Stopped-flow fluorescence kinetics of bovine α_2-antiplasmin inhibition of bovine midiplasmin

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In the conversion of bovine plasminogen to bovine plasmin not only the expected urokinase-catalysed cleavage of Arg-557–Val-558, and the following autocatalytic cleavage separating the N-terminal peptide 1–77 from the heavy chain of plasmin, but also a cleavage at Arg-342–Met-343 between kringles 3 and 4 is seen. Here, kinetic studies of the interaction of bovine α_2-antiplasmin with bovine plasmin were performed on isolated bovine midiplasmin (lacking kringles 1–3) and on bovine plasmin containing all of the activation products from the bovine plasminogen. A series of experiments using stopped-flow fluorescence fast kinetics as well as conventional techniques suggests a reaction model in accordance with the one known for the human system. First, a tight complex (K_i in the nanomolar range) is formed in a fast reaction step; and second, a tightening of this complex occurs in a slow reaction step. The final complex is indeed so tight ($K_i \leqslant$ pM), that the reaction for many practical purposes is legitimately considered irreversible. The stopped-flow method allows for the determination of reliable values of the second-order rate constant for the fast association step. At pH 7.4 and 25 °C, $k_{+1} = 1.7 \times 10^6$ M^{-1} s^{-1} was obtained in the absence and $k_{+1} = 0.9 \times 10^6$ M$^{-1} \cdot$ s^{-1} in the presence of the kringles 1–3 domain of bovine plasmin. In contrast to this, substantial reductions of k_{+1} were seen in the presence of concentrations of 6-aminohexanoic acid corresponding to lysine-binding-site interactions and far too low to be attributed to active-site interactions with the bovine plasmins (for each, $K_i = 42$ mM). All in all, the data indicated that the lysine-binding site(s) not of kringle 1, but of midiplasmin (those of kringles 4 and 5) are regulating the inhibition reaction.

INTRODUCTION

α_2-Antiplasmin (α_2AP), the primary inhibitor of plasmin, is present in plasma at a concentration of ~ 1 μM (Mori and Aoki, 1976). α_2APs from two species have been characterized. Thus the primary structure of human α_2AP and the N-terminal and reactive-site sequences of bovine α_2AP are known (Holmes et al, 1987; Christensen and Sottrup-Jensen, 1992). α_2AP is a member of the serpin family (Travis and Salvesen, 1983); it has an acceptor site for activated coagulation factor XIII mediated cross-linking to fibrin during blood coagulation (Tamaki and Aoki, 1982) at Gln-14 (new numbering, Christensen and Sottrup-Jensen, 1992; Bangert et al., 1993; Enghild et al., 1993), and contains a C-terminal extension with affinity to lysine-binding sites (LBSs) in plasmin(ogen). Purification of α_2AP is typically performed by affinity chromatography on immobilized plasminogen or plasminogen fragments containing kringle 1 (Mori and Aoki, 1976; Wiman et al., 1979). In human α_2AP, Lys-475 and the C-terminal Lys-491 appear to be involved (Sugiyama et al., 1988), indicating that the effect cannot be assigned to any single lysine-binding site and therefore may involve the weak lysine-binding sites of plasmin(ogen) (Christensen, 1984; Christensen and Mølgaard, 1992) as reported earlier (Christensen and Clemmensen, 1978). In plasma, plasmin stems from the proenzyme plasminogen (for reviews, see: Castellino and Powell, 1981; Ponting et al., 1992). The primary structures of human, bovine, porcine and mouse plasminogen are highly homologous (Sottrup-Jensen et al, 1977; Schaller et al., 1985; Marti et al., 1985; Schaller et al., 1987; Petersen et al., 1990; Friezner Degen et al., 1990) (overview by Schaller and Rickli, 1988). The conversion of native human plasminogen to plasmin catalysed by plasminogen activators requires cleavage of only one peptide bond (residues 561–562), but is followed by an autocatalytic cleavage at residues 78–79 (Summaria et al., 1975; Christensen, 1977), and results in a disulphide-bonded two-chain plasmin molecule with a heavy chain (residue 79–561) containing kringles 1–5 with the LBSs and a light chain (residues 562–791) containing the catalytic serine proteinase domain. Kringles 1, 4 and 5 possess LBSs (Sottrup-Jensen et al., 1977; Lerch et al., 1980; Llinás et al., 1983; Christensen, 1984; Thewes et al., 1987) that mediate important interactions of the plasmin(ogen) and the other proteins of the fibrinolytic system, but are not essential for the actual catalytic activity of plasmin. Miniplasmin of which kringles 1–4 has been removed from the proenzyme with elastase (Sottrup-Jensen et al., 1977) before conversion with plasminogen activator shows catalytic properties very similar to that of full-length plasmin (Christensen et al., 1979), but its rate of association with α_2AP is 10–35-fold slower than that of full-length plasmin (Wiman et al., 1978; Christensen et al., 1979). Association rates, dependent on temperature and buffer composition, have been reported as (7×10^5)–(2×10^7) M$^{-1} \cdot$ s^{-1} for plasmin and $\sim 5 \times 10^5$ M$^{-1} \cdot$ s^{-1} for miniplasmin (Christensen and Clemmensen, 1977; Wiman et al., 1978; Christensen et al. 1979; Longstaff and Gaffney, 1991; Lijnen et al., 1992).

