Biochimica et Biophysica Acta, 526 (1978) 194—201

BBA 68525

ALLOSTERIC EFFECTS OF SOME ANTIFIBRINOLYTIC AMINO ACIDS ON THE CATALYTIC ACTIVITY OF HUMAN PLASMIN

ULLA CHRISTENSEN

Chemical Laboratory IV, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen (Denmark)

(Received January 20th, 1978)

Summary

The effects of L-lysine, 6-aminohexanoic acid, and *trans*-4-aminomethylcyclohexane-1-carboxylic acid on the catalytic activity of plasmin (EC 3.4.21.7) have been investigated. The kinetics of the plasmin-catalysed hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester in the presence of these compounds have been studied at a number of different concentrations of the three modifiers. They each exert two effects on the reaction, an activation and an inhibition, the concentration dependencies of which are markedly different. They must therefore arise from two different interactions between plasmin and the modifier. The inhibition is competitive, so that it most probably results from direct interaction at the catalytic site. The activation is kinetically non-competitive. The experimental observations seem to be explained best by assuming that L-lysine and certain analogous compounds function as both allosteric modifiers and competitive inhibitors of plasmin.

Introduction

Plasmin (EC 3.4.21.7) is the serine proteinase which converts fibrin into soluble products in the last step of physiological fibrinolysis. 6-Aminohexanoic acid and some analogous compounds are well-known inhibitors of fibrinolysis [1,2]. Indeed most compounds and reactions of the fibrinolytic system have been reported to be influenced by the presence of such amino acids [1—5]. Their effects on plasmin and plasmin-catalysed reactions have been studied by several investigators and recently evidence that plasmin possesses at least one site other than the catalytic site which interact with these antifibrinolytic amino acids has been obtained [6—10]. Little is known about the

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

effects which second site interaction might have on the catalytic activity of plasmin although Castellino and co-workers [9,11] have concluded that plasmin undergoes a conformational change when exposed to antifibrinolytic amino acids at concentration levels where binding seems to occur only at second site(s), and in a single case have observed a stimulating effect on the catalytic activity.

In the work described here the effects of L-lysine, 6-aminohexanoic acid, and *trans*-4-aminomethylcyclohexane-1-carboxylic acid on the plasmin-catalysed hydrolysis of Bz-Arg-OEt have been studied kinetically. It has been found that each acts both as an activator and an inhibitor of the reaction. The activation results in higher values of the catalytic constants, while the inhibition, which is first observed at higher concentrations of the modifiers, leads to higher values of the apparent Michaelis constants. The effects are completely independent. The observations are consistent with the assumption that (at least) two modifier molecules are bound at different plasmin sites, one of which is an allosteric site and the other of which is the catalytic site.

Materials and Methods

Human plasminogen was purified as previously described [12]. Human plasmin was prepared from NH_2-terminal glutamic acid plasminogen on a column of urokinase-substituted Sepharose 4B [13], the eluate was concentrated by ultrafiltration, and the concentration of plasmin was determined by titration [14].

L-Lysine · HCl was obtained from Merck, Darmstadt, G.F.R., 6-aminohexanoic acid from Fluka, Buchs, Switzerland, and Bz-Arg-OEt from Calbiochem, La Jolla, Calif., U.S.A. *Trans*-4-aminomethylcyclohexane-1-carboxylic acid was provided as a gift from Kabi Vitrum A/S, Copenhagen, Denmark.

The kinetic parameters of the plasmin-catalysed hydrolysis of Bz-Arg-OEt in the presence of various concentrations of each of the antifibrinolytic amino acids in turn were determined. The measurements and calculations were made in an analogous manner to some described earlier [13]. Each pair of kinetic parameters was calculated from 24 initial velocities measured at eight substrate concentrations: 0.1, 0.2, 0.33, 0.5, 0.67, 1, 2 and 3 mM. The concentration of plasmin in the reaction mixtures was 0.2 μM. The solvent was 0.05 M Tris · HCl/0.1 M NaCl, pH 7.8, and the temperature was 25°C.

