a] Mangel et al. (1990). [b] 0.05 M 6-aminohexanoic acid.

Kratky, 1982). An estimate of the shape can be obtained by model building in which the objective is to find the shape of uniform scattering-length density whose calculated scatter most closely matches the experimentally derived scatter. The shapes tested were a sphere, a cylinder, ellipsoids of revolution, and a Debye random coil (Kirste & Oberthur, 1982). The main physical difference between a Debye random coil and a highly elongated cylinder or ellipsoid is that the Debye random coil represents an average of an ensemble of many different conformations arising from flexibility. All of the parameters including the radius of gyration were allowed to float. Buffers in D_2O were used, because they have a much better signal-to-noise ratio due to the lower incoherent scattering of D_2O compared to H_2O.

The higher angle scattering data from Lys-plasminogen in the absence and presence of 0.05 M 6-aminohexanoic acid are compared in Figure 3 to that calculated from a prolate ellipsoid and a Debye random coil. A similar analysis of the higher

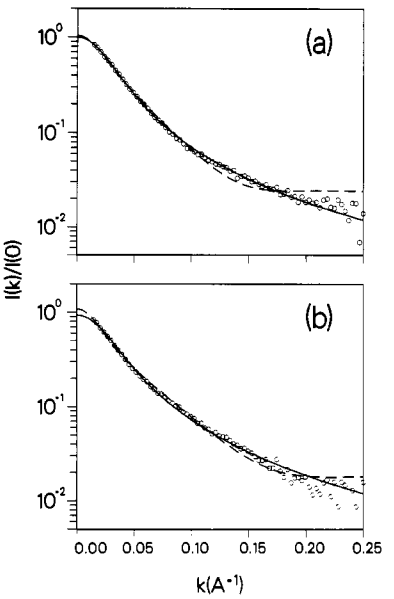

FIGURE 3: Comparison of the higher angle scattering data of Lys-plasminogen to model structures. The models are a prolate ellipsoid (dashed line) and a Debye random coil (solid line). Lys-plasminogen in the absence (A) and in the presence (B) of 0.05 M 6-aminohexanoic acid.

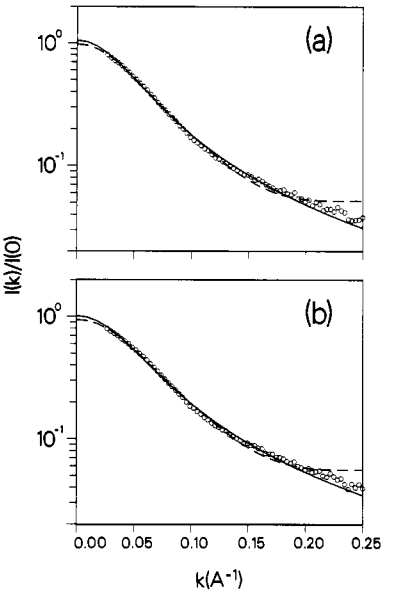

FIGURE 4: Comparison of the higher angle scattering data of K1-3 to model structures. The models are a prolate ellipsoid (dashed line) and a Debye random coil (solid line). K1-3 in the absence (A) and in the presence (B) of 0.05 M 6-aminohexanoic acid.

angle scattering data from K1-3 is shown in Figure 4. None of the data from Lys-plasminogen or K1-3 fit at all well to regular solids.

The reduced χ^2 values for the various fits are listed in Table II. They indicate that, for Lys-plasminogen in the absence or presence of 0.05 M 6-aminohexanoic acid, the best fits were to a Debye random coil. The second best fits were to an elongated ellipsoid, but in these cases the reduced χ^2 values were 5-fold higher in the absence and 2.7-fold higher in the the presence of 0.05 M 6-aminohexanoic acid. For K1-3, the higher angle scattering data in the absence or presence of 0.05 M 6-aminohexanoic acid fit equally well either to an elongated ellipsoid or to a Debye random coil.