Eqn. 1 illustrates the currently accepted reaction model for the inhibition of human plasmin by α_2AP. It was described by Christensen and Clemmensen (1977, 1978), Wiman et al. (1978) and, more recently, by Longstaff and Gaffney (1991):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI^* \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI \quad (1)$$

where E is the enzyme, I the inhibitor, EI* a first tight enzyme–inhibitor complex and EI the final, even tighter complex.

Abbreviations used: 6-AHA, 6-aminohexanoic acid; α_2AP, α_2-antiplasmin; LBS, lysine-binding site.

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In previous studies conventional kinetic methods have been used to determine the fast association (k_{+1}) as well as the slow tightening step (k_{+2}). In this paper, the kinetics of the reaction of bovine plasmin and α_2AP was investigated using the stopped-flow technique to study the fast association by monitoring a change in intrinsic protein fluorescence occurring as the complex forms. Full-length bovine plasmin was not obtainable, because the kringles 1–3 fragment of the bovine plasminogen is cleaved off, probably autocatalytically by plasmin, during the urokinase-catalysed activation of bovine plasminogen. We have isolated and characterized the bovine midiplasmin (lacking kringles 1–3), and have used it as well as the total activation mixture of bovine plasminogen (bovine plasmin) in the studies of the inhibition reaction. A hitherto overlooked importance of kringle 5 and/or 4 in the inhibition of plasmin is discussed.

MATERIALS AND METHODS

Lysine–Sepharose was prepared according to March et al. (1974). S-Sepharose FF was from Pharmacia. Urokinase–Sepharose was prepared by coupling urokinase to CNBr-activated Sepharose according to the instructions from Pharmacia (13333 PU/ml of gel) (PU, Plough Units; 1 PU = 1.4 CTA units; CTA, Committee of Thrombolytic Agents). Substrates H-D-Val-Leu-Lys-*p*-nitroanilide (VLKpNA) and pyro-Glu-Phe-Lys-*p*-nitroanilide (< EFKpNA) were from Kabi–Pharmacia. 4′-Nitrophenyl-4-guanidinobenzoate was from Boehringer.

Proteins

Bovine plasmin

Bovine plasminogen was isolated from bovine plasma by affinity chromatography on lysine–Sepharose essentially as described by Deutsch and Mertz (1970) and converted to plasmin by incubation with 0.25 vol. of urokinase–Sepharose in 50 mM Tris/HCl, 1 mM L-lysine, 20% (v/v) glycerol, pH 7.4, for 6 h at room temperature and centrifuged. The supernatant was the bovine plasmin used here. Its concentration of active plasmin (8 μM) was determined by titration with α_2AP, which was itself titrated with trypsin. As shown below (Figure 1), the preparation of bovine plasmin thus obtained consists of a mixture of the N-terminal fragment (residues 1–77), kringles 1–3 (residues 78–342) and midiplasmin (kringles 4 and 5 disulphide-bound to plasmin light chain, residues 343–557 plus 558–786). As discussed below, kringles 1–3 and midiplasmin do not appear to form an association complex.

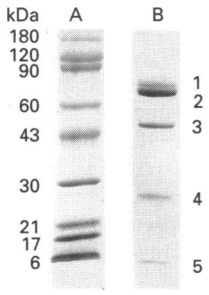

Figure 1 SDS/PAGE analysis of partially activated bovine plasminogen

Non-reduced electrophoresis was carried out using a 10–20% (w/v) polyacrylamide gel: lane A, molecular-mass markers; lane B, bovine plasminogen partially activated with urokinase. Protein bands were identified by partial N-terminal sequence analysis and from size. Band 1, D_1LLD, bovine plasminogen; band 2, R_{78}IYL, Arg_{78}-plasminogen; band 3, two sequences, M_{343}DVP and I_{558}VGG, midiplasmin; band 4, R_{78}IYL, kringles 1–3; band 5, D_1LLD, peptide 1–77.