Results and Discussion

The effects of the modifiers L-lysine, 6-aminohexanoic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid on the plasmin-catalysed hydrolysis of Bz-Arg-OEt have been investigated at pH 7.8, 25°C. Initial velocities obtained at various concentration of Bz-Arg-OEt and at any given fixed concentration of a modifier obey the Michaelis-Menten rate equation (Eqn. 1),

$$v(M) = k_c(M)e_\circ/(1 + K(M)/s) \quad (1)$$

where $v(M)$ is the initial velocity, $k_c(M)$ is the catalytic constant and $K(M)$ is the apparent Michaelis constant at modifier concentration M and substrate concen-

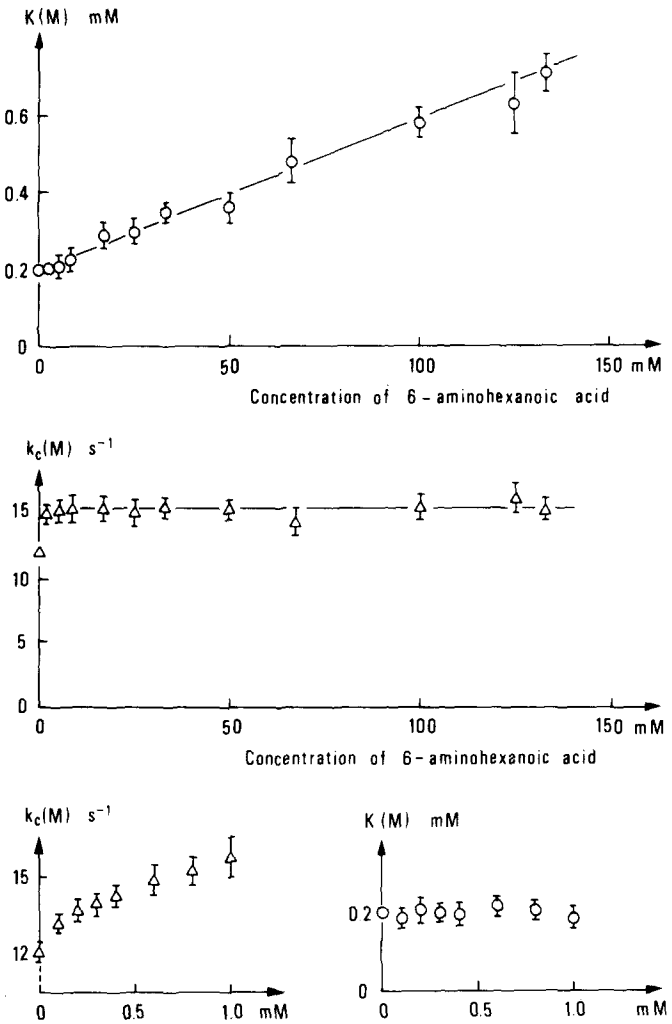

Fig. 1. The kinetic parameters of plasmin-catalysed hydrolysis of Bz-Arg-OEt in the presence of 6-aminohexanoic acid ($k_c(M)$ (s^{-1}) and $K(M)$ (mM)) plotted against the concentration of 6-aminohexanoic acid (mM). Experimental conditions: buffer 0.05 M Tris · HCl/0.1 M NaCl, pH 7.8, 25°C. $K(M)$ and $k_c(M)$ and their standard errors were determined by fitting to Eqn. 1 the results of 24 initial velocity measurements for which the modifier concentration was constant and the substrate concentration was varied in the range 0.1—3 mM [13].

tration s, and where e_o is the concentration of plasmin. The variation of the kinetic parameters with concentration of antifibrinolytic aminolytic amino acid is shown in Figs. 1—3. In each case at a rather low concentration of modifier the catalytic constant increases with modifier concentration, but eventually reaches a value which is constant for higher concentrations. The threshold value of the catalytic constant is the same for all three modifiers. In contrast to these observations the apparent Michaelis constant in each case is constant in the low concentration range but increases with increasing concentration at higher concentrations. The general effect on the overall catalytic activity of plasmin in

