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On the Kinetics of the Reaction between Human Antiplasmin and Plasmin

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The reaction between antiplasmin (A) and plasmin (P) proceeds in at least two steps: a very fast reversible second-order reaction followed by a slower irreversible first-order reaction, and may be represented by: $P + A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PA \xrightarrow{k_2} PA'$. The two forms of plasmin with different affinities for lysine-Sepharose (plasmin I and plasmin II) react at different rates with antiplasmin. The k_1 is $(3.8 \pm 0.3) \times 10^7$ M^{-1} s^{-1}, for the reaction between plasmin I and antiplasmin, and $(1.8 \pm 0.2) \times 10^7$ M^{-1} s^{-1} for plasmin II and antiplasmin. This reaction rate is one of the fastest so far described for protein-protein interactions. The k_1 for trypsin and antiplasmin is $(1.8 \pm 0.2) \times 10^6$ M^{-1} s^{-1}, which is similar to the reaction rates of trypsin with trypsin inhibitors. The dissociation constant of the reversible step is $(1.9 \pm 0.3) \times 10^{-10}$ M for plasmin I with antiplasmin and $(1.8 \pm 0.3) \times 10^{-10}$ M for plasmin II with antiplasmin. The second step has a half-time of approximately 165 s for both types of plasmin, corresponding to a k_2 of 4.2×10^{-3} s^{-1}. Plasmin which has 6-aminohexanoic acid bound to its lysine binding site or the substrate D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide bound to its active site does not react, or reacts very slowly, with antiplasmin.

From these findings we postulate that plasmin formed in the circulating blood is rapidly neutralized by antiplasmin, but plasmin bound to fibrin is not. This hypothesis may provide a molecular basis for the mechanism of thrombolysis *in vivo*.

Plasmin formed in human plasma is mainly inactivated by a recently discovered protease inhibitor which has been provisionally named antiplasmin, α_2-plasmin inhibitor or primary plasmin inhibitor [1–4]. Antiplasmin has been purified to homogeneity and found to be a single chain glycoprotein with a molecular weight of about 70000 [3,5]. The purified protein reacts rapidly with plasmin forming a very stable stoichiometric 1:1 complex with the B chain of plasmin [3,5].

The present paper deals with the kinetics of the reaction between plasmin and antiplasmin.

MATERIALS AND METHODS

Antiplasmin

Human antiplasmin was purified to homogeneity by affinity chromatography on plasminogen-Sepharose followed by chromatography on DEAE-Sephadex and concanavalin–Sepharose as described previously [5]. The activity of this preparation is very stable even after one week at room temperature at pH 7.3 [5]. A stock solution of antiplasmin was prepared by dissolving lyophilized antiplasmin in 0.1 M sodium phosphate buffer, pH 7.3, to a concentration of about 2 μM. The exact concentration was determined by titration against plasmin of known concentration (see below). The solution was then diluted to 1.5 μM, divided into aliquots and stored at −90 °C until use.

Plasminogen and Plasmin

Plasminogen was prepared as previously described [6]. Plasminogen types I and II were separated by 6-aminohexanoic acid gradient elution from lysine-sepharose as described by Brockway and Castellino [7]. Activation of these two plasminogen forms to plasmin types I and II was performed with urokinase-Sepharose [6]. The concentration of plasmin was determined by active-site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate as described by Chase and Shaw [8]. Stock solutions of plasmin 1.0 μM or 10 μM in phosphate buffer, pH 7.3, containing 25% glycerol, were divided into aliquots and kept at −90 °C until use.

Abbreviation. DVal-Leu-Lys-Nan, D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide.

Enzymes. Plasmin (EC 3.4.21.7); trypsin (EC 3.4.21.4); urokinase (EC 3.4.99.26).

Trypsin

Trypsin, treated with L-1-tosylamido-2-phenylethyl chloromethylketone, was obtained from Sigma Chemical Company (St Louis, Mo, U.S.A.). It was dissolved in 1 mM HCl, and its concentration determined by active-site titration. The stock solution was then diluted to 1.0 μM with 1 mM HCl divided into aliquots and stored at −20 °C.

