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STUDIES ON THE NATURE OF THE CATALYTICALLY ESSENTIAL IONIZING GROUP OF PLASMIN WITH p*K* 8.4

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Summary

A fully carbamylated derivative of plasminogen having no free amino groups has been prepared and converted by urokinase to an active enzyme, called carbamyl plasmin A, with a single free NH_2-terminal amino group (Val-561). Carbamyl plasmin A was shown to possess a catalytically essential ionizing group having p*K* 8.6. Carbamylation of the free NH_2-terminal amino group of carbamyl plasmin A led to complete loss of catalytic activity. The results of solvent perturbation studies of normal plasmin (EC 3.4.21.7) indicate that the group with p*K* 8.4 is a neutral acid group.

It is suggested that the catalytically essential ionizing group of plasmin having a p*K* of 8.4 is the α-ammonium group of the NH_2-terminal Val-561 of the light chain of plasmin, forming an ion pair with a COO^- group of an aspartate or glutamate residue.

Introduction

Kinetic investigations of plasmin (EC 3.4.21.7)-catalysed hydrolysis of synthetic substrates [1,2] have shown that plasmin possesses at least two catalytically essential ionizing groups, one having a p*K* of 6.5, presumably that of the Asp-His-Ser system of the active site, and the other having a p*K* of 8.4. The conversion of plasminogen to plasmin involves hydrolysis of the peptide bond Arg-560-Val-561 of plasminogen [3,4]. In chymotrypsin and trypsin, there seems to be no doubt that one of the catalytically essential ionizing groups is the $-NH_3^+$ group of the NH_2-terminal isoleucine (e.g. Ref. 5). The group with p*K* 8.4 in plasmin could, by analogy with chymotrypsin and trypsin, be the free α-amino function of Val-561.

The present work describes the preparation of some carbamyl derivatives of plasminogen and plasmin and the results of a number of kinetic experiments designed to investigate the nature of the group of plasmin with pK of 8.4.

Materials and Methods

D-Val-Leu-Lys-pNA (S-2251) was a gift from Kabi Vitrum A/S (Copenhagen, Denmark). Bz-Arg-OEt was obtained from Calbiochem (La Jolla, CA, U.S.A.), 4′-Nitrophenyl-4-guanidinobenzoate-HCl from Merck (Darmstadt, F.R.G.), potassium cyanate from Fluka AG (Buchs, Switzerland), dansyl chloride and dansyl amino acids from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and urokinase from Leo (Copenhagen, Denmark). Buffer chemicals used were analytical grades available commercially.

NH_2-terminal glutamic acid plasminogen (Glu-plasminogen) was purified from stored human plasma as previously described [6]. The plasminogen from a pool of several batches was precipitated with 50% $(NH_4)_2SO_4$ at 4°C. The suspension was allowed to stand for 2 h before the precipitate was removed by centrifugation at 5000 × g for 30 min at 4°C. The plasminogen was redissolved in water and then 99.9% of the remaining salt was removed by four successive ultrafiltrations (40 ml → 5 ml) in an Amicon cell with a PM 30 membrane. Finally, the material was freeze-dried.

The following three carbamylated derivatives of plasmin were prepared: carbamyl plasmin A was made from plasminogen which was carbamylated and subsequently subjected to urokinase-catalysed conversion to carbamyl plasmin A. Carbamyl plasmin B was prepared by carbamylation of normal plasmin and carbamyl plasmin C was prepared by recarbamylation of carbamyl plasmin A.

Carbamylation of the proteins was carried out in the reaction vessel of a pH stat (Radiometer ABU 13, TTT 60) for 48 h at 25°C, essentially as described by Stark [7]. pH was kept at 8.0 by automatic addition of 0.1 M acetic acid. When the starting material was Glu-plasminogen two samples of approx. 50 mg (approx. 500 nmol) of freeze-dried material were each dissolved in 1.5 ml water and mixed with 1.5 ml 50% (v/v) N-ethylmorpholine/water, which was previously adjusted to pH 8.0 with glacial acetic acid. Then, approx. 130 mg KOCN was added to one of the samples, the other was run as a carbamyl blank. After 48 h, the mixtures plus approx. 5 ml wash solution (0.1 M sodium phosphate buffer, pH 7.6) were transferred to Sephadex G-25 columns (40 cm × 177 mm^2) equilibrated with 0.1 M sodium phosphate buffer (pH 7.6) at 4°C. After removal of samples for gel electrophoresis and for dansylation experiments, the eluates were used for preparation of plasmin (carbamyl blank) and carbamyl plasmin A. The material was passed through a column (35 mm × 177 mm^2) of urokinase covalently attached to Sepharose 4B at 22°C (0.1 M sodium phosphate buffer, pH 7.6) [1,8]. The eluate (1.5-ml fractions) was collected at 4°C in tubes containing 0.5 ml glycerol.

