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Biochim. Biophys. Acta, 38 (1960) 252–258

THE ACTIVE CENTER OF PLASMIN

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(Received May 20th, 1959)

SUMMARY

Plots of the variation of pK_m and log V_{max} with pH for plasmin, determined in the absence and presence of either Ni^{++} or Ca^{++}, indicate that the active center of this enzyme is functionally identical to trypsin and that both of these enzymes differ significantly from thrombin. As with trypsin, some five groups are implicated in the active center or are affected by ES complex formation and subsequent decomposition; however, the probability exists that one or more of these groups in plasmin differ chemically from their functionally identical analogues in trypsin. Data are presented on the storage stability of plasmin and trypsin which also serve to emphasize their individuality.

INTRODUCTION

The studies reported here are a continuation of earlier research designed to identify the groups and group functions in the active centers of trypsin, thrombin and plasmin. The last report on trypsin and thrombin[1] presented data resulting from the application of the theoretical, kinetic treatment elaborated by DIXON[2] and by LAIDLER[3]. This report now extends that treatment to plasmin and makes comparison both with the previous results on thrombin and trypsin and with rechecked data on trypsin. Additional data on both the storage stability of plasmin and trypsin and as evidence of their non-identity are presented.

Abbreviation: TAME = N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester.

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MATERIALS AND METHODS

Measurement of enzyme activity and the determination of K_m

The measurement of enzyme activity was performed by the null point titration procedure which has been previously described[4]. 2 ml reaction volumes were employed. 15-μl aliquots of trypsin solutions and 60-μl aliquots of plasmin solutions were used. Suitable controls were run. K_m's were determined by the method of LINEWEAVER AND BURK[5]. Five to seven points were used to establish each curve. In most cases the graphical determination of K_m leads to values for the constant which could vary by as much as $\pm$ 1.0·10^{-3}. The error on the logarithmic scale would vary with the absolute value of K_m. As most values of K_m were numerically equal to 5·10^{-3} or above, a maximal variation on the logarithmic scale of $\pm$ 0.079 units can occur. For most values of K_m, the error is smaller and falls in the region of $\pm$ 0.04 logarithmic units. The conditions under which K_m was determined are as follows: 38°; initial substrate concentrations from 0.006 M to 0.02 M; initial enzyme concentration: for plasmin —2.92 Th units/ml, for trypsin —4.12 Th units/ml; above pH 6.75 veronal buffer (0.01 M) was used; below pH 6.75 succinate buffer (0.01 M) was employed. The Th unit of tryptic enzyme activity has been previously defined[4] and slightly amended[1]. This unit is as applicable to plasmin as it is to trypsin and thrombin.

Substrate and enzymes

The substrate, TAME, was a product of H. and M. Chem. Co., Santa Monica, Calif. Trypsin was a twice crystallized product and gift from Armour & Co. Plasmin was prepared from fresh beef blood as described in the next paragraph. Trypsin was stored at 0–4° in a 0.01 M HCl solution (pH 2.3). Plasmin was stored in the deep freeze as a lyophilized powder. Stock solutions of plasmin were prepared by dissolving suitable quantities in 0.015 M tris buffer at pH 7.4; these solutions were stored at 0–4°. Comments on the storage stability of these enzymes are given elsewhere in this report.

The preparation of plasmin

The method is a conglomerate of the procedures of other investigators[6,7] with significant revision from that earlier used[8], thus making a procedural recitation appropriate.