Another indication that Lys-plasminogen behaves as a Debye random coil comes from analyzing the scattering data according to the method of Kratky (1963). In Kratky plots

Table II: Reduced χ^2 Values for the Best and the Second-Best Fits of the Scattering Data of the Different Forms of Plasminogen to Model Structures

molecule	best fit	reduced χ^2	second best fit	reduced χ^2
Lys-pmg (−) 6-AHA[a]	Debye coil	1.74	ellipsoid (244 × 46 × 46 Å)	8.7
Lys-pmg (+) 6-AHA	Debye coil	3.2	ellipsoid (200 × 38 × 38 Å)	8.7
K1-3 (−) 6-AHA	ellipsoid (124 × 38 × 38 Å)	14.86	debye coil	14.87
K1-3 (+) 6-AHA	ellipsoid (113 × 37 × 37 Å)	11.82	debye coil	13.6
Glu-pmg (−) 6-AHA	ellipsoid[b] (146 × 56 × 56 Å)	27.7	cylinder (106 × 52 Å)	39.0
Glu-pmg (+) 6-AHA	Debye coil	4.6	ellipsoid (338 × 48 × 48 Å)	76.8

[a] 0.05 M 6-aminohexanoic acid. [b] The fit to a Debye coil had a reduced χ^2 value of 257.

[$k^2I(k)$ vs k] for a structure that behaves as Debye random coil, as kR_g becomes large, $I(k)$ should approach k^{-2} asymptotically. Thus the curve should reach a plateau. The Kratky plots for Lys-plasminogen in the absence or presence of 0.05 M 6-aminohexanoic acid appear to approach k^{-2} asymptotically (Figure 5B). The slight slope for Lys-plasminogen in the absence of 6-aminohexanoic acid is possibly from a small residual amount of incoherent scattering. The concentration of Lys-plasminogen was very low, which resulted in a low signal-to-noise ratio at high k. Alternatively, there may have been a small amuont of Glu-plasminogen present, although this could not be observed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1972) or by polyacrylamide gel electrophoresis at pH 3.2 in the presence of urea (Panyim & Chalkley, 1969). Thus, it is not clear whether the differences between Lys-plasminogen in the absence or presence of 0.05 M 6-aminohexanoic acid in Figure 5B are significant in terms of the shape or flexibility of the molecules.

As a control, we analyzed the scattering data from Glu-plasminogen according to the method Kratky (1963). We had previously shown that the scattering data of Glu-plasminogen in the absence of 6-aminohexanoic acid gave a very poor fit to a Debye random coil, suggesting that it is not extended and flexible but is compact like most globular proteins (Mangel et al., 1990). As predicted, the data in the Kratky plot in Figure 5A did not approach k^{-2} asymptotically. We had also previously shown that the scattering data from Glu-plasminogen in the presence of 0.05 M 6-aminohexanoic acid gave an excellent fit to a Debye random coil, and as predicted the data in the Kratky plot in Figure 5A approached k^{-2} asymptotically.

The reduced χ^2 values for the various fits for the scattering data from Glu-plasminogen are listed in Table II and are consistent with our previous conclusions about the closed and open conformations. In the absence of 6-aminohexanoic acid, the best fit was to a prolate ellipsoid with dimensions of 146 × 56 × 56 Å. The reduced χ^2 value for a fit to a Debye random coil was 9.3-fold greater. The second best fit, which had a slightly higher reduced χ^2 value than the best fit, was to a cylinder with dimensions of 106 × 52 Å. In the presence of 0.05 M 6-aminohexanoic acid, the best fit was to a Debye random coil. The second best fit, to an ellipsoid with dimensions of 338 × 48 × 48 Å, had a reduced χ^2 value 17-fold greater.

