Glycosylation at Asn-289 facilitates the ligand-induced conformational changes of human Glu-plasminogen

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Abstract Glu-plasminogen exists in two major glycoforms (I and II). Glycoform I contains carbohydrate chains linked to Asn-289 and Thr-346, whereas glycoform II is glycosylated only at Thr-346. Disparities in carbohydrate content lead to differences in the important functional properties of the zymogen, e.g. the kinetics of activation. The kinetics of the large ligand-induced conformational changes of each of the Glu-plasminogen glycoforms have been studied using stopped-flow fluorescence. The results are in accordance with a conformational change governed by positive co-operative binding at two weak lysine-binding sites. Additional glycosylation at Asn-289 in Glu-plasminogen I results in a two-fold increase in the overall dissociation constant of a ligand, *trans*-4-aminomethyl-cyclohexane carboxylic acid. This effect stems directly from the reaction step during which the conformational changes occur. This implies a higher population of Glu-plasminogen I in the open conformation even in the absence of ligands, and thus accounts for a higher rate of activation of Glu-plasminogen I, in comparison with Glu-plasminogen II.

Key words: Glu-plasminogen, conformation of; Glu-plasminogen, glycoforms; Lysine binding site; AH site; Stopped-flow kinetics; Fibrinolysis, regulation of

1. Introduction

The serine protease plasmin (EC 3.4.21.7) participates in a wide variety of physiological and pathological processes including vascular thrombolysis, tumour cell invasion and angiogenesis (for reviews see [1,2]). It has been established that activation of plasminogen, the inactive precursor to plasmin, is enhanced upon binding to ligands, such as antifibrinolytic amino acids, and substrates of plasmin, such as fibrin and components of the extracellular matrix, which presumably interact with the lysine binding sites of plasminogen and exert their effects by changes in the conformation of plasminogen. The conformational changes of plasminogen have been extensively studied over the past 30 years [3–15]. Lysine-analogous ligands, such as 6-aminohexanoic acid (6-AHA) and *trans*-4-aminomethylcyclohexane carboxylic acid (*t*-AMCHA), are known to induce a large-scale conformational change: from a 'closed' compact structure to an 'open' elongated structure [10,11,14,15]; the latter is likely to be similar to the structure of plasminogen when bound to fibrin [16]. We have recently shown, by kinetic [13] and structural experiments [15], that positive co-operative binding of at least two ligands to Glu-plasminogen is required to induce a fully extended conformation of the molecule.

Although the 'AH site' [17] of K5 binds both C-terminal lysine analogues and AH ligands with relatively low affinity [17–19], it appears to be the predominant binding site of plasminogen for both fibrin and cultured endothelial cells [20–22]. It is thought that ligand-induced conformational changes are due to initial binding via K5, which induces the actual change of conformation and facilitates the rapid binding of a second ligand to the LBS of K4 [13,15,23].

Two major glycoforms of plasminogen have been isolated from human plasma on the basis of their differing affinities for lysine-Sepharose [24–26]: type I possesses an *N*-linked oligosaccharide at Asn-289 and an *O*-glycosidally linked oligosaccharide at Thr-346, whereas type II is glycosylated at Thr-346 only. The presence of the oligo-saccharide structure at Asn-289 is known to result in reduced affinities of plasminogen K1 for fibrin and α_2-PI [20,27] and of plasminogen K4 for monocytoid U937 cells [28]. Furthermore, it appears to cause faster activation of Glu-plasminogen by streptokinase and urokinase-type plasminogen activator [29].

These functional disparities between plasminogen glycoforms prompted us to investigate the kinetics of the ligand-induced conformational change processes for Glu-plasminogen forms I and II using the stopped-flow fluorescence kinetic technique, previously demonstrated for a mixture of the two glycoforms [12,13]. From the present experiments it is concluded that glycosylation at Asn-289 affects the overall equilibrium, but significantly affects neither the individual binding constants for the two allosteric sites, nor the forward rate of conformational change, whereas the presence of the carbohydrate chain at Asn-289 inhibits the rate of the reverse rate of the change. Thus, the open conformation is favoured in the presence of this oligosaccharide.