Midiplasmin

Midiplasmin was prepared by incubating 18.5 mg of plasminogen with 4200 PU of urokinase for 1 h in 34 ml of 10 mM Tris/HCl, pH 7.4, at 37 °C. After the addition of 50 ml of cold (4 °C) 50 mM sodium acetate, pH 5.2, also used for column equilibration, the material was applied on a column of S-Sepharose FF (1 cm × 9.5 cm) and eluted by a gradient of NaCl (0–0.5 M in equilibration buffer, total volume 420 ml) at a flow rate of 4 ml/min. The yield of midiplasmin was 85%. Fractions containing pure midiplasmin were dialysed against 10 mM sodium acetate, pH 5.2, concentrated to 13.7 μM and stored at −20 °C.

α_2AP

α_2AP was isolated from bovine plasma as described by Christensen and Sottrup-Jensen (1992). Its reactive-site concentration was determined using 4′-nitrophenyl-4-guanidinobenzoate-titrated trypsin (20 nM) and the trypsin substrate VLKpNA (93 μM). Trypsin was from Sigma. Urokinase was from Immuno Danmark A/S.

N-terminal sequence analysis

Proteins were separated by SDS/PAGE (Laemmli, 1970) and transferred to ProBlott membranes by electroblotting (Matsudaira, 1987). Edman degradations were done in an AB 477A sequenator using the Donblot reaction and conversion cycles. The initial yield was ~ 50 pmol.

Fluorescence titration

Fluorescence emission spectra of α_2AP, bovine plasmins and mixtures thereof were recorded from λ = 300–400 nm in 50 mM Tris/HCl, 0.1 M NaCl, pH 7.4, at 25 °C (this buffer was also used in all kinetic experiments) in a Perkin-Elmer LS-50 fluorometer. The excitation wavelength was 280 nm and the excitation and emission slits were each 5 nm. Inner filter effects were negligible. Titrations were performed by adding 12.5 μl aliquots of plasmin (~ 1 μM) to 2500 μl of α_2AP (0.05 μM). After incubation for at least 5 min, the spectra ($F[C]$) were recorded. Control spectra of inhibitor alone ($F[0]$) and plasmin alone at various concentrations ($F[E]$) were used to calculate the relative fluorescence change, ΔF, thus:

$$\Delta F = \frac{F[C]-(F[O]+F[E])}{F[C]} \quad (2)$$

where $F[C]$, $F[0]$ and $F[E]$ are the integrated intrinsic protein fluorescence intensities at the actual enzyme concentrations.

Stopped-flow kinetic experiments

Fast kinetic experiments were performed in a Hi-Tech Scientific PQ/SF-53 spectrofluorometer equipped with a high intensity xenon arc lamp. The excitation wavelength was 280 nm, the slit width 5 nm. The light emitted from the reaction mixture passed through a WG 320 filter before detection in the photomultiplier. The time course of intrinsic fluorescence intensity changes of the fast reaction step of the inhibition reactions was recorded for 10 s after mixing of equal concentrations of α_2AP and bovine plasmin. The mixing time was < 1 ms. The final enzyme and inhibitor concentrations were 2, 1.5, 1.16, 0.68, 0.48 and 0.23 μM. In each experiment, 400 pairs of data were recorded,

and sets of data from at least four experiments were averaged and analysed with the Hi-Tech HS-1 Data Pro Software. The regression analysis used is based on the Gauss–Newton procedure. Background experiments showed each initial fluorescence intensity to be equal to the sum of that of plasmin and that of α_2AP each obtained in the absence of the other compound. After recording 10 s of the reactions, control measurements were made 6 times at 5 min intervals; such measurements showed no further changes of the fluorescences.

Steady-state kinetic experiments

Kinetic constants for bovine plasmin- and midiplasmin-catalysed substrate hydrolysis were determined from series of initial rate measurements and calculated using the non-linear regression analysis software Enzfitter (Leatherbarrow, 1987). The two enzyme preparations showed no differences and the kinetic parameters were as follows: $K_m = 0.15$ mM, $k_{cat.} = 6\ s^{-1}$ for VLKpNA and $K_m = 85\ \mu M$, $k_{cat.} = 20\ s^{-1}$ for < EFKpNA. Also, the same weak active-site interaction with 6-aminohexanoic acid (6-AHA) that manifests itself as competitive inhibition of substrate catalysis was obtained using VLKpNA as the substrate. K_i was found to be 42 mM for bovine midiplasmin as well as for our bovine plasmin.

