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Kinetic Properties of the Primary Inhibitor of Plasmin from Human Plasma

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The interaction of human plasmin with the newly discovered α_2-plasmin inhibitor was investigated. It was found from rate measurements that the reaction involves the rapid formation of a first enzyme–inhibitor complex, followed by the slow irreversible transition to another complex. L-Lysine influences the first step, but not the second.

The existence of a hitherto unknown plasmin inhibitor in human plasma has been demonstrated (Müllertz, 1974; Collen *et al.*, 1975). The inhibitor has been purified and has been found to be an α_2-glycoprotein of approx. mol.wt. 60000. It is present in human plasma at a concentration of approx. 1 μM. The reaction between the inhibitor and plasmin has been characterized as fast and irreversible (Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976). The present paper deals with the kinetics of this reaction.

Materials and Methods

Human plasminogen with an *N*-terminal lysine residue was purified as previously described (Christensen & Müllertz, 1977). Human plasmin was prepared from plasminogen with *N*-terminal lysine on a column of urokinase-substituted Sepharose 4B (Christensen, 1975). The primary inhibitor of plasmin* was partially purified from human plasma as earlier described (Müllertz & Clemmensen, 1976). The inhibitor constituted 12% (w/w) of the protein in the resulting product, which was free of all the known proteinase inhibitors and of plasminogen, plasmin and fibrinogen. Such preparations were dialysed against 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.7, and then were applied to a column (25 mm × 65 mm) of plasminogen-substituted Sepharose 4B containing 0.6 μmol of plasminogen. After washing with the buffer the inhibitor was eluted with a solution of 0.02 M-6-aminohexanoic acid in 0.1 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.7 at 4°C. After dialysis against 1 mM-dithiothreitol in the same buffer, the protein was concentrated to approx. 2 μM by ultrafiltration. It showed only one precipitate in crossed immunoelectrophoresis with rabbit immunoglobulin against human serum proteins. The procedure was analogous to that described by Moroi & Aoki (1976).

All kinetic experiments were run at least twice, at 25°C. Solutions were prepared in 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.8. The initial velocities of plasmin-catalysed hydrolysis of Bz-Arg-OEt† were measured as described earlier (Christensen, 1975). Plasmin and the primary inhibitor of plasmin were incubated as follows: the desired amounts of the buffer solution, inhibitor stock solution (1.07 or 3.09 μM) and, where appropriate, 1.5 M-L-lysine were first mixed. Plasmin stock solution (2.78 μM) was added ($t = 0$, total volume 1160 or 1550 μl) and, after the chosen incubation time [$t = t(i)$], 40 or 50 μl of 20 mM-Bz-Arg-OEt was added, the reaction mixture (1200 or 1600 μl) was poured into a semi-micro cuvette and the measurements were started. For measurements at zero time Bz-Arg-OEt was added before plasmin. Measurements were made at 0, 0.25 and 0.5 M concentrations of L-lysine (l) with total concentrations of plasmin (p_0) in the range 50–500 nM, at concentrations of inhibitor (i_0) in the range 0–500 nM. The concentration of the plasmin stock solution was determined by titration with 4′-nitrophenyl-4-guanidinobenzoate (Chase & Shaw, 1969). The concentrations of inhibitor solutions were determined from the initial velocities obtained with i_0/p_0 in the range 0–0.15 when $t(i) = 60$ s.

Results and Discussion

The initial velocity, v, of plasmin-catalysed hydrolysis of Bz-Arg-OEt is proportional to the concentration of plasmin free to react, p (in nM). It is $8.73 \times p$ nmol/s with 0.67 mM-Bz-Arg-OEt at pH 7.8 and 25°C (Christensen, 1975). If v_0 is the initial velocity obtained with no inhibitor present, but at the

* The primary inhibitor of plasmin is identical with α_2-antiplasmin (Collen, 1976) and α_2-plasmin inhibitor (Moroi & Aoki, 1976).

† Abbreviation: Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester.

same total concentration of plasmin, p_0, at which v is determined, then $v/v_0 = p/p_0$.