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Abbreviations: 6-AHA, 6-aminohexanoic acid; *t*-AMCHA, *trans*-4-aminomethyl-cyclohexane carboxylic acid; LBS, lysine binding site; K1–K5, kringle-domains of Glu-plasminogen; AH ligand, amino hexyl ligand; α_2PI, α_2-proteinase inhibitor; AH site, amino hexyl site as defined in [17]

2. Materials and methods

2.1. Reagents

Lysine-Sepharose 6FF and AH-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden), 6-AHA from Aldrich-Chemie (Steinheim, Germany). *t*-AMCHA was kindly provided as a gift from Kabi (Stockholm, Sweden). All other chemicals were analytical grade from either Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

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2.2. Proteins

Human Lys-plasminogen and aprotinin was provided from Novo Nordisk (Gentofte, Denmark). Human Glu-plasminogen was prepared essentially as described in [30] from outdated frozen human plasma. Glu-plasminogen was separated from minor amounts of partially degraded plasminogen on AH-Sepharose 4B as in [31]. The Glu-plasminogen I and II were then separated by linear 6-AHA gradient elution (0–5 mM) of lysine-Sepharose 6FF-adsorbed plasminogen, leading to two partially resolved peaks. Pools were made from the leading edge of peak I and the trailing edge of peak II, containing Glu-plasminogen I and II, respectively. To ensure that essentially no 6-AHA ([6-AHA] < 1 pM) was present in the Glu-plasminogen preparations, these were extensively dialysed against buffer (0.05 M Tris-HCl/0.1 M NaCl/pH 7.8). The concentration of Glu-plasminogen was determined spectrophotometrically using an $A_{280}^{1\%}$ value of 16.2 [32]. The identity of the proteins were verified by ELISA and SDS-PAGE. It was confirmed by high performance capillary electrophoresis that the preparations of Glu-plasminogen I and II were not cross-contaminated (data not shown). Protein preparations were stored in 0.05 M Tris-HCl/0.1 M NaCl/pH 7.8 at −20°C.

2.3. Stopped-flow kinetic experiments

Stopped-flow kinetic experiments were performed at 25°C in 0.05 M Tris-HCl/0.1 M NaCl/pH 7.8 using a Hi-Tech Scientific PQ/SF-53 spectrofluorimeter equipped with a high-intensity xenon arc lamp and a fluorescence-enhancement device. The excitation wavelength was 280 nm, and the slit 5 mm. Light emitted from the reaction mixtures was subjected to a WG 320 (320 nm cut off) filter before reaching the detector.

For each of the two Glu-plasminogen glycoforms a series of experiments using a range of ligand concentrations was performed. Mixing of protein sample (Glu-plasminogen I or II, 1.8 μM, final concentrations) with ligand (*t*-AMCHA, 0.1–50 mM, final concentrations) was achieved in less than 1 ms. Subsequently, the intrinsic protein fluorescence was measured as a function of time (up to 50–500 ms). For each experiment 400 data points were recorded, and data sets from five or six experiments performed under identical conditions were averaged.

3. Results

3.1. Theory

Large positive fluorescence changes were observed upon addition of *t*-AMCHA to both Glu-plasminogens I and II. The time courses of the fluorescence changes were well described by a single exponential function as previously reported for the glycoform mixture, Eq. 1 [12,13]:

$$\Delta F([L],t) = \Delta F([L],\infty)\{1-\exp(-k_{obs}([L])t)\} \quad (1)$$

where $k_{obs}([L])$ is the observed first-order rate constant, and $\Delta F([L],t)$ is the relative fluorescence change observed at ligand concentration, [L], and time, *t*. $\Delta F([L],\infty)$ is the relative fluorescence change observed at equilibrium ($t \rightarrow \infty$). Values of $\Delta F([L],\infty)$ and $k_{obs}([L])$ were calculated for each employed concentration of *t*-AMCHA from fitting Eq. 1 to the experimental data.