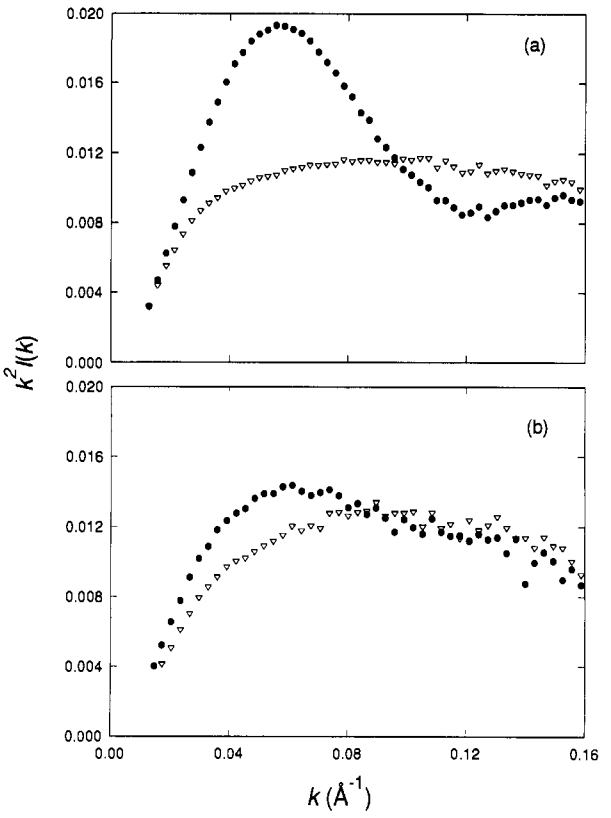

FIGURE 5: A Kratky plot of the scattering in D_2O from Glu-plasminogen (A) and Lys-plasminogen (B) without (●) and with (▽) 0.05 M 6-aminohexanoic acid. $I(k)$ is the scattered intensity as a function of $k = 4\pi \sin \theta/\lambda$, where 2θ is the scattering angle and λ is the mean wavelength.

Discussion

Previously (Mangel et al., 1990), we concluded that the shape of native Glu-plasminogen is in the form of a prolate ellipsoid with an axial ratio of 2.6:1, a radius of gyration of 39 Å, and a maximal dimension of 150 Å (Tables I and II). Upon occupation of a weak lysine-binding site by the presence of 0.05 M 6-aminohexanoic acid, the shape of this closed form changes to an open form best described by a Debye random coil with a radius of gyration of 56 Å and a maximum dimension of 240 Å. There are no intermediate conformations between the closed and open forms of Glu-plasminogen.

Here we showed that Lys-plasminogen was in the open form regardless of occupation of its lysine-binding sites and that it was in a form best described by a Debye random coil with a radius of gyration of 51 Å (Figures 1 and 3 and Tables I and II). Thus Lys-plasminogen has a shape similar to that of Glu-plasminogen in the presence of 0.05 M 6-aminohexanoic acid.

The fragment K1–3 had a radius of gyration of about 30 Å and a shape best described equally by either an elongated prolate ellipsoid or a Debye random coil (Figures 2 and 4 and Tables I and II). This fragment is less than half the size of Glu- or Lys-plasminogen, and it is difficult to distinguish between an elongated ellipsoid and a flexible structure consisting of only three domains. From our data, it is not clear whether the three domains are free to move with respect to each other or are constrained.

For the shape analysis with the higher angle scattering data, all the variables were allowed to float. The best fit for Lys-plasminogen was to a Debye random coil with R_g values of 52.6 Å in the absence and 49.8 Å in the presence of 0.05 M 6-aminohexanoic acid. These values were close to those ob-

tained independently from the Guinier plots (Figure 1 and Table I). Similarly, the R_g values for K1–3 obtained from modeling, 32.2 Å in the absence and 29.9 Å in the presence of 0.05 M 6-aminohexanoic acid, were close to those obtained from the Guinier plots (Figure 2 and Table I). Also, the values for $I(0)$ from the modeling studies of Lys-plasminogen and K1–3 were in good agreement with those obtained from the Guinier plots.