Kinetics of inhibition progress

To characterize the slow step and determine the rate (k_{+2}) of the expected rearrangement of the first complex to a more tightly bound final complex of α_2AP and the bovine plasmins, the residual activities of plasmin in equimolar α_2AP–plasmin reaction mixtures, containing sufficient amounts of the proteins to leave measurable amounts of residual free plasmin after the formation of the first complex, were determined over a long time range in two ways. (a) Mixtures of 375 μl of plasmin and 375 μl of α_2AP (each 2 or 1 μM final concentration) were preincubated. At different times, 750 μl of < EFKpNA (447.5 or 85 μM final concentration) was added and the residual plasmin activity determined for the initial rates. (b) Alternatively, the release of product was followed after manual mixing of all three components and the rate corresponding to the residual plasmin activity at chosen points of time was determined from the slope (the first time derivative) of the recorded curve, which was calculated using the PECSS software of the Perkin–Elmer spectrophotometer.

In both types of experiments, an exponential decrease of the plasmin activity corresponding to a slow first-order reaction step occurring after the fast initial equilibration of the first step was seen. The zero-time values of the fitted exponential equations are the points that correspond to the initial values of the second reaction step for a theoretical immediate equilibration of the first step. They were used to calculate K_1, assuming full equilibration between substrate, enzyme and inhibitor (with respect to the first step) during that part of the slow step on which the fitted exponentials were based. This assumption is apparently valid because K_1 values varied by < 10%.

RESULTS

Conversion of bovine plasminogen to plasmin

The urokinase-catalysed conversion of bovine plasminogen to plasmin was followed by non-reduced SDS/PAGE analysis (Figure 1). From partial sequence analysis of the material in bands 1–5 three peptide bonds in plasminogen were found to be cleaved: Lys-77–Arg-78, Arg-342–Met-343 and Arg-557–Ile-558. In Figure 1, band 1 is intact residual plasminogen, band 2 is Arg-78-plasminogen, band 3 midiplasmin (kringles 4+5 disulphide-bound to plasmin light chain), band 4 kringles 1–3 (residues 78–342), and band 5 the N-terminal fragment (residues 1–77). The cleavage sites were identified on the basis of the primary structure of bovine plasminogen (Schaller et al., 1985). The kinetic experiments were performed on the entire mixture of bovine plasmin fragments as well as on purified midiplasmin.

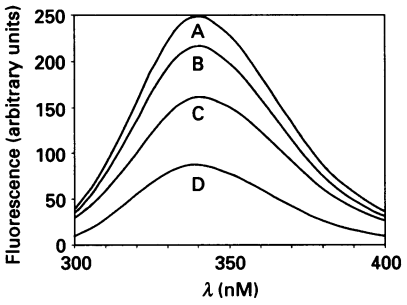

Figure 2 Fluorescence spectra of plasmin and α_2AP

Fluorescence spectra were recorded from 300 to 400 nm in 50 mM Tris/HCl, 0.1 M NaCl, pH 7.4, at 25 °C after excitation at 280 nm. A, sum of C+D; B, complex of plasmin (50 nM) and α_2AP (50 nM); C, plasmin (50 nM); and D, α_2AP (50 nM).

Fluorescence spectra

Fluorescence emission spectra were obtained of α_2AP, bovine plasmin and the α_2AP–plasmin complex (Figure 2). Emission maxima were all at 340 nm. As expected from the molar content of Trp residues, the intensity of α_2AP (trace D) was smaller than that of plasmin (trace C). Interestingly, the fluorescence intensity of the sum of the uncomplexed components (trace A) exceeded the fluorescence of the complex (trace B), showing a substantial fluorescence intensity decrease in the complex (18 ± 1%; mean ± S.D., $n = 4$) and no further changes after incubation much longer than the 5 min used here. This allowed for direct titration of bovine plasmin with α_2AP as well as direct monitoring of the first step of the α_2AP–plasmin reaction in the absence of a plasmin substrate.