The reaction of plasmin with this inhibitor is very fast. At all initial concentrations of the inhibitor, i_0, in the range 0–0.6p_0, the initial velocities obtained after 20, 30, 60 and 120s incubation were the same. Since inhibition under these conditions is presumably completed in less than 20s, a lower limit for the association rate constant, k_{+1}, may be determined for the reaction:

$$P + I \xrightarrow{k_{+1}} C \qquad (1)$$

where C is a plasmin–inhibitor complex. The maximum rate of complex-formation is $-dp/dt = k_{+1}p_0i_0$. At time t therefore $p_0 - p < k_{+1}p_0i_0t$. For $p_0 - p = i_0$, $p_0 = 50$nM and $t = 20$s, this leads to $k_{+1} > 10^6 M^{-1}\cdot s^{-1}$. This is also true if the reaction is:

$$P + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C \qquad (2)$$

Since protein–protein interactions appear to be limited at rates of about 10^6–$10^7 M^{-1}\cdot s^{-1}$ (Eigen & Hammes, 1963; Gutfreund, 1972) k_{+1} is probably of this order of magnitude. An estimate of the dissociation constant was obtained by investigating the reaction in detail near the equivalence point. The results obtained at $p_0 = 424$nM are illustrated in Figs. 1 and 2. For eqn. (2) the dissociation constant is:

$$K = \frac{k_{-1}}{k_{+1}} = \frac{p(i_0 - p_0 + p)}{(p_0 - p)} = \frac{p_0\frac{p}{p_0}\left[\frac{p}{p_0} - \left(1 - \frac{i_0}{p_0}\right)\right]}{1 - \frac{p}{p_0}}$$

If it is assumed that no other reaction occurs within the first 20s and that equilibrium prevails after this incubation time, $K = 3 \pm 2$nM, when no L-lysine is present. This result is the mean value of those calculated from 75 initial-velocity measurements made at various total concentrations of plasmin.

L-Lysine strongly influences the equilibrium, but not the plasmin-catalysed hydrolysis of Bz-Arg-OEt. If it is assumed that plasmin and L-lysine form a complex, with a dissociation constant $K_l = pl/p_l$, where p_l is the equilibrium concentration of the complex, and if the inhibitor does not react with this complex, then the dissociation constant of the plasmin–inhibitor complex, K', calculated from the measured initial velocities, depends on the concentration of L-lysine thus:

$$K' = p_0\frac{\frac{p+p_l}{p_0}\left[\frac{p+p_l}{p_0} - \left(1 - \frac{i_0}{p_0}\right)\right]}{1 - \frac{p+p_l}{p_0}} = \frac{p\left(1 + \frac{l}{K_l}\right)i}{c}$$

$$= K\left(1 + \frac{l}{K_l}\right) \text{ for } l \gg p_0$$

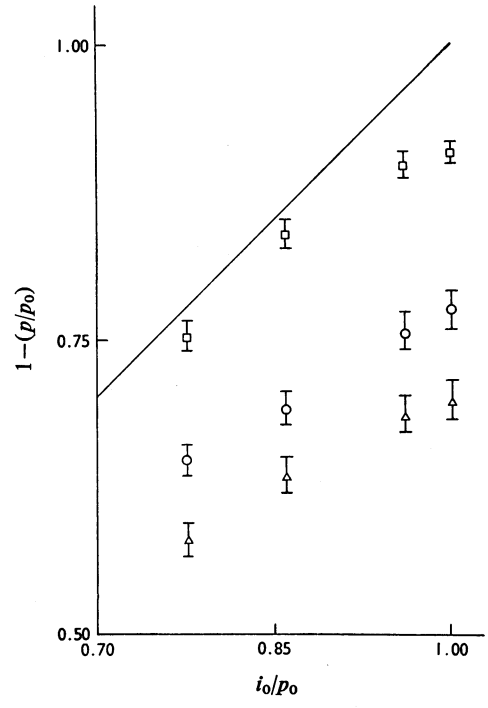

Fig. 1. *Inhibition of plasmin by the primary inhibitor of plasmin*

Incubation time was 20s. The total concentration of plasmin, p_0, was 424nM. The total concentration of the inhibitor, i_0, was in the range 0.7–1.0p_0. Then $1-(p/p_0)$ is the fraction of plasmin actually inhibited; i_0/p_0 is the fraction of plasmin that would be inhibited if the enzyme–inhibitor complex did not dissociate; i_0/p_0 is shown by the line. Plasmin activities were measured as initial velocities of Bz-Arg-OEt hydrolysis; 50μl of Bz-Arg-OEt was added to the incubation mixture (1550μl) at 20s. Buffer was 0.1M-NaCl/0.05M-Tris/HCl, pH7.8 at 25°C. Concentrations of L-lysine were: □, 0; ○, 0.25M; △, 0.5M. Bars indicate S.E.M. based on two to five experiments.