Reagents

The synthetic peptide substrate DVal-Leu-Lys-Nan was a kind gift from Dr Göran Claesson, AB Kabi (Mölndal, Sweden). *p*-Nitrophenyl-*p'*-guanidinobenzoate was purchased from Nutritional Biochem. Corp. (Cleveland, Ohio, U.S.A.).

Determination of Plasmin and Trypsin Activity

This was performed with DVal-Leu-Lys-Nan using substrate concentrations ranging from 0.12 mM to 3.0 mM. All experiments were carried out in 0.1 M phosphate buffer, pH 7.3, at 25 °C, using a volume of 2 ml in a cuvette with 1-cm path-length. The change in absorbance at 410 nm was automatically recorded with a Beckman 25 double-beam spectrophotometer with a five-fold increased sensitivity. The lower limit for determination of plasmin concentration with this substrate under these conditions is about 50 pM.

The Michaelis-Menten constant, K_m, for plasmin and trypsin was determined as 0.29 mM and 0.9 mM respectively.

Determination of Antiplasmin Activity

To a solution of plasmin (final concentration 40 nM) was added antiplasmin to final concentrations of between 5 and 50 nM. The mixtures were incubated for 30 s and the residual plasmin activity was measured after addition of DVal-Leu-Lys-Nan to a final concentration of 0.30 mM. A line was obtained which only deviated from linearity close to the 1 : 1 molar ratio of antiplasmin to plasmin. The exact antiplasmin concentration was determined by extrapolation of the line to zero residual plasmin concentration.

Determination of the Kinetic Constants of the Plasmin-Antiplasmin Reaction

Plasmin and antiplasmin in different concentrations ranging from 5.0−400 nM for plasmin and 7.5−600 nM for antiplasmin were mixed and residual plasmin activity was measured with the peptide substrate DVal-Leu-Lys-Nan after incubation at 25 °C for 4 s to 30 min.

The plasmin-antiplasmin reaction was also studied in the presence of DVal-Leu-Lys-Nan (0.12−3.0 mM). In this case the absorbance at 410 nm was automatically recorded during the experiment. The residual plasmin concentration at a certain time could be determined from the slope of the curve at that time. This system, with 0.3 mM DVal-Leu-Lys-Nan, was also used to study the effect of 6-aminohexanoic acid (1−10 μM) on the plasmin-antiplasmin reaction. The change in absorbance at 410 nm was measured accurately (recorder scale 0−0.025 absorbance). In order to obtain reproducible results it was necessary to use perfectly clean cuvettes and carefully filtered dust-free reagents.

RESULTS

The disappearance of plasmin after addition of excess antiplasmin did not follow first-order kinetics (Fig. 1). Most of both plasmin I and plasmin II was very rapidly inactivated but the process only slowly proceeded towards completion. This time course of the reaction may be explained by a kinetic model composed of two successive reactions of which the first is rapid and reversible and the second slower and irreversible. This model can be represented by:

$$P + A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PA \xrightarrow{k_2} PA'$$

in which P represents plasmin, A antiplasmin, PA the reversible complex formed in the first-step and PA' the irreversible complex formed in the second step.

Determination of k_1

The reaction between antiplasmin and plasmin I and II was studied in a highly diluted system using short incubation times as described under Materials and Methods.

Plasmin (final concentration 5.6 nM) and antiplasmin (final concentration 8.4 nM) were rapidly mixed in a cuvette and incubated for 4 or 8 s. Then DVal-Leu-Lys-Nan (final concentration 0.6 mM) and 6-aminohexanoic acid (final concentration 0.1 mM) were rapidly added and the change in absorbance at 410 nM recorded within 5 s. The addition of substrate and 6-aminohexanoic acid caused an approximately 10-fold reduction in the reaction rate between plasmin and antiplasmin.