4 l of fresh beef blood was collected in 250 ml of a 1.85 % potassium oxalate plus 0.5 % oxalic acid solution. The hematocrit was centrifuged off (2,600 × g, cold) resulting in 1.2 l of plasma; 40 ml of 5.3 % $CaCl_2$ was added and the plasma was refrigerated overnight. However, no clot formed. Addition of 1,000 NIH units of thrombin caused clotting in 5 min. The fibrin was centrifuged off (2,600 × g) leaving about 800 ml of serum, which was mixed with 200 ml of 0.15 M tris buffer at pH 7.4 and placed overnight in the deep freeze. In the morning, the frozen solution was thawed at room temperature and centrifuged to remove undesirable material. The solution was divided into two parts and each part was shaken with 100 ml of chloroform for 1 h at room temperature. The chloroform was removed; the resulting serum adjusted to pH 5.5 with dilute sulfuric acid and refrigerated overnight. The precipitate that formed was centrifuged off (2,600 × g, cold) and dissolved in 100 ml of 0.015 M

tris buffer, pH 7.4. The protein solution thus obtained was devoid of plasmin activity. It was treated with 2 mg of trypsin for 1 h, followed by 20-fold dilution and adjustment to pH 5.5 with dilute sulfuric acid. After a night's refrigeration, a white precipitate formed. The supernatant was decanted; the residue was retrieved by centrifugation (10,500 × *g*, cold, 20 min) and dissolved in 50 ml of 0.015 *M* tris buffer at pH 7.4. This solution had no fibrinolytic activity. 20 mg of trypsin (20 mg commercial preparation = 10 mg of enzyme) was added and the solution was incubated (25°; 1.5 h), followed by 20-fold dilution, adjustment to pH 5.5 and overnight refrigeration. After decanting most of the supernatant, the plasmin precipitate was centrifuged off (2,600 × *g*, 25°) and redissolved in 30 ml of 0.015 *M* tris buffer, pH 7.4. Solution was fairly rapid and complete after a night of refrigeration. This solution had potent fibrinolytic activity; a 0.1-ml aliquot prohibited clot formation of an aliquot of fibrinogen solution which readily clotted in its absence. The plasmin solution was diluted to 600 ml, and on adjustment to pH 5.5 with 0.1 *N* H_2SO_4 soon became cloudy. It was placed in the refrigerator for 4 h. The precipitate was centrifuged off (2,600 × *g*); dissolved in 25 ml of distilled water and lyophilized for 8 h. A white, fluffy powder was obtained (0.62 g); total activity: 4,000 Th units (equals 6.75 Th units/mg of dry preparation). This value represents about 3 to 3.5 times greater activity than the previous preparation[8]. In the above procedure, the pretreatment with 2 mg of trypsin was insufficient to produce activation, but may have been instrumental in acting as a "protein and inhibitor cleansing agent" thus permitting the subsequently added trypsin to act more fully and to produce greater activation of plasminogen to plasmin.

RESULTS AND DISCUSSION

Storage stability of plasmin and trypsin

Plasmin stored as a lyophilized powder in a screw-top bottle in the deep freeze (—15°) was stable for at least two months. However, the requirement for *in vacuo* storage at this temperature stated by JACKSON AND MERTZ[7] proved unnecessary. As dry storage of the enzyme is undesirable for its daily use, the stability of plasmin in solution was investigated. Fig. 1 contains the results which are obvious upon inspection; affirming the already known stability of trypsin[4,9] at pH's in the vicinity of 2.3. The data serve as a guide for the storage of these enzymes under conditions which will assure a relatively constant level of activity (within the bounds of experimental error) for reasonable lengths of time. In addition, the observation that plasmin is unstable at a pH where trypsin is stable (pH 2.3) while, to the contrary, being stable at a pH (7.4) where trypsin is unstable, adds another line of evidence to the six items previously listed[8] to support the conclusion that trypsin and plasmin are remarkably similar but definitely not identical enzymes. Striking emphasis of this conclusion in terms of the active center is presented in the material that follows.

The active center of plasmin

This discussion presumes a familiarity with the kinetic analyses of DIXON[2] and LAIDLER[3] and with the previous report on trypsin and thrombin[1].

Fig. 2 presents plots of the pK_m against pH and of the corresponding $\log_{10}V_{max}$ *vs.* pH for the reaction between TAME and plasmin. These curves are hereinafter referred to as the pK_m or the log V curves.