50% of carbamyl plasmin A was converted to carbamyl plasmin C, 50% of plasmin (carbamyl blank) was converted to carbamyl plasmin B.

Dansylation experiments on the carbamylated proteins were made to check their contents of free amino groups. Dansylations and hydrolysis of the dansylated proteins were performed essentially as described by Gros and Labouesse

[9], and identification of the dansylated amino acids essentially as described by Weiner et al. [10]. Samples containing at least 10 nmol of protein were used. Only approx. 1 nmol is required for identification of a dansylated amino acid.

The concentrations of plasminogen (after conversion to plasmin), plasmin and carbamyl plasmin solutions were determined by titration with 4'-nitrophenyl-4-guanidinobenzoate [6,11].

The kinetic experiments and the analysis of the resultant initial velocities were performed essentially as previously described [1]. The hydrolysis of Bz-Arg-OEt catalysed by the carbamyl plasmins were investigated at various pH values in the range 7—9. Also solvent perturbation studies of the reaction of normal plasmin with D-Val-Leu-Lys-pNA were performed in the pH range 7—9. The kinetic parameter, $k_c/K_{m(app)}$ was determined from the steady-state initial velocities measured at low substrate concentrations. The high-pH profiles of the $k_c/K_{m(app)}$ parameter were determined in a neutral acid buffer system, 25 mM 4-hydroxyphenylsulfonate, sodium salt, and in a cationic acid buffer system, 25 mM Tris-HCl in the presence and absence of 10% (v/v) dimethylformamide.

Results and Discussion

Carbamyl plasminogen

Fully carbamylated plasminogen with no free amino groups was obtained when carbamylation of plasminogen was performed as described above. No ϵ-*N*-dansyl lysine or α-*N*-dansyl amino acids were seen in the dansylation experiments. Fully carbamylated plasminogen was converted to an active enzyme by urokinase. This enzyme was called carbamyl plasmin A.

Carbamyl plasmin A, B and C

The dansylation experiments showed that carbamyl plasmin A possessed only one free amino group, the α-amino function of a valine residue. Since this new NH_2-terminal amino acid is formed by urokinase-catalysed hydrolysis of a peptide bond of a plasminogen derivative the bond cleaved is most probably Arg-560-Val-561, which is the only bond of native plasminogen cleaved by urokinase.

Titrations with 4'-nitrophenyl-4-guanidinobenzoate of the carbamyl plasmin A preparation showed that the amount of active enzyme was 60% of that of the corresponding plasmin preparation (carbamyl blank). Indicating, that carbamyl plasminogen is a good urokinase substrate, although not as good as normal plasminogen.

The steady-state kinetics of carbamyl plasmin A were studied in the pH range 7—9 using Bz-Arg-OEt as the substrate, 25° C. Table 1 shows the values of the kinetic parameters obtained. The pH profile of the $k_c/K_{m(app)}$ parameters is shown in Fig. 1. The carbamylation of the amino groups of plasmin causes an increase of approximately 0.2 unit in the pK value of the catalytic group ionizing at high pH. The conversion of positively charged amino groups to neutral carbamides increases the net negative charge of the protein. Therefore a suppression of the separation of charges involved in dissociation of a proton from the protein with resultant increase of the pK value is expected. Thus the catalytically essential ionizing group of plasmin with pK = 8.4 shows a slight increase

TABLE I

KINETIC PARAMETERS FOR CARBAMYL PLASMIN A-CATALYSED HYDROLYSIS OF Bz-Arg-OEt

Temperature, 25°C. Buffers: 0.05 M Tris-HCl, 0.1 M NaCl, pH 7—9. Carbamyl plasmin A is a plasmin derivative in which all, but one of the amino groups are converted to carbamides. The free amino group is that of the NH_2-terminal Val-561 of the light chain of plasmin. Values of $k_c/K_{m(app)}$ are equal for carbamyl plasmin A and normal plasmin [1]. But the values of k_c and $K_{m(app)}$ of carbamyl plasmin A are all less than those of plasmin. Presumably the deacylation steps but not the acylation steps of the reaction are affected when plasmin is carbamylated.

