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Reaction of human α_2-antiplasmin and plasmin
Stopped-flow fluorescence kinetics

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Abstract The interaction of human plasmin with human α_2-antiplasmin was measured in the presence and absence of lysine-binding ligands using the corresponding active site fluorescence changes. The stopped-flow method allows for direct determination of reliable values of the second order rate constant for the fast association step of plasmin and α_2-antiplasmin in the absence of another interacting compound, e.g. a plasmin substrate. At pH 7.4, 25°C, $k_1 = 2.2 \times 10^7$ M^{-1} s^{-1} was obtained. Substantial reductions in k_1 were seen in the presence of *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid at concentrations corresponding to lysine-binding site interactions at kringle 4 of plasmin; at saturation the rate constant is reduced 20-fold, whereas the effect of saturation of kringle 1 is only a 2-fold reduction. It is thus found that the interaction of α_2-antiplasmin with the lysine-binding site of kringle 1 is of little importance compared with that of kringle 4 in regulating the inhibition reaction of plasmin with α_2-antiplasmin. Similar results were recently obtained for the bovine plasmin-bovine α_2-antiplasmin reaction (Christensen et al. (1995) Biochem. J. 305, 97–102).

Key words: Plasmin; α_2-Antiplasmin; Stopped-flow fluorescence; Kinetics; Lysine-binding site

1. Introduction

α_2-Antiplasmin, the primary inhibitor of plasmin (α_2AP), is present in plasma at a concentration of about 1 μM [1]. α_2AP from two species has been characterized. Thus, the primary structures of human α_2AP and of bovine α_2AP are known [2–4]. α_2AP is a member of the serpin family [5]; it has an acceptor site for activated coagulation factor XIII mediated cross-linking to fibrin during blood coagulation [6] at Gln-14 (new numbering [3,7,8]), and contains a C-terminal extension with affinity to lysine-binding sites in plasmin(ogen). Purification of α_2AP is typically performed by affinity chromatography on immobilized plasminogen or plasminogen fragments containing kringle 1 [1,9]. In human α_2AP Lys-475 and the C-terminal Lys-491 appear to be involved [10], indicating that the effect cannot be assigned to any single lysine-binding site (LBS) and therefore may involve the weak lysine-binding sites of plasmin(ogen) [11,12] as reported earlier [13]. In plasma, plasmin stems from the proenzyme plasminogen (for reviews see [14,15]). The primary structures of human, bovine, porcine, and mouse plasminogen are highly homologous [16–21] (overview [22]). 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 autocatalytic cleavage at residues 78–79 [23,24], and results in a disulfide-bonded two-chain plasmin molecule with a heavy chain (residue 79–561) containing kringles 1–5 with the lysine-binding sites (LBS) and a light chain (residues 562–791) containing the catalytic serine proteinase domain. Kringles 1, 4 and 5 possess LBS [11,16,25–27] that mediate important interactions of the plasmin(ogen) and other proteins of the fibrinolytic system, but are not essential for the actual catalytic activity of plasmin. Miniplasmin, of which kringles 1–4 have been removed from the proenzyme with elastase [16] before conversion with plasminogen activator, shows catalytic properties very similar to those of full-length plasmin [28], but its rate of association with α_2AP is 10–35 fold slower than that of full length plasmin [28,29]. Association rates, dependent on temperature and buffer composition, have been reported in the range of 7×10^5–2×10^7 M^{-1} s^{-1} for plasmin and about 5×10^5 M^{-1} s^{-1} for miniplasmin [28–32].