The residual plasmin concentration was determined from the initial slope of the curve. The rate constant k_1 was determined using the classical equation for second order kinetics:

$$k_1 t = \frac{1}{c_A^o - c_P^o} \ln \frac{c_P^o (c_A^o - c_P^o + c_P)}{c_A^o c_P}$$

where c_A^o and c_P^o are the initial concentrations of antiplasmin and plasmin respectively.

The values of k_1 for the reaction between antiplasmin and plasmin I and between antiplasmin and plas-

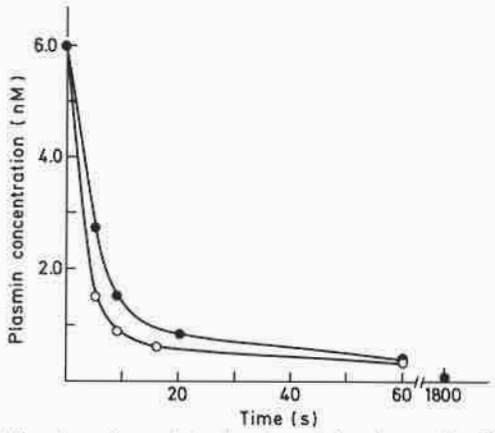

Fig. 1. *Time dependence of the plasmin-antiplasmin reaction.* Plasmin (final concentration 6.0 nM) and antiplasmin (final concentration 9.0 nM) were mixed and incubated at 25 °C. At different time intervals residual plasmin activity was measured after addition of the plasmin substrate DVal-Leu-Lys-Nan to a final concentration of 0.6 mM and of 6-aminohexanoic acid to a final concentration of 0.1 mM. Plasmin I (○——○); plasmin II (●——●)

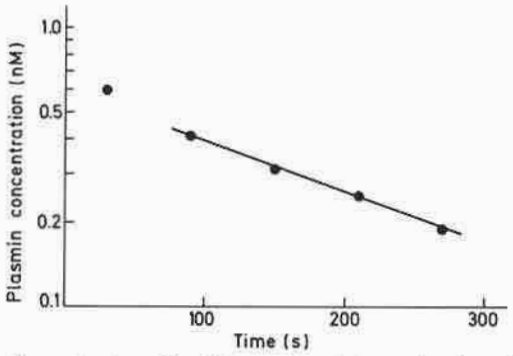

Fig. 2. *Determination of* k_2 *of the reaction between plasmin and antiplasmin.* Plasmin II (final concentration 200 nM) and antiplasmin (final concentration 300 nM) were allowed to react for 30 s at 25 °C, DVal-Leu-Lys-Nan was added to a concentration of 0.3 mM and the change in absorbance measured at 410 nm. The residual plasmin concentration was calculated from the slope of the curve. Plasmin I and II behaved very similarly in this system

min II were significantly different and were found to be $(3.8 \pm 0.3) \times 10^7$ M^{-1} s^{-1} and $(1.8 \pm 0.2) \times 10^7$ M^{-1} s^{-1}, (mean ± S.D.) respectively. This is one of the most rapid reactions between two protein molecules ever described [9].

Determination of the Dissociation Constant K

This was performed by mixing a solution of plasmin I or plasmin II (final concentration 25 nM) in sodium phosphate buffer with antiplasmin to a final concentration of 37.5, 50 or 100 nM. After incubation for 20 s DVal-Leu-Lys-Nan was added to a concentration of 0.6 mM. The change in absorbance at 410 nm was automatically recorded and K calculated from the residual plasmin activity using the equation

$$K = \frac{c_P c_A}{c_{PA}} = \frac{c_P (c_A^o - c_P^o + c_P)}{c_P^o - c_P} .$$

K was found to be $(1.9 \pm 0.3) \times 10^{-10}$ M for the reaction of plasmin I with antiplasmin and $(1.8 \pm 0.3) \times 10^{-10}$ M for the reaction of plasmin II with antiplasmin.