Stopped-flow kinetics

The kinetics of the reactions of α_2AP with plasmin and with midiplasmin were investigated by mixing series of equimolar concentrations of the two proteins using the stopped-flow technique. A typical stopped-flow trace of the resulting decrease in fluorescence intensities is shown in Figure 3. Also, the fitted curve corresponding to eqn. 3, which describes second-order reaction kinetics at equal concentrations of the two reactants, is illustrated in Figure 3:

$$\Delta F(t) = \Delta F(\infty)\left(1 - \frac{1}{1 + k_{obs.} \cdot E_o \cdot t}\right) \quad (3)$$

$\Delta F(t)$ is the relative fluorescence change at time t, $\Delta F(\infty)$ is the limit of value for $t \rightarrow \infty$ corresponding to completion of the current reaction step; E_o is the concentration of bovine plasmin or midiplasmin equal to that of α_2AP; and $k_{obs.}$ is the observed second-order rate constant. Other equations corresponding to first-order single exponential or mixed-type biexponential reactions showed poor fits to the data, whereas eqn. 3 not only fitted well but also resulted in essentially identical $k_{obs.}$ values at all concentrations of enzyme and inhibitor with plasmin as well

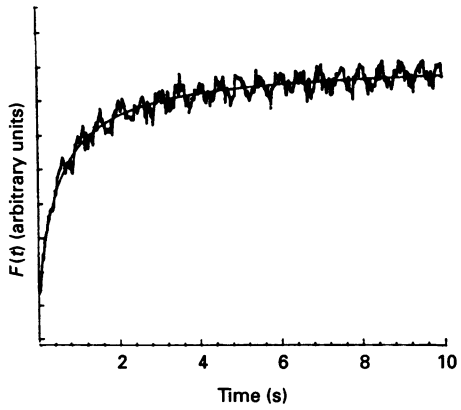

Figure 3 Typical time course of the stopped-flow fluorescence signal resulting from the inhibition of plasmin by α_2AP

Midiplasmin (2 μM) and α_2AP (2 μM) were mixed and the fluorescence followed for 10 s. The curve shows the result of the fit to the data of eqn. 3 that describes second-order kinetics. The average of four traces is shown. Series of such experiments were performed at concentrations of enzyme and inhibitor in the range 2.0–0.23 μM.

Table 1 Second-order rate constants for inhibition of bovine plasmin by α_2AP

Stopped-flow fluorescence measurements were used to determine the association rate constants for the inhibition of plasmin and midiplasmin by α_2AP in the absence ($k_{obs.}$) and at two concentrations ($k_{6\text{-AHA}}$) of 6-AHA. Equimolar concentrations (2.0–0.23 μM) of enzyme and inhibitor were mixed within 1 ms and the resultant change in fluorescence followed for 10 s. A curve describing second-order kinetics was fitted to the data and the rate constant was determined. The data represent mean ± S.E.M. of the rate constants obtained at six concentrations.

	$10^{-6} \times k_{obs.}$ ($M^{-1} \cdot s^{-1}$)	$k_{6\text{-AHA}}$ ($M^{-1} \cdot s^{-1}$)	
Plasmin type	0 mM 6-AHA	0.1 mM 6-AHA	10 mM 6-AHA
Plasmin*	0.9 ± 0.1	$(0.9 \pm 0.1) \times 10^6$	$(0.8 \pm 0.1) \times 10^5$
Midiplasmin	1.7 ± 0.2	$(1.1 \pm 0.1) \times 10^6$	$(1.0 \pm 0.1) \times 10^5$

* The bovine plasmin contains all of the activation products from plasminogen but no full-length plasmin.