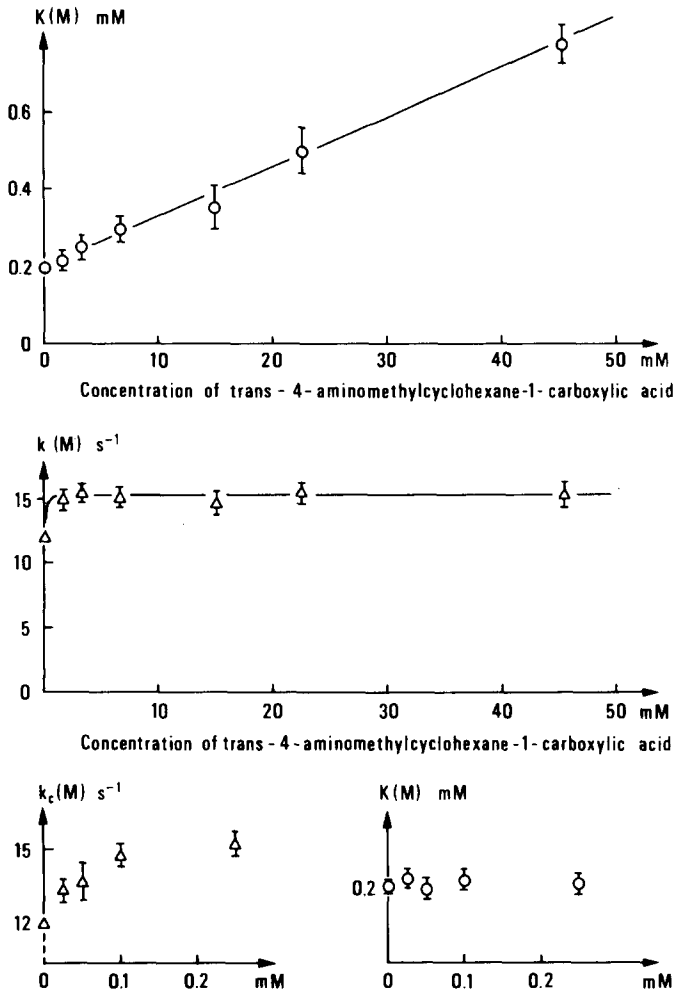

Fig. 2. The kinetic parameters of plasmin-catalysed hydrolysis of Bz-Arg-OEt in the presence of *trans*-4-aminomethylcyclohexane-1-carboxylic acid ($k_c(M)$ (s^{-1}) and $K(M)$) plotted against the concentration of *trans*-4-aminomethylcyclohexane-1-carboxylic acid. Experimental conditions: see Fig. 1.

these conditions can be described as a non-competitive activation, (so called because only the catalytic constant is affected) followed by a competitive inhibition. Since the dependence of the two effects upon modifier concentration is markedly different, it may be concluded that they are independent of each other. Modification of the catalytic activity of plasmin is unlikely to occur without direct interaction between plasmin and modifier, so (at least) two such interactions must be assumed. Fig. 4 shows a reaction scheme which includes the formation of an activated form of the enzyme upon addition of a modifier to an activation site of the enzyme, and the formation of an inhibited form of the enzyme when the modifier interacts at an inhibition site of the enzyme. An EI complex is not postulated in the scheme since no inhibition is observed at low modifier concentrations, and since at high concentrations it would be