In those pH regions where the log V curves are horizontal straight lines, k_3 (the ES decomposition constant) is unaffected by the discontinuities in the pK_m curves and, further, these discontinuities indicate ionizations which affect or are affected by the formation of the ES complex. As the guanidino group of TAME remains cationic

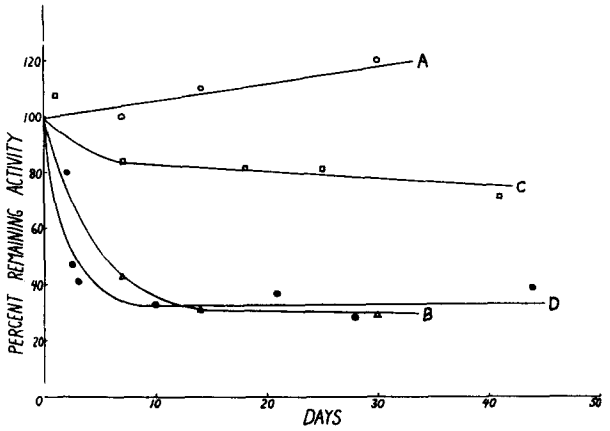

Fig. 1. Storage stability of plasmin and trypsin. Curve A, trypsin stored at pH 2.3 (HCl solution), 0–4°. Curve B, trypsin stored at pH 7.4 (0.015 *M* tris buffer), 0–4°. Curve C, plasmin stored at pH 7.4 (0.015 *M* tris buffer), 0–4°. Curve D, plasmin stored at pH 2.3 (HCl solution), 0–4°. Tests of activity conducted at 38°; pH 8.0; 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer; 2-ml reaction volume.

in the pH regions investigated here, the changes in the pK_m curves refer to dissociations on either the free enzyme or the ES complex. Further, any electrostatic combination between the substrate through its positively charged guanidino group and a negatively charged group on the enzyme would produce no alteration in total charge on ES complex formation and thus would not cause any deviation in the pK_m curve from a horizontal position. The slopes of all non-horizontal lines, shown by the dashed extensions, approximate either $+1$, $+2$, -1, or -2 values. Groups with a $+1$ slope can be described by the following reaction prototype on the sloped portion of the curve: $EH^+ + S \rightarrow ES + H^+$; on the other hand, those having a -1 slope yield a reaction prototype on the sloped portion of the curve as follows: $E + H^+ + S \rightarrow EH^+S$.

In Fig. 2, indicated groups are designated by the capital letters drawn close to the various inflection points. To avoid confusion, Table I presents the pK's, slopes and identity suggestions of the possible groups in summary form. Curves II and III, Fig. 2, are virtually identical but differ from Case I in that the pK's of several of the groups are shifted to alkaline regions about 0.6 to 0.8 pH unit. A shift of similar degree for the analogous cases has been earlier found with trypsin and thrombin[1]. The inflection at 7.30 (Cases III and II) and at 6.56 (Case I) mark a point of transition in each case from $+1$ to -2 slope and is indicative of three groups on the free enzyme, that is, one responsible for the $+1$ slope (Group A) and two (Groups B and C) responsible for the -2 slope. At pH 7.60 (Case III), 7.54 (Case II) and 6.92 (Case I) the transitions from the -2 slope to a $+1$ slope indicate three groups on the ES complex. Waves or steps in the pK_m–pH curves indicate shifts in group pK values in the free enzyme from what they are in the ES complex and *vice versa*. The curves in

TABLE I

GROUPS ON PLASMIN*

Group designations	Group pK		Slope**	Possible group identities***
	On free enzyme	On ES complex		
A	6.56§, 7.30§§, 7.30§§§		+1	Glyoxaline (histidyl)
B	6.56§, 7.30§§, 7.30§§§	6.92§, 7.54§§, 7.60§§§	−1	Glyoxaline (histidyl) or α-amino
C	Same as Group B			
D	7.75§, 8.00§§, 7.96§§§	6.92§, 7.54§§, 7.60§§§	+1	α-amino
E	9.01§, 9.07§§, 9.03§§§		−1	α-amino, sulphydryl or ε-amino

* Values taken from Fig. 2.