Fluorescence changes as a result of ligand binding to the high affinity LBS of K1 are not discussed here as this binding event is known to be independent and does not contribute to the Glu-plasminogen conformational change process [12,13].

3.2. Glu-plasminogen I

Fig. 1 shows a plot of the values of $\Delta F([L],\infty)$ for Glu-plasminogen I with various *t*-AMCHA concentrations, [L]. At high ligand concentrations ([L] > 1 mM) a 12% fluorescence change occurs. At low ligand concentrations ([L] ≤ 1 mM) the data clearly show a sigmoidal behaviour (inset i). Inset ii is a Hill plot of the same data; the line drawn has the slope with the value 2.0. Fig. 2 shows the values of the observed rate constant, $k_{obs}([L])$, plotted against the concentration of *t*-AMCHA, [L], while the inset of Fig. 2 shows a close-up of the first part of the curve ([L] ≤ 1 mM).

3.3. Glu-plasminogen II

Fig. 3 shows the values of $\Delta F([L],\infty)$ obtained for Glu-plasminogen II plotted versus *t*-AMCHA concentrations. The maximal fluorescence change was found to be 9%. As observed for glycoform I, the data at low ligand concentrations ([L] ≤ 1 mM) show a sigmoidal behaviour (inset i). The Hill

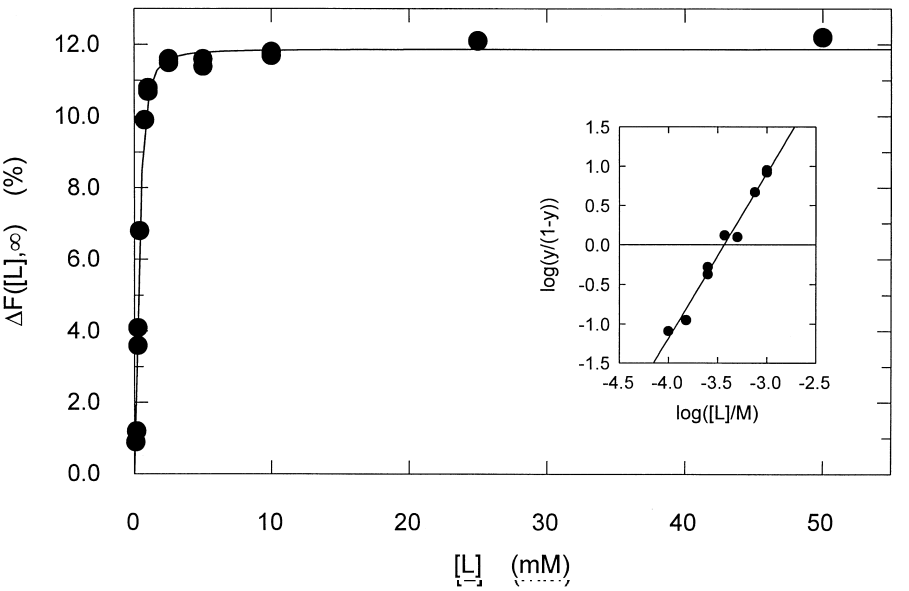

Fig. 1. Glu-plasminogen I interaction with *t*-AMCHA; the ligand dependence of the relative fluorescence changes obtained at equilibrium. Equilibrium values of the large positive change of the protein fluorescence ($\Delta F([L],\infty)$, Eq. 1) obtained after interaction of Glu-plasminogen I with *t*-AMCHA plotted against the concentration of *t*-AMCHA, [L]. The curve shown is that given by the fitted expression (equation 2 of Scheme 1). The inset shows the corresponding Hill plot, where y is the fractional saturation, $\Delta F([L],\infty)/\Delta F_{max}([L],\infty)$. The resulting parameters, $\Delta F([L],\infty)$ and the Hill slope, are given in Table 1. The experimental errors are within the size of the symbols.