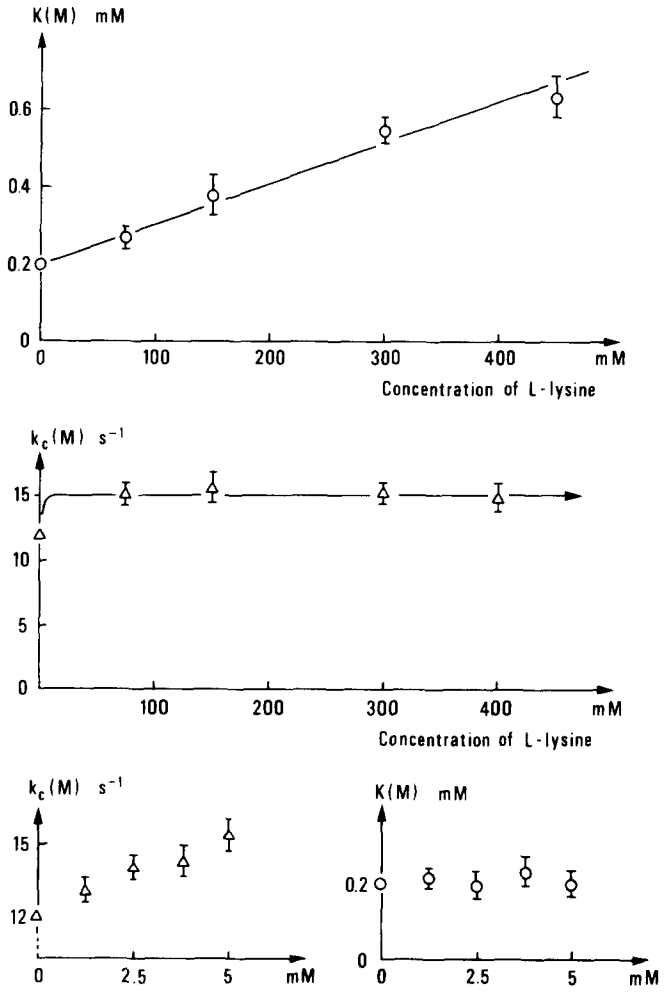

Fig. 3. The kinetic parameters of plasmin-catalysed hydrolysis of Bz-Arg-OEt in the presence of L-lysine ($k_c(M)$ (s^{-1}) and $K(M)$) plotted against the concentration of L-lysine. Experimental conditions: see Fig. 1.

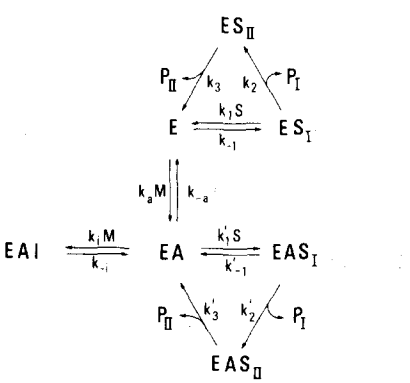

Fig. 4. Reaction scheme for an enzyme which catalyses an Ordered Uni Bi reaction, $S \rightarrow P_I + P_{II}$, in the presence of a modifier, M. Upon addition of M, the free enzyme, E, is converted to an activated form, EA. EAI is an inhibited form of the enzyme with two modifier molecules bound at different sites. It is assumed that the affinity of M for the activation site is much greater than it is for the inhibition site; consequently an enzyme form EI does not appear in the scheme.

present in only very small amounts, nearly all of the enzyme being in the activated form. At zero concentration of the modifier only the forms E, ES_I and ES_{II} are present, and the steady-state velocity of hydrolysis of the substrate is

$$v(\circ) = k_c e_\circ/(1 + K/s) \tag{2}$$

where

$$k_c = k_2 k_3/(k_2 + k_3) \tag{3}$$

and

$$K = [(k_{-1} + k_2)k_3]/[k_1(k_2 + k_3)] \tag{4}$$

If at some non-zero concentration of the modifier only the enzyme forms EA, EAS_I, and EAS_{II} were present then the steady-state velocity would be