In the Kratky plots of Lys-plasminogen (Figure 5B), the curve in the presence of 0.05 M 6-aminohexanoic acid clearly approaches k^{-2} asymptotically, but there is a slight slope to the curve of Lys-plasminogen in the absence of 6-aminohexanoic acid. Although this slight slope could be due to the experimental conditions as mentioned under Results, it could also indicate that Lys-plasminogen in the absence of 6-aminohexanoic acid is not as fully extended as it is in the presence of 0.05 M 6-aminohexanoic acid. There is some evidence that is consistent with the latter interpretation. The sedimentation coefficient of Lys-plasminogen decreases from 4.66 to 4.15 S in the presence of 0.01 M tranexamic acid (Markus et al., 1979). Under similar conditions, the sedimentation coefficient of Glu-plasminogen decreases from 5.51 to 4.15 S. Also, the lysine-binding site in kringle 4 is inaccessible to chemical modification by 1,2-cyclohexanedione in Lys-plasminogen, but it is accessible to modification in isolated K4 and in the fragment K4 + miniplasminogen (Vali & Patthy, 1982). Thus in Lys-plasminogen there may be an interaction between kringles 1–3 and the kringle 4 domain.

When we measured the secondary structure of Glu-plasminogen in the absence and presence of 0.05 M 6-aminohexanoic acid by vacuum ultraviolet circular dichroism, we observed no difference (Mangel et al., 1990). Since the structure within the domains does not change upon conversion from the closed to open form, we interpreted this to indicate that the structure of the closed form of plasminogen is formed by domain interaction and that upon conversion to the open form this interaction is abolished.

Kringles are autonomous structural, functional, and folding domains, so that it is not unreasonable that the polypeptide backbone within them does not change upon ligand binding (Patthy et al., 1984). The triple-loop structure of a kringle is rigidly held together by three disulfide bridges that permit little movement of the peptide chains. Thus the flexibility of the open form of plasminogen must arise from those parts of the polypeptide chain between domains, once domain interaction has been abolished by ligand binding. Consistent with this interpretation are the observations that the peptide chains connecting kringle 3 to kringle 4 and kringle 4 to kringle 5 are selectively attacked by elastase, thermolysin, and subtilisin (Sottrup-Jensen et al., 1978). Freedom of movement of peptide chains is a precondition for their susceptibility to limited proteolysis in native proteins (Neurath, 1975).

There is other evidence consistent with our conclusion that the domains interact in the closed form of plasminogen but not in the open form. Upon conversion from Glu- to Lys-plasminogen, the high-affinity lysine-binding site becomes weaker, from a K_d of 9 to 35 μM, and one of the weak sites increases in affinity about 20-fold, from a K_d of 5 to 0.26 mM (Markus et al., 1978a,b, 1979). Yet isolated K1 and K4 bind 6-aminohexanoic acid with high affinity, with K_d values of 16 and 36 μM, respectively (Lerch et al., 1980). Secondly, the rate of formation of an intramolecular cross-link between Lys_{204} of the kringle 2 domain and Tyr_{672} of the protease domain is significantly decreased when plasminogen is in the open form compared to when it is in the closed form (Banyai & Patthy, 1984, 1985). Furthermore, this intramolecular cross-link in Glu-plasminogen prevents conversion to the open form by the presence of 6-aminohexanoic acid or by removal of the NH_2-terminal peptide at Lys_{77}. Lastly, Glu- and Lys-plasminogens bind to anti-K4 antibodies about 20 and 4 times, respectively, less strongly than does isolated K4 (Hockschwender & Laursen, 1981).

Our observation that the open conformation can best be described as a Debye random coil implies that in this structure there is little or no interaction between the domains. Consistent with this conclusion are differential scanning calorimetric analyses of Lys-plasminogen and its fragments that show that the ΔH_{cal} of thermal denaturation of Lys-plasminogen is equal to the sum of the ΔH_{cal} of thermal denaturation of K1–3, K4, and Val_{442}-plasminogen (Castellino et al., 1981; Novokhatny et al., 1984). Secondly, a monoclonal antibody to the lysine-binding site in K4 was used to show that the dissociation constant for 6-aminohexanoic acid and kringle 4 in Lys-plasminogen was the same as that for isolated K4 (Cole & Castellino, 1984).