pH	$K_{m(app)}$(mM)	$k_c(s^{-1})$	$k_c/K_{m(app)}(mM^{-1} \cdot s^{-1})$
7.20	0.14 ± 0.011	8.4 ± 0.4	60 ± 3.1
7.58	0.14 ± 0.009	8.2 ± 0.3	59 ± 1.8
7.78	0.15 ± 0.016	8.5 ± 0.5	57 ± 3.8
8.28	0.19 ± 0.012	8.2 ± 0.3	43 ± 2.2
8.58	0.25 ± 0.02	7.9 ± 0.5	32 ± 4.1
8.87	0.38 ± 0.03	8.5 ± 0.4	22 ± 2.9

of the pK value but is not modified when all but one of the amino groups of the enzyme are carbamylated. The only possible candidate therefore is the amino group of the NH_2-terminal valine residue formed in the conversion of plasminogen to plasmin (Val-561), a sulfhydryl group or a phenolic hydroxyl group.

Two preparations of derivatives of plasmin in which the amino function of the NH_2-terminal valine residue (Val-561) was converted to a carbamide were made. Carbamyl plasmin C was fully carbamylated as seen from the dansylation experiments. The α-amino groups of carbamyl plasmin B were all carbamylated, but some ϵ-amino groups of lysine residues were not. Traces of ϵ-dansyl lysine were seen in the dansylation experiments on carbamyl plasmin B. Carbamyl plasmin B and C each failed to react with 4′-nitrophenyl-4-guanidinobenzoate and to hydrolyse Bz-Arg-OEt at a measurable rate. Kinetic experiments were

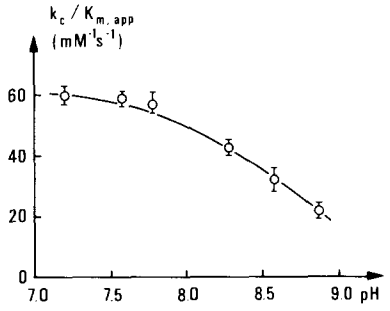

Fig. 1. The high-pH profile of the kinetic parameter, $k_c/K_{m(app)}$ for carbamyl plasmin A-catalysed hydrolysis of Bz-Arg-OEt, 25°C. Values of $k_c/K_{m(app)}$ and their standard errors ($mM^{-1} \cdot s^{-1}$) determined experimentally are plotted against pH (○). The values of K, the acid dissociation constant of a catalytically essential enzymic group and of, $k'_c/K'_{m(app)}$, the pH-independent kinetic parameter were obtained from a weighted least-squares fit of the experimentally determined $k_c/K_{m(app)}$ values to Eqn. 1.

$$k_c/K_{m(app)} = (k'_c/K'_{m(app)})/(1 + K/[H^+]) \quad (1)$$

The curve shown is that given by Eqn. 1. A pK value of 8.6 was obtained for carbamyl plasmin A. Previously a pK value of 8.4 was obtained for plasmin [1,2].

performed at pH 7.8, 25°C using concentrations of Bz-Arg-OEt up to 3 mM (15 times the value of $K_{m(app)}$ for normal plasmin) and concentrations of carbamyl plasmin B or C up to 10 μM (100 times that normally used of plasmin). The absorbances of the reaction mixtures were measured for more than 1 h, but no reaction was observed. The concentrations of carbamyl plasmin B and C were estimated from the known concentrations of the plasmin (carbamyl blank) and the carbamyl plasmin A preparations from which they were prepared.

The complete loss of activity of carbamyl plasmin B and C is in accordance with the amino group of Val-561 being the catalytically important ionizing group with pK = 8.4 of plasmin. The results do not prove this, however. It has been reported that the serine residue of the active site may be modified when chymotrypsin is carbamylated [12] and therefore this may happen also in the case of plasmin.

Solvent perturbation studies, that is investigations of the effect of solvent polarity on the pK value of a group on an enzyme, may indicate whether the ionizing group is a cationic or a neutral acid. A neutral acid has no charge when protonated and dissociation generates a negative and a positive charge. A cationic acid has a positive charge.