** Examples of reaction prototypes to fit these values are given in the RESULTS and DISCUSSION section.

*** These are only likely suggestions. Other possible identities are not excluded.

§ Case I. §§ Case II. §§§ Case III.

Fig. 2 about the points discussed above can be mentally resolved into the forms ⌝⌞ and ⌞⌜. Thus the two groups, B and C, responsible for the −2 slope on the ES complex with pK values of 7.60, 7.54 and 6.92, respectively, would then be the same as those having pK values of 7.30, 7.30 and 6.56 on the free enzyme in the same respective cases. Group D, which is responsible for the +1 slope on the ES complex at 7.60, 7.54 and 6.92 would then be the same group on the free enzyme which gives rise to pK's at 7.96, 8.00 and 7.75 in Cases III, II and I, respectively. As in the earlier case of trypsin[1], it is again important to note that according to DIXON[2], "if a substrate does not actually combine with the group in question but merely affects its ionization so that its pK is not the same in the ES complex as it is in the free enzyme, a wave will appear in the curve, etc.". Thus, Groups B, C and D are presumably not involved in ES complex formation. The additional points of inflection at pH 9.03, 9.07 and 9.01 in Cases III, II and I, respectively, represent a group, E, on the free enzyme. From the above, it appears that during ES complex formation and subsequent decomposition some five groups are either involved in the reaction; that is, are members of the active center, or their pK's are affected by the hydrolytic process. Table I contains suggestions for possible chemical identities of the various groups. It is emphasized that these are merely suggestions; other possibilities are not excluded.

The data from the log V curves indicate that Groups A, E and at least one of the three, B, C and D, have no influence on the rate of ES complex decomposition (k_3). In Case I, the +2 slope with an inflection point at 6.88 indicates that two of the three Groups, B, C and D, affect k_3. In Case II, one of these two groups exhibits virtually the same pK as in Case I, namely at 6.82; whereas the other group has its pK shifted some 0.7 pH unit to 7.52. When Ni^{++} is present (Case III), the shift observed in Case II for one of the groups is maintained and an identical inflection point to that found in the latter case is observed. However, the group with inflection points at 6.88 and 6.82 for Cases I and II, respectively, apparently loses its influence when Ni^{++} is present. It is reasonable to presume that this group interacts with Ni^{++}. This situation is virtually identical to that observed earlier in the case of trypsin[1], except that the

shifting of the pK's of both groups in the presence of Ca^{++} is observed with this enzyme.

The influence of two groups of the three, B, C and D, on the rate of ES complex decomposition, k_3, is of further interest in view of the fact that these groups, as pointed out above, do not take part in ES complex formation.

Whereas Ca^{++} ion produces virtually no change in pK_m, it activates plasmin by an augmentation of the k_3. This effect is, however, not clearly observable from the log V curves. Ni^{++} produces some decrease in pK_m, but evidences its inhibition mainly by a decrease in k_3.

Comparison of the plasmin curves with those for trypsin and thrombin

The most striking feature of the curves for plasmin in comparison with those previously given for trypsin[1] is that they are practically identical in shape, but that the pK's of several of the groups are shifted toward the alkaline region. This makes reasonable the conclusion that the active centers of these two enzymes are *functionally* identical. Assuming a chemical identity of the groups in the active centers, the observed variations in the pK's could be caused by spacial geometry or the effects of neighboring amino acids. On the other hand, it is possible that one or more of the groups in one enzyme may be chemically different from the analogous groups in the other enzyme, yet functionally identical. Thus, either a glyoxaline histidyl group or an amino group can yield identical reaction prototypes and, therefore, an identical pK_m curve in the region of the group's pK, but would most probably show different pK's. Phenomenal, too, is the fact that the magnitude of the shift of approximately 0.6 pH unit for the pK's of Groups A, B and C and 0.4 pH unit for Group D observed for trypsin when either Ca^{++} or Ni^{++} are present, compared to the case of their absence[1], finds parallel in the data for plasmin, the shift being of only slightly different