Although the open forms of plasminogen fit best to Debye random coils, this does not mean that the molecules contain random polypeptide chains or are denatured. Rather, the good fit to a Debye random coil is evidence that these molecules are extended and flexible. Since the secondary structure of the closed form of Glu-plasminogen is unaltered upon conversion to the open form by occupation of its lysine-binding sites, the conformations within the six domains of plasminogen do not change upon ligand binding. Thus the data suggest that in the open forms plasminogen consists of independent domains, the five kringles and the protease domain, that are connected by flexible linker peptides. In the closed form of Glu-plasminogen, these domains are constrained with respect to each other, perhaps by way of a crucial interaction with the NH_2-terminal peptide. This interaction can be abolished by removal of the NH_2-terminal peptide or by occupation of a weak lysine-binding site.

How do the structural differences we characterized between the closed and open forms of plasminogen result in functional differences relevant to the regulation of the activation of plasminogen? The activation of plasminogen is targeted to occur at sites to be destroyed by plasmin and regulated so that activation occurs at the target and most importantly not elsewhere. There are two characteristics of the closed form of Glu-plasminogen relevant to this. First, it is a conformation that is not readily activatable (Peltz et al., 1982). Its K_m with u-PA is much higher than the in vivo concentration of plasminogen (Sherry, 1968; Rabiner et al., 1969), so that little or no activation of plasminogen occurs under normal physiological conditions. The high K_m may be due to interactions among the kringles and the protease domain that mask the plasminogen activator binding site. Secondly, its strong lysine-binding site in kringle 1 is functional such that binding initially occurs to targets through this site and not the other lysine-binding sites, which are 500-fold weaker. For preferential activation of plasminogen at a target to be destroyed by plasmin, Glu-plasminogen need not be in the open conformation. For example, on fibrin there is a high concentration of plasminogen-binding sites for kringle 1, one per fibrin monomer (Bok & Mangel, 1985). Upon binding, the local concentration of Glu-plasminogen is greatly increased so that bound plasminogen is preferentially activated to plasmin over Glu-plasminogen in solution. This will not occur, for example, with Glu-plasminogen bound to fibrinogen, because those complexes are soluble, and therefore no high local concen-

tration of bound plasminogen accrues. Initial cleavages of fibrin by plasmin create additional plasminogen-binding sites, carboxy-terminal lysine residues, that can lead to further increases in the local concentration of plasminogen.

The open forms of plasminogen appear only after some plasmin is generated, as during the initial stages of dissolution of a fibrin clot. This plasmin begins to convert soluble or bound Glu-plasminogen to Lys-plasminogen (Suenson & Thorsen, 1988). Lys-plasminogen binds to fibrin with an even greater affinity that Glu-plasminogen (Bok & Mangel, 1985). The open form of Glu-plasminogen can presumably be formed only if the strong lysine-binding site in kringle 1 and the weak lysine-binding site in kringle 4, whose occupation leads to the conformational change (Vali & Patthy, 1982), are both occupied in the same molecule. There is evidence for bridging of one molecule of plasminogen between two molecules of fibrin (Garman & Smith, 1982; Suenson & Petersen 1989), and this might lead to occupation of the lysine-binding sites in kringle 1 and kringle 4 of the same molecule of plasminogen. The formation of Lys-plasminogen and the open form of Glu-plasminogen leads to an acceleration in the local rate of plasmin formation, because their Michaelis constants for activation by u-PA are 10-fold lower than that for the closed form of Glu-plasminogen (Peltz et al., 1982). The K_m may be lower, because the increase in flexibility of the open form may facilitate the binding of urokinase (Peltz et al., 1982). As long as plasmin remains bound to fibrin through kringle 1, it is protected from inhibition by α_2-antiplasmin (Wiman & Collen, 1978).