Eq. 1 illustrates the currently accepted reaction model for the inhibition of human plasmin by α_2AP. It was described by Christensen and Clemmensen [13,30], Wiman et al. [29] and more recently by Longstaff and Gaffney [31]:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI \quad (1)$$

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

In previous studies of the interaction of α_2AP and plasmin conventional kinetic methods have been used to determine the fast association (k_1) as well as the slow tightening step (k_2). Here the kinetics of the reaction of human 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. The inhibition of the fast association reaction step by *t*-AMCHA ($K_d = 34$ μM) shows a hitherto overlooked importance of kringle 4 in the reaction of plasmin with α_2-antiplasmin. Little effect corresponding with interaction at the LBS of kringle 1 ($K_d = 0.4$–1 μM) is seen. This is in accordance with recent findings in the bovine system [33].

2. Materials and methods

2.1. Materials

trans-4-(Aminomethyl)cyclohexane-1-carboxylic acid (*t*-AMCHA), H-D-Val-Leu-Lys-*p*-nitroanilide (VLKpNA) and pyro-Glu-Phe-Lys-*p*-nitroanilide (< EFKpNA) were from Chromogenix, Mölndal, Sweden. All other chemicals were analytic grade from either Sigma Chemical Co., St. Louis, MO, USA or Merck, Darmstadt, Germany.

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Abbreviations: *t*-AMCHA, *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid; α_2AP, α_2-antiplasmin; LBS, lysine-binding site; VLKpNA, H-D-Val-Leu-Lys-*p*-nitroanilide; <EFKpNA, pyro-Glu-Phe-Lys-*p*-nitroanilide

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2.2. Proteins

Glu-plasminogen and Lys-plasminogen were prepared as described [34]. The kringle 1–3 fragment of plasminogen was prepared from Glu-plasminogen as described by Sottrup-Jensen et al. [16]. Rabbit immunoglobulin raised against human α_2AP was from Dako, Glostrup, Denmark. Urokinase was from Wakamoto, Tokyo, Japan.

After gel filtration to remove 6-aminohexanoic acid, Lys-plasminogen (32 μM) was converted to plasmin by incubation with urokinase (0.1 μM) in 50 mM Tris-HCl, 0.1 M NaCl, 25% (v/v) glycerol, pH 7.7, for 120 min at 25°C. Its concentration of active plasmin (25 μM) was determined from rate measurements of hydrolysis of D-Val-Leu-Lys-pNA using $K_m = 150$ μM and $k_c = 15.2$ s^{-1} [35,36].

α_2AP was isolated from human plasma essentially as described by Wiman [37] with the modifications described by Bangert et al. [7]. The purified inhibitor was analysed by modified crossed immunoelectrophoresis with Lys-plasminogen in the first dimention gel, which retards the migration of α_2AP with intact C-terminus, and anti-α_2AP in the second dimention gel [38]. No non-plasminogen-binding α_2AP was detected, the preparation apparently containing only C-terminally intact α_2AP.

The concentration of α_2AP was determined by reactive site titration using a known amount of plasmin as described by Christensen and Clemmensen [30]. The amino acid sequence of the purified α_2AP and the amounts of Met^1-α_2AP (38%) and Asn^{13}-α_2AP (62%) were determined as previously described [7].

2.3. Fluorescence spectra

Fluorescence emission spectra of α_2AP, plasmin and mixtures thereof were recorded from 320 to 420 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. Various concentrations of plasmin (0.5–0.05 μM) and α_2AP (0.5–0.05 μM) were used. After incubation for at least 5 min the spectra were recorded (F[C]). Control spectra of inhibitor alone (F[0]) and plasmin alone at various concentrations (F[E]), were used to calculate the relative fluorescence change according to Eq. 2:

$$\Delta F = \frac{F[C]-(F[0]+F[E])}{F[C]} \times 100(\%) \quad (2)$$

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

2.4. 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 and 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 an appropriate time (1, 2, or 10 s) after mixing of equal concentrations of α_2AP and plasmin. The mixing time was less than 1 ms. The final concentrations were 1.1, 0.55, 0.47, 0.22, and 0.11 μM. In each experiment 400 pairs of data were recorded, and sets of data from at least three experiments were averaged and analyzed 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 1–10 s of the reactions, control fluorescence measurements were made on the aged solution in the stopped-flow cell, the last one after 20 min. Such measurements showed no further changes of the fluorescence after the initial recording. Thus, only the fast step, and not the following slow step, gives rise to changes in the protein fluorescence when plasmin and α_2AP interact.