Fig. 2. The variation of pK_m and the corresponding $\log_{10}V_{max}$ with pH for plasmin. Conditions and procedures are as described in the MATERIALS AND METHODS section. Case I, plasmin alone. Case II, plasmin plus 0.0066 M $CaCl_2$. Case III, plasmin plus 0.001 M $NiCl_2$.

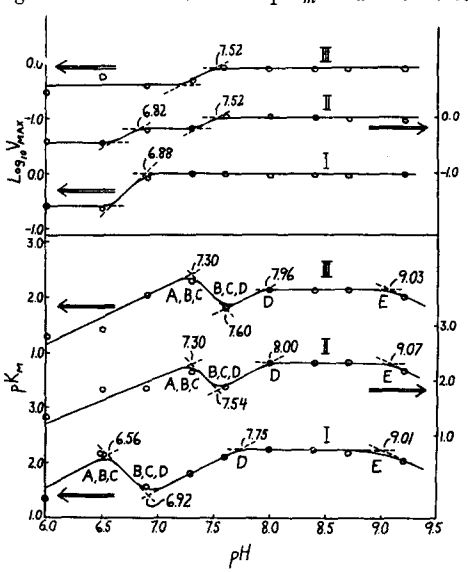

Fig. 2.

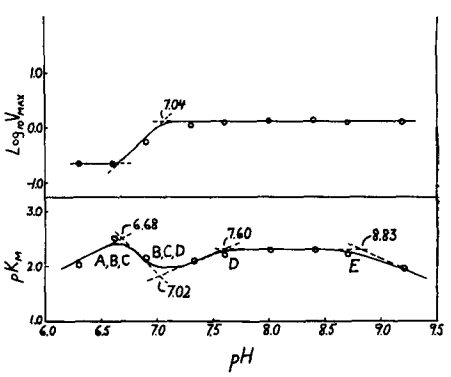

Fig. 3. The variation of pK_m and the corresponding $\log_{10}V_{max}$ with pH for trypsin in the presence of 0.0066 M $CaCl_2$. Conditions and procedures are as described in the MATERIALS AND METHODS section.

dimension (0.6 to 0.8 pH unit for Groups A, B and C and 0.2 to 0.3 pH unit for Group D). For Group E a remarkable correspondence is observed for plasmin in all three cases and the values are shifted about 0.2 unit from what they are for trypsin.

As the previous work on trypsin and thrombin involved the use of a different Model G Beckman pH meter, different standard buffer, electrodes, and other factors from those used in this study, and in view of the use of trypsin to activate plasmin, the thought was considered that perhaps the effects are really those of trypsin and that some factors in the previously used system are at variance with the same factors in the present system, thus explaining the difference between the earlier trypsin curves and the "suspect" plasmin used here (the various lines of evidence demonstrating the non-identity of trypsin and plasmin previously given[8] and as presented in an earlier paragraph in this report, notwithstanding). Therefore, the same trypsin preparation as earlier used was employed in a re-run of the case of trypsin in the presence of Ca^{++} ion. The curve that resulted, Fig. 3, is identical within experimental limits to that earlier obtained[1] (inflection points observed here at 6.68, 7.02, 7.60 and 8.83; earlier at 6.72, 7.08, 7.69 and 8.87). These data establish the validity of the earlier data on trypsin and thrombin[1] and of the present data on plasmin. Additionally, they spotlight the remarkable similarity but non-identity of plasmin and trypsin (compare Fig. 3 with Case II, Fig. 2).