Determination of k_2

Plasmin I or plasmin II (final concentration 200 or 400 nM) was mixed with antiplasmin (final concentration 300 or 600 nM). After incubation for 20 s, DVal-Leu-Lys-Nan was added to a concentration of 0.6 mM and the change in absorbance at 410 nm automatically recorded. Under these conditions, i.e. in the presence of excess antiplasmin, the concentration of the complex PA is proportional to that of the residual plasmin, c_P. The residual plasmin concentration was calculated from the slope at different times. The half-time and the rate constant k_2 of the first-order transition PA $\overset{k_2}{\rightarrow}$ PA′ were determined from the plot of log c_P against time (Fig. 2). No significant difference was found between plasmin I and plasmin II. The half-time was found to be 166 ± 13 s for plasmin I and antiplasmin and 164 ± 5 s for plasmin II and antiplasmin, corresponding to k_2 values of $(4.2 \pm 0.3) \times 10^{-3}$ s^{-1} and $(4.2 \pm 0.1) \times 10^{-3}$ s^{-1} respectively.

Effect of DVal-Leu-Lys-Nan *on* k_1 *of the Reaction between Plasmin and Antiplasmin*

Plasmin II (final concentration 5 nM) was mixed with different concentrations of DVal-Leu-Lys-Nan ranging from 0.12 – 3.0 mM. The change in absorbance at 410 nm was automatically recorded. After about 30 s antiplasmin was rapidly added (to a final concentration of 7.5 nM). The apparent k_1 values of the reaction in the presence of DVal-Leu-Lys-Nan were calculated from the slopes of the curves, determined 9 and 18 s after the addition of antiplasmin. As can be seen from Fig. 3, the substrate acts as an inhibitor of the plasmin-antiplasmin reaction. The influence of the substrate concentration on the apparent k_1 values is very similar but of opposite direction to its effect on the velocity of the plasmin-catalyzed cleavage of DVal-Leu-Lys-Nan.

Effect of 6-Aminohexanoic Acid on the Plasmin-Antiplasmin Reaction

Plasmin I or plasmin II (final concentration 5 nM) was added to a cuvette containing 0.3 mM DVal-Leu-Lys-Nan and 6-aminohexanoic acid (final concentration 1 μM to 10 mM). Addition of 6-aminohexanoic

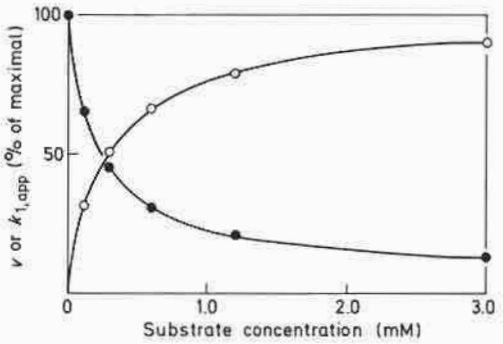

Fig. 3. *Influence of* DVal-Leu-Lys-Nan *on the reaction between plasmin II and antiplasmin and on the rate of its cleavage by plasmin.* The influence of DVal-Leu-Lys-Nan on the reaction between plasmin and antiplasmin was studied by determining the apparent k_1 values (expressed as a percentage of the value obtained in the absence of DVal-Leu-Lys-Nan, which is 1.8×10^7 M^{-1} s^{-1}) at different concentrations of DVal-Leu-Lys-Nan (●——●). The substrate dependence of the rate of the plasmin-catalyzed cleavage of DVal-Leu-Lys-Nan (expressed as percentage of maximal rate, as determined from a Lineweaver-Burke plot of the data) is also shown (○——○). Similar results were obtained for plasmin I and antiplasmin

