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Stopped-flow fluorescence kinetic studies of Glu-plasminogen

Conformational changes triggered by AH-site ligand binding

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Binding of 6-aminohexanoic acid to the AH-site, a weak lysine binding site in Glu-plasminogen, alters the conformation of the molecule. The kinetics of the binding and the accompanying conformational change are investigated at pH 7.8, 25°C. Changes of intrinsic protein fluorescence were measured as a function of time after rapid mixing in a stopped-flow apparatus. The results reflect a two-step reaction mechanism. Rapid association of Glu-plasminogen and 6-aminohexanoic acid (K_i = 44 mM) followed by the conformational change (k_3 = 69 s^{-1} and k_{-3} = 3 s^{-1}) with an overall dissociation constant K_d = 2.0 mM. Thus the conformational change is rather fast, $t_{1/2}$ = 0.01 s. Its importance for the rates of Glu-plasminogen activation reactions is discussed.

Stopped flow fluorescence, Glu-plasminogen, AH-site, 6-Aminohexanoic acid, Conformational change, Plasminogen activation

1. INTRODUCTION

Native human Glu-plasminogen (Glu-1–Asn-790) is the precursor of the fibrinolytic enzyme, plasmin (EC 3.4.21.7). Conformational changes of Glu-plasminogen mediated by ligand binding to the AH-site, a weak lysine binding site probably located in the kringle 5 domain of the molecule [1–3], are important for its binding to fibrin and its rate of conversion into plasmin catalyzed by the plasminogen activators, urokinase and tissue plasminogen activator [4–10].

In order to elucidate these important physiological processes we have undertaken studies of the Glu-plasminogen conformational changes. Here we report the results of a stopped-flow fluorescence investigation of the kinetics of the change of conformation that follows AH-site binding of 6-aminohexanoic acid to Glu-plasminogen.

2. EXPERIMENTAL

2.1. *Reagents*

Lysine-Sepharose 4B and AH-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden), 6-aminohexanoic acid from Fluka (Buchs, Switzerland). Aprotinin (Trasylol) was kindly provided as a gift from Bayer (Leverkusen, Germany). All other chemicals were anlytical grade either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Human Glu-plasminogen was prepared essentially as described in [11] from frozen human plasma kindly provided as a gift from Novo Nordisk A/S (Copenhagen, Denmark). It was separated from minor amounts of partially degraded plasminogen on AH-Sepharose 4B as in [2]. Glu-plasminogen was stored in 0.050 M Tris-HCl/0.10 M NaCl, pH 7.80 at −20°C. The approximate concentration of Glu-plasminogen was determined from absorbance measurements, using the value $E^{1\%}_{280}$ = 16.2 [12].

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2.2. *Stopped flow kinetic experiments*

Kinetic experiments were performed in a Hi-Tech Scientific PQ/SF-53 spectrofluorimeter equipped with a high intensity xenon arc lamp. The excitation wavelength was 280 nm, slit 5 nm. Energy emitted from the reaction mixtures passed a WG 320 filter before reaching the photomultiplier. Series of stopped-flow experiments were performed at 25.0°C in 50 mM Tris-HCl, 100 mM NaCl, pH 7.80. After mixing of Glu-plasminogen (1.5 μM, final concentration) and 6-aminohexanoic acid (0–200 mM, final concentrations) the intrinsic protein fluorescence intensity (arbitrary units, V) was recorded for 200 ms. Mixing was achieved in less than 1 ms. In each experiment 400 pairs of data were recorded, and sets of data from 5–6 experiments at identical conditions were averaged. Each averaged set of stopped-flow data was then fitted to a number of non-linear analytical equations using the Hi-Tech HS-1 Data Pro software. The regression analysis used is based on the Gauss-Newton procedure.

3. RESULTS

Fig. 1 shows the result of a typical stopped-flow experiment measuring the intrinsic protein fluorescence change after mixing of Glu-plasminogen and 6-aminohexanoic acid. The fitted curve illustrated is the one corresponding to a single exponential progress (Eqn 1):

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

where k_{obs} is the observed first-order rate constant, and $\Delta F(L,t)$ is the relative fluorescence change observed at the actual ligand concentration, [L], and time, t, which attains the value $\Delta F(L,\infty)$ at equilibrium. Eqn 1 showed