2.5. 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 plasmin, 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. The release of product was followed in a Perkin Elmer λ17 spectrophotometer after manually mixing of plasmin (1.1 and 0.5 μM final concentration) with a solution of α_2AP (1.1 and 0.55 μM final concentration) and H-D-Val-Leu-Lys-pNA (final concentration 1.24 mM). The rate corresponding to the residual plasmin activity was determined as a function of time from the slope (the first time derivative) of the recorded curve, which was calculated using the PECSS software of the Perkin-Elmer spectrophotometer.

In such experiments an exponential decrease in plasmin activity corresponding to a slow first order reaction step occurring after the fast initial equilibration of the first step was seen, as has also been previously reported [30]. The zero time value of the fitted exponential equation is the point that corresponds to the initial value of the second reaction step for a theoretical immidiate equilibration of the first step. It was 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.

3. Results

3.1. Fluorescence spectra

Fluorescence emission spectra were obtained for α_2AP, plasmin and the α_2AP-plasmin complex (Fig. 1). Emission maxima were all at 340 nm. Interestingly, the fluorescence intensity of the sum of the uncomplexed components (trace

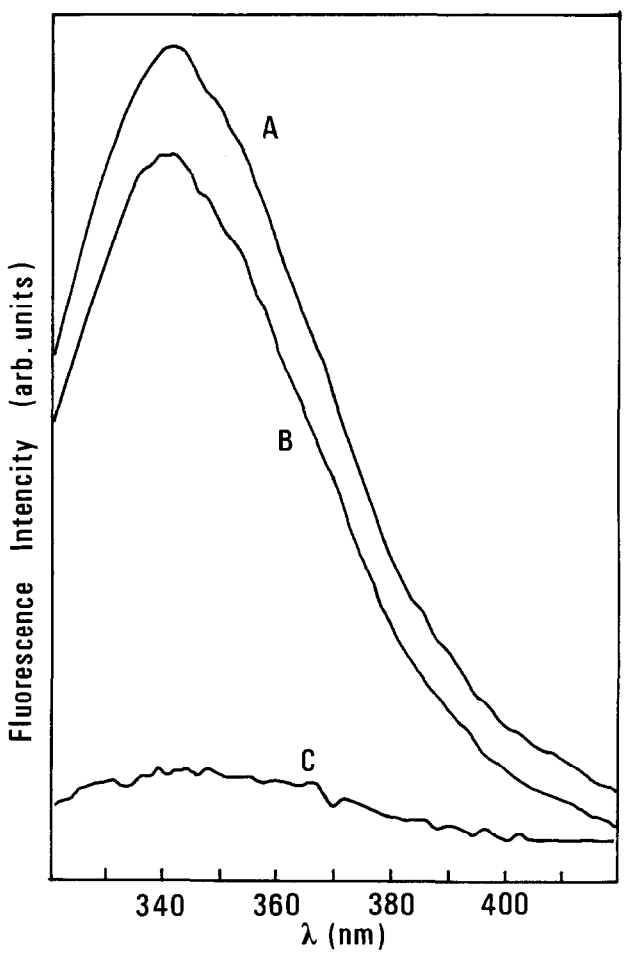

Fig. 1. Fluorescence spectra of plasmin and α_2-antiplasmin. Fluorescence spectra were recorded from 320 to 420 nm in 50 mM Tris-HCl, 0.1 M NaCl, pH 7.4 at 25°C after excitation at 280 nm. (A) Sum of spectrum of plasmin (50 nM), and spectrum of α_2AP (50 nM). (B) Spectrum of complex of plasmin and α_2AP (50 nM). (C) Difference of A and B.