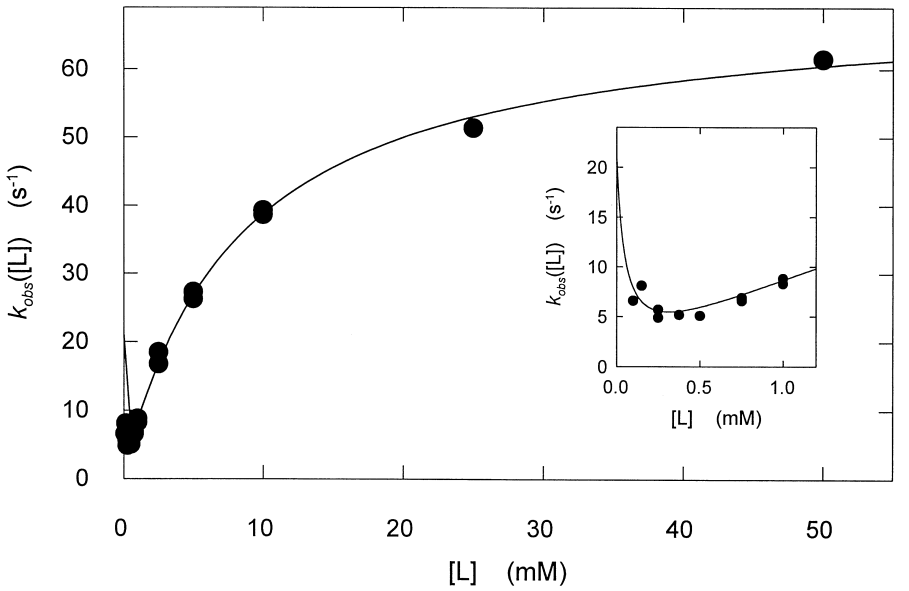

Fig. 2. Glu-plasminogen I interaction with *t*-AMCHA; the ligand dependence of the observed rate constant. The observed value of the rate constant, $k_{obs}([L])$, obtained for Glu-plasminogen I plotted against *t*-AMCHA concentration, [L]. The curve drawn is obtained from fitting equation 3 (Scheme 1) to the experimental data. The resulting parameters (K_α, K_γ, k_f and k_r) from this fit are listed in Table 1. The inset shows a close-up of the curve for [L] ≤1 mM. Experimental errors are within the size of the symbols.

plot of these data also exhibits a slope of value 2.0 (inset ii). The variation in the Glu-plasminogen II values of the observed rate constant, $k_{obs}([L])$, is shown in Fig. 4, which also contains an inset with data obtained at low ligand concentrations ([L] ≤ 1 mM).

3.4. Calculation of the kinetic parameters

The ligand concentration dependences of the fluorescence data for both Glu-plasminogen I and II exhibit similar characteristics to the data for the glycoform mixture [13]: the $\Delta F([L],\infty)$ data show a sigmoidal behaviour for both glycoforms (inset i, Figs. 1 and 3) and the slopes of the Hill plot are 2.0 (insets ii, Figs. 1 and 3). Furthermore, the profiles of the values of the observed rate constant, $k_{obs}([L])$, exhibit minima at low ligand concentrations and they approach saturating (maximum) values at high ligand concentrations (Figs. 2 and 4). These characteristics suggest that the reaction scheme previously proposed for the Glu-plasminogen glycoform mixture (Scheme 1 [13]) also is valid for each of the glycoforms individually. All other schemes previously considered [12,13] cannot account for these data. Consequently, equation 2 of Scheme 1 was fitted to the experimental values of $k_{obs}([L])$ for