Neutral acid: $RH \rightleftharpoons R^- + H^+$
Cationic acid: $RH^+ \rightleftharpoons R + H^+$

The separation of charges involved in dissociation of a neutral acid is suppressed in the presence of an organic solvent such as dimethylformamide. The pK value increases and so does the pK value of a neutral acid buffer. Therefore a higher pK value as a result of solvent perturbation is expected when a neutral acid catalytic group is studied in a cationic buffer system and no change is expected when the catalytic group and the buffer are both neutral acids or

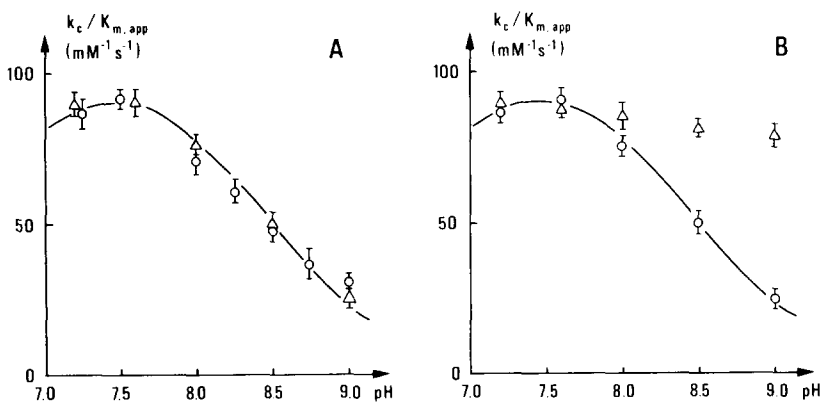

Fig. 2. Solvent perturbation of plasmin-catalysed hydrolysis of D-Val-Leu-Lys-pNA, pH 7—9, 25°C. (A) Neutral acid buffer: 0.025 M 4-hydroxyphenylsulfonate, sodium salt, 0.1 M NaCl, pH 7—9. (B) Cationic acid buffer: 0.025 M Tris-HCl, 0.1 M NaCl, pH 7—9. Values of $k_c/K_{m(app)}$ and their standard errors ($mM^{-1} \cdot s^{-1}$) determined experimentally in the absence of dimethylformamide (○) and in the presence of dimethylformamide 10% (v/v) (△) are plotted against pH. pH values were measured before addition of organic solvent. The curves shown are those given by Eqn. 1 (see Fig. 1). The normal pK value of 8.4 is obtained in the neutral acid buffer system when the organic solvent is added whereas that increases the pK value more than 0.6 unit in the cationic acid buffer system. Thus the catalytically essential group of plasmin with pK = 8.4 behaves as a neutral acid group [13].

both cationic acids, whereas a lower pK value should result when a cationic catalytic group is studied in a neutral acid buffer system [13].

The pH profiles of the kinetic parameter $k_c/K_{m(app)}$ of plasmin catalysed hydrolysis of D-Val-Leu-Lys-pNA determined in a neutral acid buffer system and in a cationic acid buffer system both in the presence and absence of dimethylformamide are shown in Fig. 2. The catalytic group of plasmin with pK = 8.4 clearly follows the pattern of a neutral acid.

It seems to be established that the catalytically important ionizing group of chymotrypsin with p$K \approx 9$ is the α-amino group of Ile-16 and that the catalytically active conformation of chymotrypsin is stabilized by a salt bridge between the NH_3^+ group of Ile-16 and the ^-OOC group of Asp-194 [5]. Kaplan and Laidler [14] reported that the group of α-chymotrypsin with pK = 9.2 seen from the variation of its pK value with the dielectric constant of the solvent appeared to be a neutral acid group and explained that by assuming that the group is only partially accessible to the solvent. A partly buried acid is shielded from the full effects of the solvent and the result deducted from the effects observed using the solvent perturbation method is unreliable in such a case. However, it does not seem quite unreasonable to this author if an ammonium group participating in a salt bridge behaves as a neutral acid, since dissociation of the proton then does involve the separation of a positive and a negative charge.

Although we cannot completely exclude the possibility that the plasmin group studied here is either a sulfhydryl or a phenolic hydroxyl group, we propose from the results shown that the catalytically important group of plasmin which is active in acylation in the protonated form and dissociates with pK = 8.4 is the α-amino group of Val-561 participating in a salt bridge, the formation of which is one of the events when plasminogen is converted to plasmin.

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