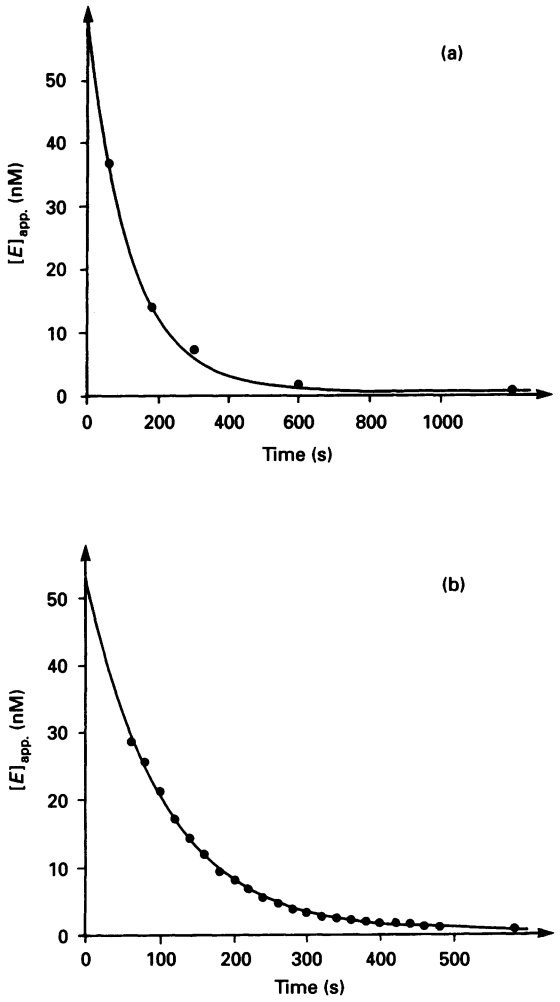

Figure 4 Representative time courses of inhibition progress

The amount of uncomplexed plasmin ($[E]_{app.}$) in mixtures of α_2AP and plasmin was determined as a function of incubation time after equilibration of the fast reaction step. The curves describe the single exponential function that was fitted to the actual data. (**a**) Samples from an incubation mixture were withdrawn at different times and residual plasmin activity assayed. (**b**) α_2AP, substrate and plasmin were manually mixed (10–20 s) and residual plasmin activities were determined from the slope of the recorded curve at chosen times. Here, resulting k_{+2} values were: (**a**) 8.1×10^{-3} and (**b**) 9.6×10^{-3}.

as with midiplasmin (Table 1), thus confirming the second-order nature of the reaction over a range of reactant concentrations spanning an order of magnitude. $k_{obs.}$ thus represents k_{+1} of eqn. 1, the second-order rate constant of the fast association of bovine plasmin and α_2AP. Partly because of instrumental differences, but also because of the concentration ranges employed, the expected relative fluorescence changes obtained in the stopped-flow experiments are less than those of the titration experiments, here 5 versus 18%. The fluorescence change nevertheless solely accompanies the formation of a first association complex of α_2AP and the bovine plasmin or midiplasmin, and no further changes were seen when measurements were made in the stopped-flow fluorimeter at reaction times much greater than that corresponding to 5–6 (concentration-dependent) half-times of the first association reaction, i.e. after its completion.

As is seen from Table 1, the presence of kringles 1–3 reduces k_{+1}. In the absence of 6-AHA k_{+1} of midiplasmin is twice that of bovine plasmin. It is well known from affinity purifications of α_2AP on plasminogen and fragments thereof that α_2AP interacts with the LBSs of kringle 1. One would thus expect some inhibitory effect of the kringles 1–3 fragment on the reaction of α_2AP and midiplasmin, an effect that would disappear in the presence of 6-AHA at concentrations sufficient to saturate the LBSs of kringle 1 (K_d = 0.01 mM; Christensen and Mølgaard, 1992). The difference between plasmin and midiplasmin is actually disappearing (Table 1), but strong effects of 6-AHA that cannot be explained from the current theories are seen, e.g. a ~10-fold reduction of the rate constants at 10 mM concentration of 6-AHA. The competitive active-site-directed effect of 6-AHA can account for only a 1.25-fold reduction (K_i = 42 mM) and, further, the effect is seen whether or not the LBSs of kringle 1 are present. The expected dependence of $k_{obs.}$ on the concentration of 6-AHA ([I]), taking into consideration the competitive inhibition of the plasmins as well as one LBS interaction, is given by:

$$k_{6\text{-AHA}} = k_{obs.}(1 + [I]/K_{LBS})(1 + [I]/K_i) \quad (4)$$

Table 2 Kinetic constants obtained of the slow step of the inhibition of bovine plasmin by α_2AP

Representative curves are shown in Figure 4. For details of the methods (A or B) used, see the Materials and methods section.

	Conditions		Results		
Method	[S] (μM)	E_o (μM)	$[E]_{app.}$ (nM)	$10^3 \times k_{+2}$ (s^{-1})	K_{+1} (nM)
A	447.5	1.98	74	9.8	0.45
B	447.5	1.98	81	8.6	0.55
A	85	1.98	44	8.3	0.50
B	85	1.98	46	8.0	0.54
A	447.5	0.97	57	8.1	0.56
B	447.5	0.97	52	9.6	0.47
A	85	0.97	31	8.0	0.51
B	85	0.97	30	9.1	0.48

where K_{LBS} and K_i are the corresponding dissociation constants. With $K_i = 42$ mM, an overall value of K_{LBS} for bovine plasmin and midiplasmin of $\sim$ 0.2–0.6 mM results from our data. This is in the range for interaction with the weak of LBS and more than two orders of magnitudes larger than the reported K_{LBS} of 6-AHA and kringle 1 (Christensen and Mølgaard, 1992).