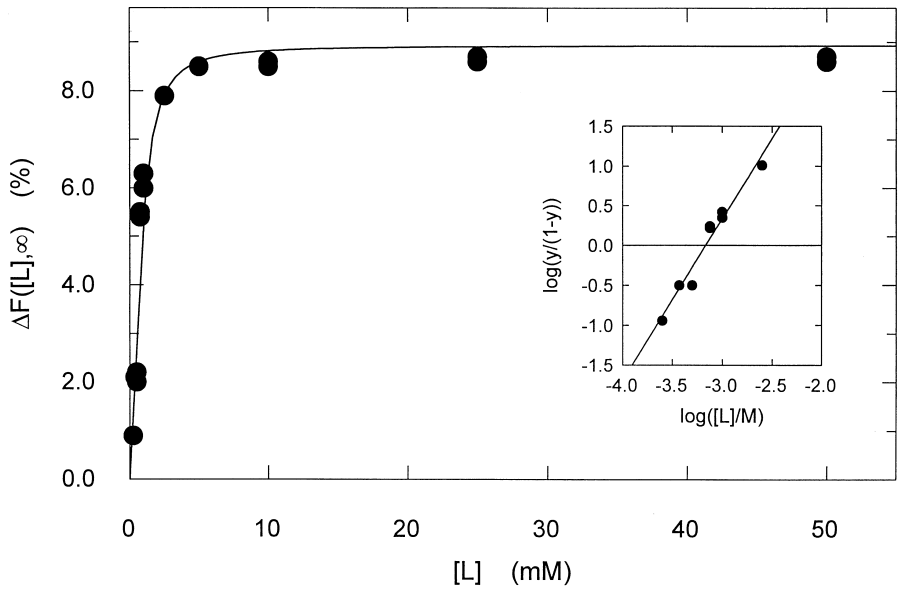

Fig. 3. Glu-plasminogen II interaction with *t*-AMCHA; the ligand dependence of the fluorescence changes obtained at equilibrium. Equilibrium values of the large positive change of the protein fluorescence ($\Delta F([L],\infty)$, Eq. 1 obtained after interaction of Glu-plasminogen II with *t*-AMCHA plotted against the concentration of *t*-AMCHA, [L]. The curve shown is that given by the fitted expression (equation 2 of Scheme 1). The inset shows the corresponding Hill plot, where y is the fractional saturation, $\Delta F([L],\infty)/\Delta F_{max}([L],\infty)$. The resulting parameters, $\Delta F([L],\infty)$ and the Hill slope, are given in Table 1. The experimental errors are within the size of the symbols.

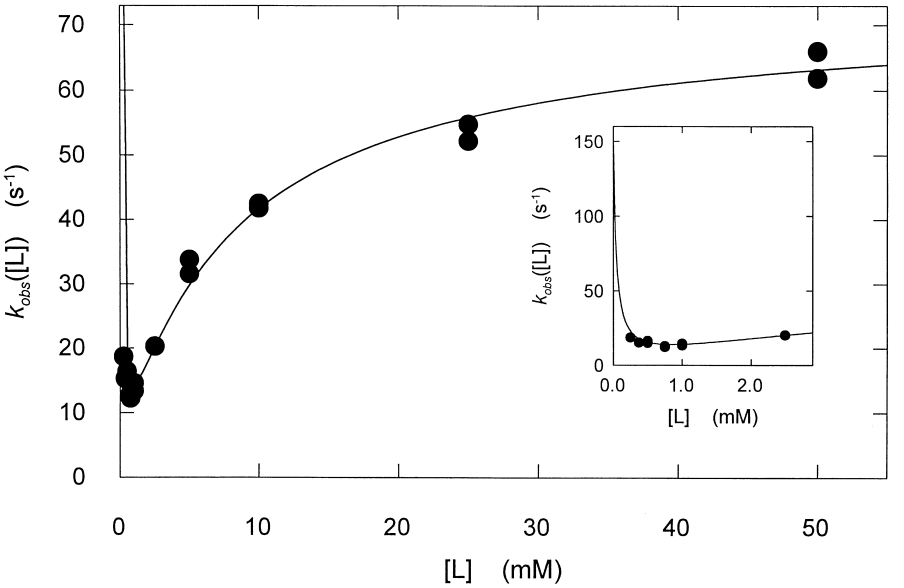

Fig. 4. Glu-plasminogen II interaction with *t*-AMCHA; the ligand dependence of the rate constant. The observed value of the rate constant, $k_{obs}([L])$, obtained for Glu-plasminogen II plotted against *t*-AMCHA concentration, [L]. The curve drawn is obtained from fitting equation 3 (Scheme 1) to the experimental data. The resulting parameters (K_α, K_γ, k_f and k_r) from this fit are listed in Table 1. The inset shows a close-up of the curve for $[L] \leq 1$ mM. Experimental errors are within the size of the symbols.

both glycoforms. The values of the dissociation and rate constants obtained from these fits are presented in Table 1. Similarly, the results on the maximal fluorescence changes, $\Delta F([L],\infty)_{max}$, for the two glycoforms, listed in Table 1, were obtained from fitting equation 3 to the values of $\Delta F([L],\infty)$.