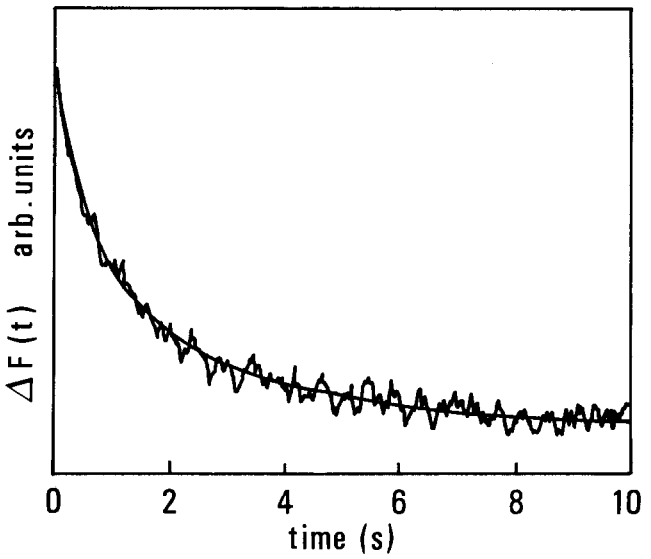

Fig. 2. Typical time courses of the stopped-flow fluorescence signal resulting from the inhibition of plasmin by α_2AP. Plasmin and α_2AP were mixed and the fluorescence followed for 10 s. The average of 3 traces is shown.The curve is that resulting from the best fit to the data of the equation that describes second order kinetics (Eq. 3). Series of such experiments were performed at various concentrations of enzyme and inhibitor in the presence and absence of *t*-AMCHA (0–10 mM).

A) exceeded the fluorescence of the complex (trace B), showing a substantial fluorescence intensity decrease in the complex ($13 \pm 1\%$) and no further changes after incubation for periods much longer than the 5 min used here. This allows direct monitoring of the first step of the α_2AP reaction with plasmin in the absence of a plasmin substrate.

3.2. Stopped-flow kinetics

The kinetics of the reactions of α_2AP with plasmin was 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 Fig. 2. Also, the fitted curve corresponding to Eq. 3, which describes second order reaction kinetics at equal concentrations of the two reactants, is illustrated.

$$\Delta F(t) = \Delta F(\infty)\left(1 - \frac{1}{1 + k_1 \cdot E_o \cdot t}\right) \quad (3)$$

$\Delta F(t)$ is the relative fluorescence change at time t, $\Delta F(\infty)$ is the limit value for $t \rightarrow \infty$ corresponding to completion of the current reaction step, and E_o is the concentration of plasmin equal to that of α_2AP. k_1 is the observed second order rate constant. The fluorescence change solely accompanies the formation of a first association complex of α_2AP and plasmin, no further changes being 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. There was reasonable internal agreement between the values obtained for k_1, 2.2×10^7 M^{-1} s^{-1} being the mean value from all the measured data in the absence of *t*-AMCHA. The presence of *t*-AMCHA, however, affects the observed rate constant significantly (Fig. 3). The concentration dependence of this effect of *t*-AMCHA results in a final 20-fold decrease of the rate constant in the concentration range 0–1 mM. These concentrations are so low that active site interaction ($K_i = 16$ mM [39]) is negligible; the effect, however, corresponds with interactions of *t*-AMCHA at the LBS of plasmin. The curve shown in Fig. 3 is obtained from a fit to Eq. 4:

$$\Delta k(I) = A + B/(1 + K_{LBS}/I) \quad (4)$$

where $\Delta k(I)$ is the difference in the observed rate constant in the absence and of *t*-AMCHA at concentration I; $k_1(0) - k_1(I)$; A is a constant: the initial decrease corresponding to saturation of the LBS of kringle 1; B is another constant: the maximal value of the further decrease obtained as a function of the degree of saturation of a LBS with dissociation constant, K_{LBS}. The best fit resulted in $K_{LBS} = 34$ μM (accuracy 15%), a B value of $0.1 \times k_1(0)$, where $k_1(0) = 2.2 \times 10^7$ M^{-1} s^{-1} is the value obtained in the absence of *t*-AMCHA, and an A value of $0.47 \times k_1(0)$. This clearly indicates an important interaction with the LBS of kringle 4 ($K_{LBS} = 36$ μM of kringle 4 of Lys-plasminogen [40]) leading to a 10-fold rate loss, whereas saturation of kringle 1, $K_{LBS} = 0.4$–2 μM [11,40,41] results in only a 2-fold decrease in the rate.

3.3. Inhibition progress kinetics

Time course experiments measuring residual plasmin activity after completion of its fast association reaction with α_2AP to obtain the equilibrium constant of the first complex, and the rate of the slow reaction, clearly confirmed the pattern originally discussed for the reaction of human plasmin and α_2AP [30]. To characterize the slow step the residual plasmin catalysed rate of substrate hydrolysis was measured in the

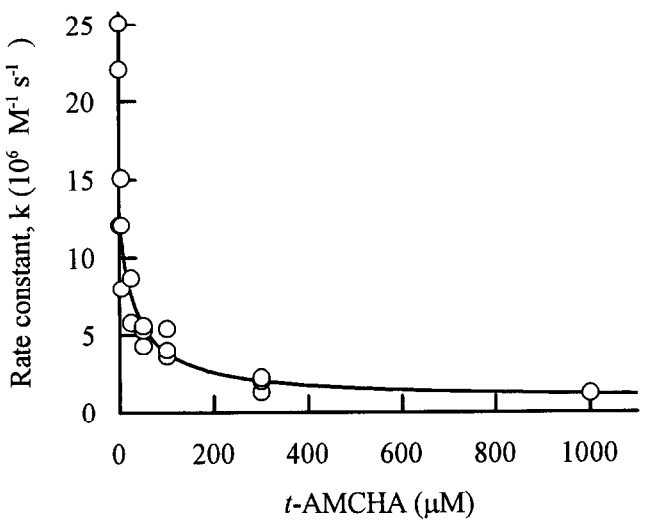

Fig. 3. Dependence of the observed second order rate constant on the concentration of *t*-AMCHA. The decrease in the second order rate constant from 2.2×10^7 $M^{-1}s^{-1}$ in the absence of *t*-AMCHA to a limit value of 1.1×10^6 M^{-1} s^{-1} in the presence of LBS-saturating concentrations of *t*-AMCHA is illustrated. The curve shown is that resulting from the best fit to the data of Eq. 4 (see text), that describes an initial decrease, A (fitted value $0.47 \times k_1(0)$), best explained as an effect of saturation of the LBS of kringle 1, followed by a decrease, B (fitted value $0.1 \times k_1(0)$) that becomes fully effective when a LBS with K_{LSB} (fitted value 34 μM) is saturated with *t*-AMCHA, best explained as an effect of saturation of the LBS of kringle 4.

presence of substrate and α_2AP. The time dependence of the rates was obtained as the first time derivative of recorded (10 points per s) curves (not shown). The results showed single exponential decays of the rates with a first order rate constant of the slow step, $k_2 = 2 \times 10^{-3}$ s^{-1} (accuracy approx. 5%) and no effects of *t*-AMCHA on the slow step were observed. This is in agreement with previous observations [30]. The resultant value of K_1 in the absence of *t*-AMCHA was determined to be $(1 \pm 0.3) \times 10^{-10}$ M.

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. Only under extreme experimental conditions, e.g. in the presence of α_2-macroglobulin, which actually forms a covalent isopeptide bonded complex with plasmin [42] may dissociation of the final plasmin-α_2-antiplasmin complex be detected. This 'irreversibility' is thus due to an extremely slow reverse reaction in the second reaction step (k_{-2}).