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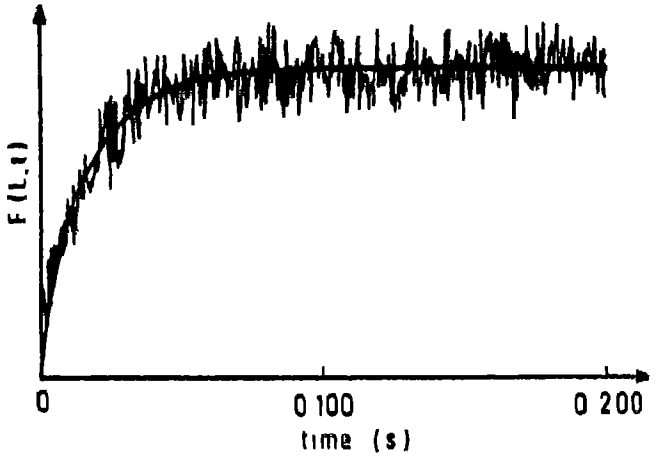

Fig. 1. A typical time course obtained after mixing of Glu-plasminogen and 6-aminohexanoic acid in the stopped-flow fluorimeter. In this example the final concentrations were: 1.5 μM Glu plasminogen and 100 mM 6-aminohexanoic acid. The fitted curve corresponds to an overall first-order progress (Eqn 1 in the text)

the most accurate fit to the measured data at all concentrations of 6-aminohexanoic acid employed.

The values of k_{obs} and $\Delta F(L,\infty)$ for the interactions of Glu-plasminogen with 6-aminohexanoic acid, obtained from fits to Eqn 1, were analysed according to the two plausible reaction models, Schemes I and II (Fig. 2).

Figs 3 and 4 show the dependences of k_{obs} and $\Delta F(L,\infty)$, respectively, on the concentration of 6-aminohexanoic acid. Obviously the dependence of k_{obs} on the ligand concentration is non-linear and not in agreement with Scheme I.

Scheme II on the other hand is in good agreement with the experimental results as seen from Figs 3 and 4. The kinetic parameters obtained were $K_1 = (44 \pm 4)$ mM, $k_{-2} = (3 \pm 1)\ s^{-1}$, $k_2 = (69 \pm 4)\ s^{-1}$, $K_2 = (0.04 \pm 0.01)$, and $K_d = (2.0 \pm 0.3)$ mM.

It is further worth noticing that low concentrations of 6-aminohexanoic acid ($< 10^{-3}$ M) did not give rise to significant changes of the intrinsic protein fluorescence of Glu-plasminogen (Fig. 4).

4 DISCUSSION

Conformational changes of Glu-plasminogen mediated by binding of ligands to the AH-site, as defined in [1], are generally considered important for its rate of conversion into plasmin [4–10]. Several laboratories have studied the overall kinetic and binding properties of the Glu-plasminogen activation systems, but the rate of the conformational change has hitherto not been determined.

In this paper we report the results of a series of stopped-flow kinetic experiments in which the change of intrinsic protein fluorescence in Glu-plasminogen

SCHEME I

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL$$

$$K_D = \frac{[P][L]}{[PL]} = \frac{k_{-1}}{k_1}$$

$$k_{obs} = k_1[L] + k_{-1} \qquad (1\ 1)$$

$$\Delta F(L,\infty) = \frac{\Delta F_{max}}{1 + \frac{K_D}{[L]}} \qquad (1\ 2)$$

SCHEME II

$$P + L \underset{}{\overset{K_1}{\rightleftharpoons}} PL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL'$$

$$K_1 = \frac{[P][L]}{[PL]}, \quad K_2 = \frac{[PL]}{[PL']}, \quad K_D = \frac{K_1 K_2}{1 + K_2}$$

$$k_{obs} = \frac{k_2}{1 + \frac{K_1}{[L]}} + k_{-2} \qquad (2\ 1)$$

$$\Delta F(L,\infty) = \frac{\Delta F_{max}}{1 + \frac{K_1 K_2}{[L](1 + K_2)}} \qquad (2\ 2)$$