4. Discussion

Conformational changes of Glu-plasminogen, mediated by binding of ligands to its weak LBSs, enhance the rate of its conversion into plasmin [6,32–38]. It has also been shown that the degree of glycosylation of the plasminogen molecule modulates its functional properties. Not only do the two Glu-plasminogen glycoforms differ with respect to affinity to lysine-Sepharose and fibrin, but they also display differences in kinetics of activation catalyzed by urokinase and streptokinase [15,24,27,29]. A plausible explanation for these disparities is that the presence of the oligosaccharide at Asn-289 in Glu-plasminogen type I affects the rate of the conformational changes.

In order to investigate this hypothesis the kinetics of the ligand-induced conformational changes were characterised for each of the two Glu-plasminogen glycoforms. As shown previously [13] the technique of stopped-flow intrinsic protein fluorescence spectroscopy allows the determination of dissociation and rate constants for the process of ligand-induced conformational changes of Glu-plasminogen. The lysine analogue, *t*-AMCHA, was chosen as a model ligand for these studies due to its known ability to induce large changes in intrinsic fluorescence [13].

Table 1
Equilibrium and kinetic constants of the positive cooperative binding of *t*-AMCHA

	Glu-plasminogen I	Glu-plasminogen II
Hill slope	2.0 ± 0.1	2.0 ± 0.1
$\Delta F_{max}([L],\infty)$ (%)	11.9 ± 0.1	8.8 ± 0.3
K_α (mM)	8.2 ± 0.6	7.7 ± 0.9
K	0.3 ± 0.2	2.0 ± 0.8
K_β (mM)	0.05 ± 0.04	0.04 ± 0.02
k_f (s^{-1})	70 ± 2	74 ± 3
k_r (s^{-1})	21 ± 14	150 ± 60
K_D (overall) (mM)	0.34 ± 0.01	0.74 ± 0.03

The maximal positive fluorescence changes at saturating *t*-AMCHA concentrations, $\Delta F([L],\infty)$, were found to be 11.9% for Glu-plg I and 8.8% for Glu-plg II, in agreement with results for mixtures of glycoforms [7,12,13]. The fluorescence changes occur at those ligand concentrations which are known to cause large-scale conformational changes within Glu-plasminogen [39] indicating that observed fluorescence changes directly reflect large-scale changes in conformation.

For each of the glycoforms the experimental values of $k_{obs}([L])$ and $\Delta F([L],\infty)$ are well described by equations 2 and 3, Scheme 1. This implies a positive co-operative process for each glycoform individually: binding of one ligand (step i) induces a conformational change (step ii), which facilitates the binding of an additional ligand (step iii). No significant variations were observed between values of either K_α or K_β values for each glycoform (Table 1). In contrast to this, a difference was found for the equilibrium constant of the actual conformational change (step ii). This arises, not from dissimilar values of the forward rate constant k_f, but from a large difference in values of the reverse rate constant, k_r. Consequently, it is concluded that the presence of the oligosaccharide at Asn-289 causes a destabilization of the open conformation of ligand-bound Glu-plasminogen.

Values of the kinetic parameters obtained for the two glycoforms are in agreement with those previously obtained for the glycoform mixture [13] except for a discrepancy between K_β values. The reason for this discrepancy remains unknown. However, we note that K_β is very sensitive to changes of k_r in the fitting procedure used here.