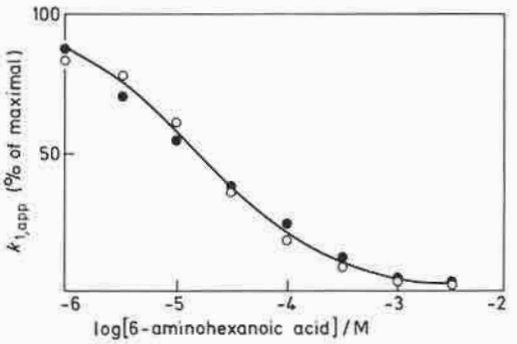

Fig. 4. *Influence of 6-aminohexanoic acid on the reaction between plasmin and antiplasmin.* The apparent k_1 values, determined in the presence of 0.3 mM DVal-Leu-Lys-Nan and of 6-aminohexanoic acid in different concentrations, are expressed as a percentage of the value obtained in the absence of 6-aminohexanoic acid. Plasmin I and antiplasmin (●——●); plasmin II and antiplasmin (○——○)

acid did not change the reaction rate of plasmin with this substrate. Then antiplasmin was added to a final concentration of 7.5 nM and the change in absorbance recorded. The apparent k_1 values were calculated from the slope of the curves before 50 % of the plasmin had been inactivated. Fig. 4 shows that 6-aminohexanoic acid inhibits the plasmin-antiplasmin reaction at a very low concentration. A 50 % reduction of k_1 was obtained at a 6-aminohexanoic acid concentration of 20 μM.

The Reaction of Antiplasmin with Trypsin

Trypsin and antiplasmin (final concentrations 10 nM and 15 nM) were mixed and incubated for 30, 60 and 90 s. Then DVal-Leu-Lys-Nan was added to a final concentration of 0.9 mM and the initial change in absorbance at 410 nm was recorded and used for calculation of the residual trypsin activity. The rate constant, k_1, was calculated as $(1.8 \pm 0.3) \times 10^6$ M^{-1} s^{-1}. 6-Aminohexanoic acid in concentrations up to 0.1 M did not affect the rate constant of the trypsin-antiplasmin reaction.

DISCUSSION

Recently a new protease inhibitor which inactivates plasmin very efficiently was discovered in human plasma. It has been called antiplasmin [1, 2, 5], α_2-plasmin inhibitor [3] or primary plasmin inhibitor [4]. It acts by rapidly forming a very stable 1 : 1 stoichiometric complex with the B chain of plasmin [3, 5]. Using dodecyl sulphate/polyacrylamide gel electrophoresis, we were unable to detect any peptide bond cleavage during the reaction between plasmin and antiplasmin [5] when the reaction was carried out in an excess of inhibitor. However, if plasmin was in excess, a peptide with a molecular weight close to 14000 was released after reduction. This peptide is probably identical with the peptide described by Moroi and Aoki [3], Collen [2] and Müllertz and Clemmensen [4]. Preliminary data indicate that it originates from the NH_2 terminus of the plasmin B chain (Wiman and Collen, unpublished results) rather than the C terminus of antiplasmin as has been suggested [2–4].

In the present study we have investigated the kinetics of the interaction of plasmin with antiplasmin by using a synthetic chromogenic tripeptide substrate DVal-Leu-Lys-Nan. We were initially unable to describe the time course of the reaction by a simple kinetic model owing to the fact that the plasmins derived from the two plasminogen forms with different affinity to lysine-Sepharose [7] reacted differently. Therefore all subsequent experiments were carried out on the separated plasmins.

The disappearance of plasmin after addition of excess antiplasmin did not follow first-order kinetics but could be adequately described by a kinetic model composed of a fast reversible complex formation followed by a slower irreversible intramolecular transition. Such a kinetic model has been used previously to describe protease-inhibitor reactions [11]. The rate constants for the complex formation between plasmin I and plasmin II and antiplasmin were found to be 3.8×10^7 M^{-1} s^{-1} and 1.8×10^7 M^{-1} s^{-1} respectively. This is among the fastest protein-protein reactions ever described [9]. Such high rate constants are close

to the value theoretically possible for a diffusion-controlled process. The reaction rate between plasmin and antiplasmin is more than 10-fold faster than any of the protease-inhibitor reactions described so far. It is also 10-fold faster than the reaction between trypsin and antiplasmin. It is very likely that the lysine-binding site of plasmin plays an important role in determining the rate of the reaction. Indeed, plasmin which has its lysine-binding site(s) occupied by 6-aminohexanoic acid (final concentration 1 – 10 mM, Fig. 4) or trypsin, which has no lysine-binding site, react at least 10-fold slower than free plasmin with antiplasmin.