4. Discussion

In human plasminogen the kringle 1–3 fragment does not bind intact fibrin [16,43,44], but may participate in the enhanced binding during plasmin-catalysed fibrin degradation, when C-terminal lysine residues, that serve as ligands for such binding, are formed [11,45]. 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 [11,12,15,33,46,47].

A large change in relative fluorescence is seen when plasmin and α_2AP associates (Fig. 1). Stopped-flow fluorescence measurements showed that this change occurs in the fast step of the reaction (Fig. 2). This allowed for determinations of values of the association rate constant, k_1, under conditions unaffected by the presence of substrates. The stopped-flow mixing technique allows measurement after just 1 ms, and thus not when most of the fast reaction has taken place as happens when one is using manual mixing [9,28-31]. The association rate shows up to 20-fold reduction in the presence of *t*-AMCHA 1 mM (Fig. 3). The concentration dependence shows that the rate reduction is only 2-fold in the range 5–25 μM, when the LBS of kringle 1 is saturated, whereas saturating the LBS of kringle 4 with *t*-AMCHA results in a further 10-fold reduction. The effect of *t*-AMCHA on the reaction allows us to estimate the dissociation constant K_{LBS} (34 μM) for the interaction between the LBS and *t*-AMCHA. This range is consistent with a binding site for *t*-AMCHA in Lys-plasminogen with $K_d = 36$ μM described by Markus et al. [40], originally located at kringle 4 [16]. Since kringle 5 alone as in miniplasmin reveals no kinetic effects on the inhibition by α_2AP [28], and since the active site is inhibited only at very high concentrations of *t*-AMCHA ($K_i = 16$ mM [39]), we conclude that kringle 4 is of importance in the regulation of the inhibition of plasmin by α_2AP. The kringle 1–3 fragment of plasminogen indeed binds α_2AP as is known from the affinity purification of α_2AP [3,48], and effects of lysine-analog ligands on the reaction of α_2AP with plasmin have in general been attributed to the LBS of kringle 1 [48]. This generally accepted belief is not supported. Our findings suitably explain that not only the C-terminal lysine residue, but also Lys-475 [10] of human α_2AP seem important in the reaction, if kringle 4 as well as kringle 1 is involved in the regulation of the α_2AP-plasmin reaction. The involvement of two LBS parallels the finding that the LBS of kringles 4 and 5 are allosterically involved in the ligand-induced conformational change of Glu-plasminogen that governs its rate of activation [12].

Plasmin reacts with α_2AP in a manner in which a first association complex, the formation of which is dependent on LBS-interactions, is converted into a much tighter second complex in a slow reaction step. The dissociation constant for the first association complex, $K_1 = 1 \times 10^{-10}$ M, and the rate constant for tightening of the complex, $k_2 = 2 \times 10^{-3}$ s^{-1}, were obtained. These values are consistent with results of Christensen and Clemmensen [13,30], Christensen et al. [28] and Longstaff and Gaffney [31].

Recently, Longstaff and Gaffney [31] argued that lysine analogs such as *t*-AMCHA should not act by blocking a second site for the inhibitor on the enzyme. However, in our experiments (Fig. 3) the active site inhibition ($K_D = 16$ mM) cannot account for the effect on k_1, which must be explained in terms of binding of *t*-AMCHA to a secondary site (LBS). This model is also supported by the work of Sugiyama et al. [10] who analyzed the effect of C-terminal peptides from α_2AP on the reaction with plasmin and showed the importance of the C-terminus of α_2AP for fast inhibition (k_1) of plasmin. It should be noted, however, that blocking of the LBS merely slows the reaction down; it is not stopped and is still rather fast with $k_1(I) = 1.1 \times 10^6$ M^{-1} s^{-1} at 1 mM concentration of *t*-AMCHA.

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