$$v(M) = k'_c e_\circ/(1 + K'/s) \tag{5}$$

where

$$k'_c = k'_2 k'_3/(k'_2 + k'_3) \tag{6}$$

and

$$K' = [(k'_{-1} + k'_2)k'_3]/[k'_1/(k'_2 + k'_3)] \tag{7}$$

Further if the two reactions

$$E + M \underset{K_a}{\rightleftharpoons} EA \quad \text{and} \quad EA + M \underset{K_i}{\rightleftharpoons} EAI$$

are assumed to be in equilibrium, and if the steady-state approximation is applied to the reactions in which the substrate is hydrolysed, then the reaction scheme (Fig. 4) leads to the steady-state rate equation

$$v(M) = k_c(M)e_\circ/(1 + K(M)/s) \tag{8}$$

where the kinetic parameters are functions of the modifier; the catalytic constants is

$$k_c(M) = k_c \frac{1 + k'_c MK/k_c K_a K'}{1 + MK/K_a K'} \tag{9}$$

and the apparent Michaelis constant is

$$K(M) = K \frac{1 + M(1 + M/K_i)/K_a}{1 + MK/K_a K'} \tag{10}$$

where M is the concentration of the modifier, K_a ($= k_{-a}/k_a$) is the dissociation constant of EA, K_i ($= k_{-i}/k_i$) is the dissociation constant of EAI and where $K_a << K_i$. k_c, K, k'_c, and K' are defined by Eqns. 3, 4, 6 and 7, respectively.

It can be seen from Eqn. 9 that the catalytic constant, $k_c(M)$, which is equal to k_c for $M = 0$, eventually attains the value k'_c as M is increased. Here $k'_c > k_c$ and therefore $k_c \leqslant k_c(M) \leqslant k'_c$.

When $M \approx K_a << K_i$, the apparent Michaelis constant, $K(M)$, becomes (Eqn. 10)

$$K(M) = K \frac{1 + M/K_a}{1 + MK/K_a K'} \tag{11}$$

Since for this range of M the results show that $K(M) = K$ it must be concluded that $K' = K$.

When $M \approx K_i >> K_a$

$$K(M) = K'\left(1 + \frac{M}{K_i}\right) \tag{12}$$

and the results show that

$$K(M) = K\left(1 + \frac{M}{K_i}\right) \tag{13}$$

indicating again that $K' = K$. Thus there is no change in the apparent Michaelis constant, but an increase of the catalytic constant, when plasmin is converted to the activated form (with kinetic parameters k'_c and K'). All three modifiers give rise to the same final value of the catalytic constant, k'_c, therefore the three antifibrinolytic amino acids may induce the same activated form of plasmin.

Estimates [13] of the disscociation constants of the plasmin · modifier complexes are given in Table I. All the K_a values were obtained in the concentration range where the following have been postulated or shown to occur: a conformational change of plasmin [9,10], interaction of 6-aminohexanoic acid with some site on the heavy chain of plasmin [10], strong inhibition of only the plasmin-catalysed hydrolysis of fibrin and not of other substrates [2,4,7]. It seems probable that formation of a plasmin · modifier complex occurs concurrently with a change of the plasmin molecule into a more active conformation, and that modifier is attached at some site(s) on the heavy chain and not at the catalytic site. The effect of attachment at the catalytic site, i.e. competitive inhibition, is observed at much higher concentrations and the catalytic site has been shown to be associated exclusively with the light chain of plasmin.

The allosteric concept embodies the attachment of a modifier at a site other than the catalytic site, with consequent modification of the enzyme. Accordingly plasmin seems to be an allosteric enzyme with L-lysine and certain analogous compounds as modifiers. Allosteric site interactions seem to be of special importance to what really may be regarded as specific plasmin reactions, namely the known physiological reactions. For example, plasmin catalysed hydrolysis of fibrin is strongly inhibited by antifibrinolytic amino acids at concentrations corresponding to those at which allosteric site interactions occur,

TABLE I

ESTIMATED VALUES OF THE DISSOCIATION CONSTANTS OF PLASMIN · MODIFIER COMPLEXES, pH 7.8, 25°C

K_a ($= k_{-a}/k_a$ (Fig. 4)) is the dissociation constant of the activated form of plasmin; that is, the plasmin · modifier complex with the modifier attached at an allosteric site of plasmin. K_i ($= k_{-i}/k_i$ (Fig. 4)) is the dissociation constant of the inhibited form of plasmin; that is, the plasmin · modifier complex with the modifier attached at the catalytic site of plasmin.