The equilibrium (dissociation) constant was estimated to be 1.9×10^{-10} M for plasmin I with antiplasmin and 1.8×10^{-10} M for plasmin II with antiplasmin. Similar values have been obtained for other protease-inhibitor complexes [12]. From the values of the equilibrium constant K and the rate constant of the complex formation k_1, the rate constant of the first-order complex dissociation k_{-1} was calculated to be 7.0×10^{-3} s^{-1} for plasmin I with antiplasmin and 3.2×10^{-3} s^{-1} for plasmin II with antiplasmin.

The second step of the reaction follows first-order kinetics with a half-time of about 165 s for both plasmin forms, corresponding to a rate constant k_2 of 4.2×10^{-3} s^{-1}. These results indicate that k_{-1} and k_2 are of the same order of magnitude, which means that a true equilibrium will never be reached.

Very recently Christensen and Clemmensen [13] reported on the kinetic properties of the primary plasmin inhibitor, which is identical to antiplasmin. They have also proposed a reaction mechanism composed of two steps: a fast reversible reaction followed by a slow irreversible one. They were, however, unable to determine the rate constant of the reaction, but estimated the equilibrium constant to be about 2×10^{-9} M and k_2 to 6.5×10^{-3} s^{-1}. With the use of a better plasmin substrate which allowed us to measure very small concentrations of plasmin in a short time we were able to determine the constants of the reaction more accurately.

The first step of the process is clearly dependent on the presence of a free lysine-binding (site(s) and active site in the plasmin molecule. Indeed, plasmin molecules which have bound the substrate to their active site or 6-aminohexanoic acid to their lysine-binding site(s), do not react or react only very slowly with antiplasmin. Christensen and Clemmensen have recently reached the same conclusion [13]. The influence of the substrate concentration on the apparent k_1 values is very similar but of opposite direction to its effect on the velocity of the plasmin-catalyzed cleavage of D-Val-Leu-Lys-Nan. This suggests that antiplasmin reacts with the active site of the plasmin molecule. The reaction rate between plasmin and antiplasmin is decreased to 50% at a 6-aminohexanoic acid concentration of about 20 μM. This is more than 10-fold lower than the dissociation constants reported for the plasminogen – 6-aminohexanoic acid complex [7, 10]. As can be seen from Fig. 4, the influence of 6-aminohexanoic acid on the rate constant of the plasmin-antiplasmin reaction does not appear to be the result of a single association reaction between plasmin and 6-aminohexanoic acid. This discrepancy might be explained by the fact that several lysine-binding sites seem to exist in plasminogen [14, 15] and that they differ in the strength with which they bind 6-aminohexanoic acid. This is also supported by the finding of a biphasic activation of plasminogen at different concentrations of 6-aminohexanoic acid [16].

The same lysine-binding site(s) that is (are) involved in the plasmin antiplasmin reaction also seem to be responsible for the plasmin(ogen)-fibrin interaction. Indeed, the binding of plasminogen to fibrin which is mediated through the lysine-binding site [17] is decreased to 50% at a 6-aminohexanoic acid concentration of 80 μM [18]. Furthermore it has recently been shown that antiplasmin interferes with the binding of plasminogen to fibrin [19].

These lysine-binding site(s) seem(s) to be of great importance for the specificity and effectiveness of the fibrinolytic system. From the data presented in this work it might indeed be speculated that plasmin molecules bound to and involved in fibrin degradation are only slowly inactivated by antiplasmin, whereas plasmin molecules which are circulating in plasma are rapidly inactivated. From the concentration of antiplasmin in plasma and the rate constants of the plasmin-antiplasmin reaction, the half-time of free plasmin in plasma was calculated to be 25 – 50 ms. This hypothesis may provide a molecular basis for the mechanism of thrombolysis *in vivo*.

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