From this point of view, the closed form of Glu-plasminogen is designed for targeting, and it is activated only if there is a high local concentration of plasminogen-binding sites, a criterion not met by soluble proteins but one met by higher ordered structures such as fibrin (Suenson & Thorsen, 1982; Bok & Mangel, 1985), the extracellular matrix (Knudsen et al., 1986) and the cell surface (Plow et al., 1986). The open forms of plasminogen occur only under highly specific conditions: kringle 1 in Glu-plasminogen must be bound and the structure to which kringle 4 binds must be within a restricted radius of that binding site. Lys-plasminogen appears only after a small amount of plasmin has accrued at the target. Thus the open forms are designed to accelerate the rate of activation of plasminogen at the target.

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^{31}P ENDOR Studies of Xanthine Oxidase: Coupling of Phosphorus of the Pterin Cofactor to Molybdenum(V)†

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ABSTRACT: ^{31}P ENDOR spectra are described for three different molybdenum(V) species in reduced xanthine oxidase samples. The spectra were not affected by removing the FAD from the enzyme, implying that this is located at some distance from molybdenum. Furthermore, in confirmation of the work of J. L. Johnson, R. E. London, and K. V. Rajagopalan [(1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6493-6497], NMR and chemical analysis of the phosphate content of highly purified xanthine oxidase showed there are only three phosphate residues per subunit of the enzyme. It is concluded that the ENDOR features are due to hyperfine coupling of the phosphate group of the pterin cofactor to the molybdenum atom. Evaluation of the dipolar component of the coupling has permitted estimation of the molybdenum-phosphorus distances as 7-12 Å. This implies that the cofactor is in an extended conformation in the enzyme molecule. Less detailed ^{31}P ENDOR data on sulfite oxidase are consistent with a similar conformation for the cofactor in this enzyme.

The molybdenum enzymes are found in organisms from bacteria to man and display a variety of roles. They have been studied for many years by using a number of physical and biochemical techniques (Bray, 1988). There are, however, as yet no X-ray crystallographic data on the enzymes, and amino acid sequence information is only just beginning to contribute [cf. Wootton et al. (1991)]. Major contributions to our understanding of the mechanisms by which molybdenum enzymes function have been made by EPR spectroscopy of molybdenum(V). However, despite the extensive literature, structural information on the enzymes, even on the immediate environment of the metal, is still in need of expansion. This is partly because ligand hyperfine splittings from some nuclei may be too small to be resolved in EPR spectra. These atoms therefore remain undetected, and structural analysis from their couplings is not possible.

The improved resolution of electron nuclear double resonance (ENDOR)[1] spectroscopy, its capacity to distinguish different nuclear types and to provide interatomic distances [cf. Hughes et al. (1990)], offers an ideal tool to extend considerably the information available through EPR spectroscopy. An improved insight into the disposition of particular ligands and the general structure of the metal ion binding site should be forthcoming from such studies. In this report, attention is focused on the extraction, from frozen solution ENDOR spectra, of structural data on the phosphorus atoms near the molybdenum in milk xanthine oxidase, the most extensively studied of the molybdenum enzymes. The molybdenum center of xanthine oxidase, at the molybdenum(V) oxidation level, can exist in a considerable number of different and clearly defined states. Some of these are derived from the active enzyme and some from inactive species. Each species gives rise to a characteristic EPR signal, and signals are distinguished by names such as Rapid, Inhibited, and Desulfo Inhibited. Structural information is available for each of these species, derived largely from EPR work involving substitution with stable isotopes (Bray, 1988). In the present work, we have used ENDOR spectroscopy to study in detail coupling of ^{31}P nuclei to molybdenum(V) in several of these species. We employed xanthine oxidase both in its normal form and as the deflavoenzyme, from which FAD has been removed (Bray, 1975). Less comprehensive studies have also been made on chicken liver sulfite oxidase. Such data have not been readily obtainable by other physical techniques,

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[1] Abbreviation: ENDOR, electron nuclear double resonance.

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