For comparison with thrombin, the earlier remarks on trypsin[1] are equally applicable to plasmin. Thus, the difference in the manner by which Ca^{++} causes its activation toward thrombin as compared to trypsin is valid for thrombin as compared to plasmin. Also, Ni^{++} inhibits all three enzymes in the same general manner, apparently acting identically toward plasmin as toward trypsin. The similarity between Group D in trypsin and Group B in thrombin is likewise applicable to Group D in plasmin. As with trypsin, plasmin has two groups involved in ES complex formation (Groups A and E) and at least two involved in the subsequent hydrolysis (two of the triad, B, C and D). This situation is duplicated with thrombin except that only one group is involved in the subsequent hydrolysis[1], though, as noted above for both trypsin and plasmin, the influence of one of their two groups involved in the subsequent hydrolysis is lost when Ni^{++} is present. Thus for all three enzymes only one group appears essential for the hydrolytic step.

It has been mentioned above that the two groups of the three, B, C and D, whose pK's appear on the log V curves, have their pK's shifted in the case of trypsin in the presence of Ca^{++} ion, whereas only one of the two has its pK shifted in the case of plasmin in the presence of the same ion. A possible explanation of this difference may be that the group which does not shift in plasmin, but does in trypsin, is functionally identical but chemically different in each enzyme. Also, it may indicate that the two of the three groups, B, C and D, which are concerned in this matter are both functionally and chemically identical in trypsin, but only functionally identical in plasmin.

It has been demonstrated that trypsin soy bean inhibitor inhibits both trypsin and plasmin but not thrombin despite the fact that all three enzymes hydrolyze TAME and other similar substrates[10]. The data of Fig. 2 and that earlier presented[1] permit an explanation of this observation. Obviously, as plasmin and trypsin have functionally identical active centers, which differ from thrombin's, they have a group(s) in common which reacts with the soy bean inhibitor that is not present in

thrombin. Groups B and C in plasmin and trypsin appear to have no counterpart in thrombin and may well be the group(s) involved.

The observation that plasmin and trypsin have functionally identical active centers although the group pK's are different may be evidence of at least one mechanism whereby biological activity is controlled by nature. For plasmin as compared to trypsin, the pK's are all shifted from 0.2 to 0.8 pH unit toward the alkaline side. Thus, at the blood pH of 7.4, much less of any molecular concentration of plasmin would be in the active form than a corresponding molecular concentration of trypsin.

The data also explain the earlier observation that over a short interval of time (5 min) plasmin and trypsin are relatively stable to acid treatment whereas thrombin is rapidly destroyed under the same conditions[8].

GLADNER AND LAKI[11] and DIXON *et al.*[12] have demonstrated the presence of the amino acid sequence, gly·asp·ser·gly, in thrombin and trypsin which they claim is part of the active centers of these enzymes. In view of the identity of the pK_m curves for plasmin and trypsin, this sequence may also be present in plasmin. Further, the pK_m curves presented both here and in the earlier work on thrombin and trypsin[1] would only have uncovered the action of the seryl hydroxyl group. However, the data on all three enzymes, while not excluding the function of such a group, do indicate that the active centers of thrombin, trypsin and plasmin are considerably more complicated than the single tetrapeptide "thread" mentioned above.

As a final conclusion, the data indicate that in terms of the active center, plasmin is functionally identical but slightly different from trypsin, while both differ to a relatively greater extent from thrombin. Although all three enzymes hydrolyze the same substrate types, the additional possession of clot catalyzing properties by thrombin may be the resultant either of a group it possesses which has no counterpart in plasmin or trypsin (Group C in thrombin)[1] or that the at least two groups in plasmin and trypsin (B and C) having no seeming counterpart in thrombin prevent these enzymes from catalyzing the clotting process.

ACKNOWLEDGEMENT

This work was done during the tenure of an Advanced Research Fellowship and Grant from the American Heart Association.

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