Inhibition progress kinetics

Time-course experiments measuring residual plasmin activity after completion of its fast association reaction with α_2AP, to possibly obtain the equilibrium constant of the complex, revealed that a further slow reaction took place. The pattern clearly was the same as that originally discussed for human plasmin (Christensen and Clemmensen, 1977). To characterize the slow step, residual plasmin-catalysed rates of substrate hydrolysis were measured in two ways. (a) Equimolar amounts of enzyme and inhibitor were pre-incubated and samples withdrawn at different times and assayed (Figure 4a). (b) Alternatively, such samples were followed after the time of manual mixing (10–20 s) in the presence of substrate and rates were obtained from tangents to the curve [the calculation of each point of the first time derivative of the recorded (10 points/s) curve is based on 25 data points] (Figure 4b). The results showed single exponential decays of the rates with a first-order rate constant for the slow step, $k_{+2} = 9 \times 10^{-3}$ s^{-1} (accuracy $\sim$ 5%), as is summarized in Table 2. With micromolar concentration range of enzyme and inhibitor employed, equilibrium prevails in the first association reaction within 10 s (Figure 2), the half-time of the slow step is 78 s. Therefore the (extrapolated) zero-time values of the single exponential function that were obtained from a fit to the slow-reaction-step data provide a good measure of the theoretical value of residual plasmin at equilibrium of the first reaction, before any further conversion has taken place. Accordingly, K_1 may be obtained thus:

$$K_1 = \frac{k_{-1}}{k_{+1}} = \frac{[E][I]}{[EI^*]} \quad (5)$$

where the free, residual plasmin concentration at zero time of the second reaction step ([E] of eqn. 5) is determined from the initial value of the fitted exponential equation, as at that time [EI] = 0. If, further, the effect of the presence of substrate (S) is taken into consideration, $[E] = [E]_{app.}/(1+[S]/K_m)$, $[EI^*] = E_o - [I]$ and $[I] = [E]_{app.}$. The resultant value of K_1 was $(5 \pm 0.5) \times 10^{-10}$ M. Table 2 shows the results of the individual experiments. Because $k_{+1} = 10^6$ $M^{-1} \cdot s^{-1}$, as determined from the stopped-flow experiments (Table 1), $k_{-1} = 5 \times 10^{-4}$ s^{-1} according to eqn. 5.

As is known from previous reports on the reaction of human plasmin with α_2AP, the final complex formed is so tightly bound that the reaction may be considered irreversible unless in extreme experimental conditions, where sufficient perturbation of the equilibrium allows detection of dissociation of the complex in due time, e.g. in the presence of α_2-macroglobulin, which actually forms a covalent isopeptide-bonded complex with plasmin (Shieh et al., 1989). This 'irreversibility' is attributable to an extremely slow reverse reaction in the second reaction step (k_{-2}). Bovine plasmin shows the same phenomenon, the second reaction step apparently goes to completion and the concentration of residual plasmin at final equilibrium is very, very low. Estimated from the infinity value of the fitted exponential equation for $t \rightarrow \infty$ (Figures 4a and 4b), the corresponding estimate of an upper limit of the overall inhibition constant is $K_{I,AP} \leqslant 5 \times 10^{-12}$ M:

$$K_{I,AP} = \frac{[E][I]}{[EI^*] + [EI]} \quad (6)$$

Hence, from

$$k_{-2} = \frac{k_{+1}k_{+2}}{\frac{k_{-1}}{K_{I,AP}} - k_{+1}} \quad (7)$$

an upper limit value of k_{-2} is $\leqslant 4.5 \times 10^{-6}$ s^{-1}.

DISCUSSION

In the conversion of human plasminogen to plasmin, the peptide bond Arg-561–Val-562 is cleaved. Also, an autolytic cleavage occurs at residues Lys-77–Lys-78, resulting in Lys-plasmin (Summaria et al., 1975). This pattern is seen also for bovine plasminogen (Figure 1), but here an additional cleavage takes place at Arg-342–Met-343 between kringle 3 and 4. Separation of the kringles 1–3 fragment and midiplasmin (the kringles 4–5 plus light-chain fragment) was obtained by cation-exchange chromatography in a non-denaturing buffer system. No strong interaction thus exists between the kringles 1–3 fragment and the rest of the plasmin molecule.