Fig. 2. To explain the results on the relative fluorescence changes in the reaction of Glu-plasminogen, P, with 6-aminohexanoic acid, L, two reaction schemes are considered. Scheme I assumes a simple interaction with the formation of only one complex, PL, giving rise to the intrinsic protein fluorescence changes. Scheme II further takes into account a change of conformation of the first complex, and relates the change of fluorescence to a second complex, PL'. The corresponding concentration dependences of experimentally determined parameters, k_{obs} and $\Delta F(L,\infty)$, are given in Eqns 1 1–2 2. k_{obs} and $\Delta F(L,\infty)$ are defined in the text (Eqn 1). K_d is the overall dissociation constant, K_1 is that of the association complex, PL, and K_2 is the equilibrium constant of the two conformations of the Glu-plasminogen-6-aminohexanoic acid complexes, PL and PL', of which the individual first-order rate constants are k_2 and k_{-2}. ΔF_{max} is the maximal relative fluorescence change at saturating concentration of L

after rapid mixing with 6-aminohexanoic acid was measured (example in Fig. 1). No changes were observed at concentrations of 6-aminohexanoic acid in the range 10^{-6}–10^{-3} M. Saturation of the high affinity lysine binding site ($K_d = 10^{-5}$ M [13]) accordingly is of little importance for the induction of the gross conformational

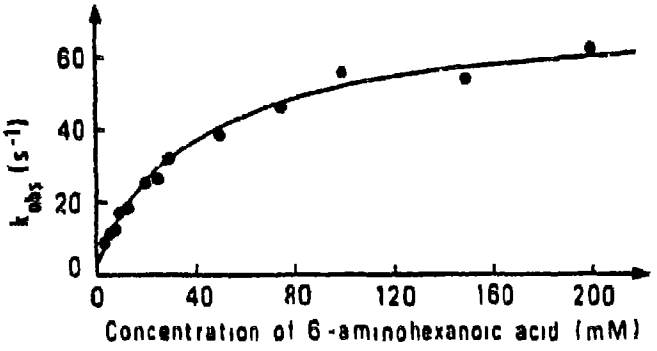

Fig. 3. The observed value of the rate constant, k_{obs}, plotted against the concentration of 6-aminohexanoic acid. The curve shown is obtained from a fit to Eqn 2.1 (Fig. 2) with the resulting parameters A_1 = 44 mM, k_{-2} = 3 s^{-1}, and k_2 = 69 s^{-1}.

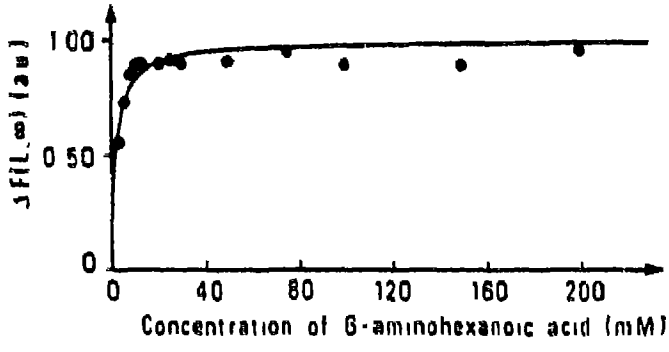

Fig. 4. The equilibrium values of the relative fluorescence change, $\Delta F(I,\infty)$, plotted as a function of the concentration of 6-aminohexanoic acid. The curve illustrated is obtained from a fit to Eqn 2.2 (Fig. 2) K_d = 2.0 mM.

change in Glu-plasminogen. This is in accordance with the results of recently published small angle scattering studies of the system [14].

The most simple reaction model that fits the observed data is Scheme II of Fig. 2, according to which the binding of the ligand involves two steps: (i) an association, which is the faster step, followed by (ii) a rate-determining, but rather fast conformational change, $t_{1/2}$ = 0.01 s. The concentration dependences (Figs 3 and 4) clearly show that the fluorescence changes involve AH-site binding. The overall dissociation constant of this 6-aminohexanoic acid-Glu-plasminogen complex is K_d = 2.0 mM in good agreement with previous AH-site results [1,13,14].

The conformational change determines the overall rate of the ligand binding process. Its rate constant, 70 s^{-1} (pH 7.8, 25°C), is approximately 20 times larger than the values of the catalytic constant of plasminogen activator catalyzed conversion of Glu-plasminogen-ligand complexes into plasmin. Although such values differ somewhat, the reported k_cs are of the order of magnitude 0.5–5 s^{-1} [6–10]. Ligands that induce conformational changes in Glu-plasminogen facilitate its activation. But as we have seen here the rate determining step(s) of the activation reaction in the presence of ligands are to be found in the actual plasminogen activator catalyzed process. The ligand induced conformational change of Glu-plasminogen is definitely not the slowest step. In the absence of ligands the situation is quite different. The same change of Glu-plasminogen conformation may be required, and when no ligand is present, that change may proceed at a very low rate and thus determine the rate of the activation.

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