Modifier	K_a (mM)	K_i (mM)
6-Aminohexanoic acid	≈0.3	58
Trans-4-Aminomethylcyclohexane-1-carboxylic acid	≈0.05	16
L-Lysine	≈2	192

and the same is true of the reaction of plasmin with what seems to be the most important physiological plasmin inhibitor [15,16], namely that which has been variously called α_2-plasmin inhibitor, antiplasmin and the primary inhibitor of plasmin [16].

Recently Landmann [7] suggested a mechanism for the plasmin-catalysed hydrolysis of fibrin which involves both a lysine-binding site and the catalytic site of plasmin. This proposal accounts satisfactorily for the special inhibitory effect which compounds such as 6-aminohexanoic acid exert on the reaction, for it assumes that formation of specific plasmin · fibrin complexes require an unoccupied lysin binding site on plasmin and that the specificity is lost if this site is occupied by an antifibrinolytic amino acid. This theory seems plausible. Efficient and specific hydrolysis of fibrin would occur if binding of fibrin residues show the same affinity pattern to plasmin sites as do the antifibrinolytic amino acids, so that binding occurs preferentially at the allosteric site(s). This would cause an alteration of plasmin into an activated form and perhaps bring a particular peptide bond of fibrin very close its catalytic site. In such circumstances the value of the apparent Michaelis constant for the interaction would be of no significance, since the catalytic site would automatically be saturated with substrate. The affinity of specific fibrin residues for the allosteric site(s) of plasmin and the catalytic constant of the activated form of plasmin would be the parameters determining the rate of hydrolysis.

References

1 Okamoto, S., Oshiba, S., Mihaca, H. and Okamoto, U. (1968) Ann. N.Y. Acad. Sci. 146, 414—420
2 Skoza, L., Tse, A.O., Semar, M. and Johnson, A.J. (1968) Ann. N.Y. Acad. Sci. 146, 659—672
3 Maxwell, R.E., Nawrochi, J.W. and Nickel, V.S. (1968) Thromb. Diath. Haemorrh. 19, 117—128
4 Lukasiewich, H., Niewiarowski, S., Worowski, K. and Lipinski, B. (1968) Biochim. Biophys. Acta 159, 503—508
5 Alkjaersig, N., Fletcher, A.P. and Sherry, S. (1959) J. Biol. Chem. 234, 832—837
6 Ambrus, C.M., Ambrus, J.L., Lassman, H.B. and Mink, I.B. (1968) Ann. N.Y. Acad. Sci. 146, 430—477
7 Landmann, H. (1973) Thromb. Diath. Haemorrh. 29, 253—275
8 Iwamoto, M. (1975) Thromb. Diath. Haemorrh. 33, 573—585
9 Brockway, W.J. and Castellino, F.J. (1971) J. Biol. Chem. 246, 4641—4647
10 Richli, E.E. and Otawsky, W.I. (1975) Eur. J. Biochem. 59, 441—447
11 Violand, B.N., Sodetz, J.M. and Castellino, F.J. (1975) Arch. Biochem. Biophys. 170, 300—305
12 Christensen, U. and Müllertz, S. (1977) Biochim. Biophys. Acta 480, 275—281
13 Christensen, U. (1975) Biochim. Biophys. Acta 397, 459—467
14 Chase, J. and Shaw, E. (1969) Biochemistry 8, 2212—2224
15 Müllertz, S. and Clemmensen, I. (1976) Biochem. J. 159, 545—553
16 Christensen, U. and Clemmensen, I. (1977) Biochem. J. 163, 389—391