Although *in vitro* experiments show nearly complete cleavage of Arg-342–Met-343 (Figure 1), two types of plasmin (a full-length form equivalent with human Lys-plasmin, and the smaller midiplasmin) might be present *in vivo*, differing in their content of LBSs. This suggests that the kringles 1–3 part of bovine plasmin(ogen) primarily has a function in the zymogen. This is probably also the case in human plasminogen, where the kringle 1–3 fragment does not bind intact fibrin (Sottrup-Jensen et al., 1977; Thorsen et al., 1981; Suensen and Thorsen, 1981), but may participate in the enhanced binding during plasmin-catalysed fibrin degradation, when C-terminal lysine residues, which serve as ligands for such binding, are formed (Christensen, 1985). The possibility of a more prominent role of the LBSs of kringles 4 and 5 rather than of the kringle-1 LBS as regulatory domains in the human system has recently received attention (Christensen, 1984; Anonick and Gonias, 1991; Rejante et al., 1991; Christensen and Mølgaard, 1992; Ponting et al., 1992).

The reaction of bovine plasmin and bovine midiplasmin with α_2AP showed essentially the same kinetic behaviour. The kinetic constants were very similar to those determined for human plasmin, having an intact heavy chain. The $\sim$ 2-fold reduction of k_{+1} for plasmin compared with midiplasmin is likely to be due to the presence of the kringle 1–3 fragment, which indeed binds

α_2AP, as shown by the affinity chromatography used in its purification (Wiman and Collen, 1978; Christensen and Sottrup-Jensen, 1992).

The large change in relative fluorescence seen during association of enzyme and inhibitor allowed us to use the direct methods of stopped-flow fluorescence measurements to follow the fast step of the reaction (Figures 2 and 3), so that the values of the association rate constant, k_{+1} (Table 1), in contrast to all of those previously reported, are free of substrate effects, which require corrections. Further, the stopped-flow mixing technique allows measurements after just 1 ms, and not when most of the fast reaction has taken place, as happens when one is using manual mixing (Christensen and Clemmensen, 1977; Wiman et al., 1978; Christensen et al., 1979; Wiman et al., 1979; Longstaff and Gaffney, 1991).

The association rates are greatly reduced in the presence of 10 mM 6-AHA (Table 1). Because kringle 5 alone, as in miniplasmin, reveals no kinetic effects on the inhibition by α_2AP (Christensen et al., 1979), and because the active site is inhibited only at high concentrations of 6-AHA (K_i 42 mM), we conclude that kringle 4 is of importance in the regulation of the inhibition of midiplasmin by α_2AP, probably in a way similar to that of full-length human plasmin. Thus, the generally accepted belief that only kringle 1 is important in α_2AP–plasmin interactions is not supported. Actually, the effects of 6-AHA seen previously in our laboratory in the human system (Christensen and Clemmensen, 1977, 1978; Christensen et al. 1979), also indicated the involvement of the weak LBS. Further, not only the C-terminal lysine residue, but also Lys-475 (Sugiyama et al., 1988) of human α_2AP seem important. The effect thus may involve kringles 4 and 5, as in the regulation of the plasminogen conformation (Christensen and Mølgaard, 1992).

The effect of 6-AHA on the reaction allows us to estimate the dissociation constant K_{LBS} (0.2–0.6 mM) for the interaction between the LBS and 6-AHA. This range is consistent with a binding site for 6-AHA in Lys-plasminogen with a K_d of 0.26 mM described by Markus et al. (1979), originally located to kringle 4 (Sottrup-Jensen et al., 1977) and recently to the combined action of the LBSs of kringles 4 and 5 (Christensen and Mølgaard, 1992). Indeed, our results suggest that the LBSs of midiplasmin (kringles 4 and 5) have a more conspicuous effect in plasmin–α_2AP interaction than previously thought.

Bovine plasmin reacts with α_2AP in a manner similar to that of human plasmin, a first association complex the formation of which is dependent on LBS interactions, and is converted into a much tighter second complex in a slow reaction step. The dissociation constant for the first association complex, $K_1 = 5 \times 10^{-10}$ M, and a rate constant for tightening of the complex, $k_{+2} = 9 \times 10^{-3}$ s^{-1} (Figure 4) were obtained. These values are consistent with results of Christensen and Clemmensen (1977, 1978), Christensen et al. (1979) and Longstaff and Gaffney (1991) for human plasmin.

Recently, Longstaff and Gaffney (1991) argued that lysine analogues such as tranexamic acid should not act by blocking a second site for the inhibitor on the enzyme. However, in our experiments (Table 1), the active-site inhibition (eqn. 4) cannot at all account for the effect of 6-AHA on k_{+1}, which must be explained in terms of a binding of 6-AHA to a secondary site (LBS). This model is also supported by the work of Sugiyama et al., (1988), who analysed the effect of C-terminal peptides from α_2AP on the reaction with plasmin, and showed the importance of the C-terminal of α_2AP for a fast inhibition (k_{+1}) of plasmin.

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