The initial velocities obtained under these conditions are measures of $(p+p_l)$, since L-lysine does not inhibit the hydrolysis of Bz-Arg-OEt, therefore $v/v_0 = (p+p_l)/p_0$. When $l = 0.25$M, $K'_{0.25} = 32 \pm 3$nM, whereas for $l = 0.5$M $K'_{0.5} = 60 \pm 4$nM. If K is taken as 3nM these lead to $K_l = 25.9$ and 26.3mM respectively.

As seen from Fig. 2 the fast-reaction step is not the only one involved. Presumably the remaining plasmin is inhibited in a slow-reaction step that follows the fast one. Analysing the reaction:

$$P + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C_1 \xrightarrow{k_{+2}} C_2 \qquad (3)$$

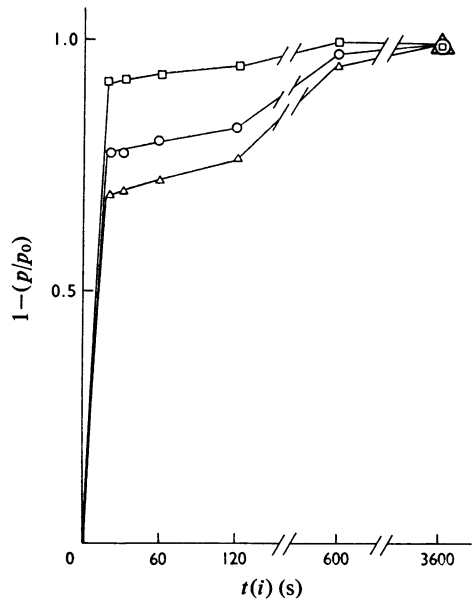

Fig. 2. *Time-dependence of the interaction of plasmin with the primary inhibitor of plasmin*

The total concentrations of plasmin and inhibitor were both 424nM ($=p_0 = i_0$). The fraction of plasmin actually inhibited, $1-(p/p_0)$, is plotted against the incubation time $t(i)$s. Plasmin activities were measured as initial velocities of Bz-Arg-OEt hydrolysis at time, $t(i)$, 50μl of 20mM-Bz-Arg-OEt was added to the incubation mixture (1550μl). Buffer was 0.1M-NaCl/0.05M-Tris/HCl, pH7.8 at 25°C. Concentration of L-lysine were: □, 0; ○, 0.25M; △, 0.5M.

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where C_1 and C_2 are two different plasmin–inhibitor complexes with the concentrations c_1 and c_2, and assuming that the first reaction step is fast and the second slow, so that $K = pi/c_1$ holds in the time range of the slow process, $-dc_1/dt = k_{+2}c_1$ and $c_1 = pi/K = p(i_0-p_0+p)/K$, since $i_0-i = p_0-p = c_1+c_2$.

Thus:

$$k_{+2} = \frac{1}{t_2-t_1} \ln \left\{ \frac{\frac{p'}{p_0}\left[\frac{p'}{p_0} - \left(1-\frac{i_0}{p_0}\right)\right]}{\frac{p''}{p_0}\left[\frac{p''}{p_0} - \left(1-\frac{i_0}{p_0}\right)\right]} \right\}$$

where p' and p'' are the concentrations of free plasmin at times t_1 and t_2 respectively. The same equation is obtained with L-lysine present in the reaction mixture if it is assumed that L-lysine only affects the reaction in the manner described above. There was reasonable internal agreement between the values obtained for k_{+2}; being the mean value from all the measured data $k_{+2} = 6.5\times10^{-3} \pm 0.8\times10^{-3}\,s^{-1}$. The half-time of the process is 107s. The assumption that only a small fraction of the C_1 present is converted into C_2 after 20s is thus not unreasonable.

These results indicate that the reaction of plasmin with the inhibitor is a two-step process in which fast reversible complex-formation is followed by a slow irreversible transition to another complex. The slow step may involve the hydrolysis of a peptide bond. Moroi & Aoki (1976) and Müllertz & Clemmensen (1976) found that plasmin splits an inhibitor–peptide bond. Since the inhibitor binds to plasminogen, but not to the L-lysine–plasmin complex, not only the active site, but also the lysine-binding site of plasmin, seem to be involved in the reaction between the primary inhibitor of plasmin and plasmin.

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