Scheme 1 describes the concerted fast process of the cooperative ligand induced conformational change of Glu-plasminogen with antifibrinolytic amino acids analogous to C-termi-

$$P + L \underset{}{\overset{K_\alpha}{\rightleftharpoons}} PL, \qquad K_\alpha = \frac{[L][P]}{[PL]} \tag{i}$$

$$PL \underset{}{\overset{K}{\rightleftharpoons}} P^*L, \qquad K = \frac{k_r}{k_f} = \frac{[PL]}{[P^*L]} \tag{ii}$$

$$P^*L + L \underset{}{\overset{K_\beta}{\rightleftharpoons}} P^*L_2, \qquad K_\beta = \frac{[L][P^*L]}{[P^*L_2]} \tag{iii}$$

$$\Delta F([L],\infty) = \frac{\Delta F_{max}([L],\infty)}{1 + \frac{KK_\beta}{[L](1+K_\beta/[L])} + \frac{K_\alpha K K_\beta}{[L]^2(1+K_\beta/[L])}} \tag{2}$$

$$k_{obs} = \frac{k_f}{1 + K_\alpha/[L]} + \frac{k_r}{1 + [L]/K_\beta} \tag{3}$$

$$K_D = (K_\alpha K K_\beta)^{\frac{1}{2}} \tag{4}$$

Scheme 1. Reaction scheme for ligand-induced Glu-plasminogen conformational change. The model shown best explains the results on the reaction of Glu-plasminogen, P, with ligands, L. The model relates the fluorescence change to the change of the Glu-plasminogen conformation and assumes a concerted reaction with three steps: (i) fast formation of the first complex, PL, (ii) change of the conformation of PL to another form, P*L, and (iii) fast formation of a 1:2 Glu-plasminogen-ligand complex, P^*L_2. The ligands both interact with weak lysine binding sites. The concentration dependences of experimentally determined parameters that correspond with this reaction scheme are given in equations 2 and 3. The parameters are defined in Eq. 1 in the text. K_D is the overall dissociation constant; K_α is that of the first complex, PL; K_β that of complex P^*L_2, for the dissociation of one ligand; and K is the equilibrium constant of step ii as defined.

nal lysine residues. As suggested previously, the finding that two weak LBSs are involved strongly indicate that the first ligand binds K5 and the second K4 [13]. Recently, it has been shown that Glu-plasminogen undergoes conformational change in more steps than those depicted in Scheme 1, and that an intermediate form, similar to that of Lys-plasminogen, which is induced in Glu-plasminogen when benzamidine binds the LBS of K5 [15], also exists on the reaction pathway. Benzamidine is an AH ligand, and binds weakly if at all to K4. It is likely, therefore, that the closed to open conformational equilibrium cannot be shifted entirely towards the open conformation with benzamidine. Scheme 1 depicts the minimum number of necessary steps, required to explain the kinetic results. Any number of further steps may exist as long as they are significantly fast kinetically that the initial conformational change (step ii) remains the rate limiting step. Indeed this appears to be the case as experiments on *t*-AMCHA-induced conformational changes of Lys-plasminogen (glycoform mixture) small fluorescence changes of a fast reaction were seen ($\Delta F_{max}([L],\infty) \approx 4\%$) and equilibrium was achieved within the dead time (≈ 1 ms) of the apparatus (results not shown). These data are in accordance with the conclusions reached in [15].

The observed different k_r values of the two glycoforms result in a two-fold difference in the overall dissociation constant, K_D (Table 1): Glu-plasminogen I displays the higher affinity for the ligand. Assuming a dynamic equilibrium between conformations, even in the absence of ligands, this result implies the existence of a higher population of open molecules for Glu-plg I than for Glu-plg II. This argument further implies that in the absence of ligand the rate of Glu-plasminogen I activation should be higher than that of glycoform II, which actually has been observed experimentally with either uPA or streptokinase as activator [29].

The results do show similar values of K_β for both glycoforms. This suggests that the binding of the second allosteric ligand to Glu-plasminogen K4 is unaffected by the Asn-289 oligosaccharide and therefore cannot solely account for the higher affinity of Glu-plg II for U937 cells [28]. Similarly, the K_α-values implies that the glycoforms possess equivalent affinity for intact fibrin, as shown experimentally [27].

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