Comparison of Some Properties of Native (Glu) and Modified (Lys) Human Plasminogen*

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A number of physical and functional properties of native human plasminogen, carrying glutamic acid as the NH$_2$ terminus, and of proteolytically modified plasminogen, having lysine in the corresponding position, are compared. In order to exclude other variables, the two preparations were made from the same fresh plasma pool. The integrity of the NH$_2$-terminal portion of the native protein was assured by the presence of bovine pancreatic trypsin inhibitor during critical steps in the purification. Carrying out the procedure in the absence of inhibitor and at room temperature yielded the modified preparation with lysine as the dominant NH$_2$ terminus. The two preparations, which had identical specific activities after activation, were compared by the following properties: (a) NH$_2$-terminal amino acid; (b) sedimentation coefficient; (c) isozyme composition by isoelectric focusing; (d) effect of L-aminocaproic acid on the activation rate by urokinase and by streptokinase; (e) activator activities (using bovine plasminogen as the substrate) of the equimolar streptokinase complexes of the native and the modified zymogen; (f) occurrence of various plasminogen and plasmin heavy chain variants during the activation of the equimolar streptokinase-plasminogen complexes (pathway of activation); and finally, (g) the binding of L-aminocaproic acid. All these measurements gave significant differences between the two kinds of plasminogen, confirming earlier data in the literature.

The comparison of the binding data for L-aminocaproic acid is considered important since it had been established that the conformational state of modified plasminogen is very similar to that of native plasminogen when in the presence of L-aminocaproic acid or L-lysine. Based on this observation, the two conformations are treated as very similar to that of native plasminogen when in the absence of ligand or of proteolytic modification. A tentative explanation given for this observation is that the conformational state of this molecule has been found to differ from that of the "native" one (Glu) in both physical and functional properties (9-11). Added interest in this transconformation, caused by the hydrolysis of a single peptide linkage, was generated when it was discovered that in the presence of L-aminocaproic acid, or L-lysine, the circular dichroic spectrum of native plasminogen in the 250 to 280 nm region becomes very similar to that of modified plasminogen (12), demonstrating that an analogous conformational transition can be obtained in the native molecule even in the absence of a bond cleavage. A similar conclusion was reached concerning the effect of these compounds on native plasminogen by sedimentation analysis (11). Functional similarities between modified plasminogen and native plasminogen in the presence of L-aminocaproic acid have also been documented (9, 13).

The present investigation started with the realization that the L-aminocaproic acid binding properties of the two kinds of plasminogen were different. In trying to establish that these differences were not due to differences in donor plasma, or to the relative age of the plasminogen preparations, we decided to prepare the two kinds of plasminogen from the same starting material, and to carry out all the determinations on paired samples simultaneously. All the determinations done on this preparation were also repeated on other native and modified preparations which, however, were of different origin. It was also necessary to carry out these studies on proteins with defined end groups, since some of the important observations in the literature were made on plasminogens

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It is now well established that the NH$_2$ terminus of human plasminogen, the circulating precursor of the proteolytic enzyme plasmin, is glutamic acid (1), and that during the isolation and purification of the proenzyme, unless the procedure is carried out in the presence of a protease inhibitor, a cleavage occurs toward the NH$_2$-terminal end of the molecule. This cleavage can occur at several sensitive sites within a short segment of the molecule approximately 70 residues removed from the NH$_2$ end. (This question has been recently reviewed by Rickli (2)). The resulting peptide of molecular weight about 7000 retains significant affinity for the remainder of the molecule (3). The new NH$_2$ terminus of the plasminogen has been most frequently identified as lysine (4), but the presence of methionine (5), valine (5, 6), and serine (7) has also been reported. The new plasminogen, which we call "modified plasminogen," has lately attracted a great deal of attention, partly because it is believed by some to represent a stage the plasminogen molecule must pass through on its way to activation to plasmin (8), partly because the conformational state of this molecule has been found to differ from that of the "native" one (Glu) in both physical and functional properties (9-11). Added interest in this transconformation, caused by the hydrolysis of a single peptide linkage, was generated when it was discovered that in the presence of L-aminocaproic acid, or L-lysine, the circular dichroic spectrum of native plasminogen in the 250 to 280 nm region becomes very similar to that of modified plasminogen (12), demonstrating that an analogous conformational transition can be obtained in the native molecule even in the absence of a bond cleavage. A similar conclusion was reached concerning the effect of these compounds on native plasminogen by sedimentation analysis (11). Functional similarities between modified plasminogen and native plasminogen in the presence of L-aminocaproic acid have also been documented (9, 13).

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whose NH$_2$-terminal groups were not determined, and other observations were made on preparations whose reported NH$_2$ termini now appear to be dubious. Finally, using the binding of e-aminocaproic acid to the two kinds of plasminogen, an approach is outlined for obtaining the free energy change for the conformational transition between Glu-plasminogen in the native conformation on the one hand and Glu-plasminogen in the modified conformation on the other, and a tentative value for the equilibrium constant for this process is suggested.

MATERIALS AND METHODS

**Plasminogen—** The zymogen was prepared by affinity chromatography according to Deutsch and Mertz (14) with the modifications described in the preceding paper (15). In brief, native plasminogen, with glutamic acid as the predominant NH$_2$ terminus, is obtained only if an inhibitor, such as pancreatic trypsin inhibitor, is present during certain critical steps of the purification. All work was done at temperatures below 15°, and dialysis against neutral buffers was avoided. The procedure for preparing modified plasminogen carrying predominantly lysine as the NH$_2$ terminus, differs from the above procedure in the absence of trypsin inhibitor and in that all steps, with the exception of the final dialysis, were carried out at room temperature. Most of the experiments reported in this paper were done on a preparation of plasminogen purified from plasma obtained from two of the authors by plasmapheresis. The fresh plasma were mixed, diluted 1:1 with 0.1 M phosphate buffer, pH 7.4, and divided into two equal halves which served for the preparation of native (A) and modified (B) plasminogen, respectively. The portion designated as A immediately received 300 μg of pancreatic trypsin inhibitor. Following the purification, protein concentrations were determined spectrophotometrically using 161.1 as the A$_{280}$, and a molecular weight of 91,000 (5). Plasminogen stock solutions (4 to 6 mg/ml of 0.01 M sodium acetate, pH 5.0) were stored at -80°.

**Streptokinase—** This activator was a product of AB Kabi, Sweden, and was kindly supplied to us by Dr. William H. Holleman of Abbott Laboratories.

**Urokinase—** The human activator was a kind gift of Dr. Alex Lesuk of Winthrop Laboratories.

**Amino End Group Analysis—** Amino end group analysis was performed by the dansyl$^1$ method of Gray and Hartley (16), as modified by Zanetta et al. (17). The thin layer chromatographic procedure of Woods and Wang (18) was used to separate the dansylated amino acids.

**Acrylamide Gel Electrophoresis—** Acrylamide gel electrophoresis in SDS was done by the method of Laemmli (19), as described earlier (20).

**Isoelectric Focusing—** Isoelectric focusing was done by the method of Radola (21) as developed and modified by LKB Instruments, Inc. and described in the brochure Application Note. Focusing was carried out at 5° for a period of 20 h at 400 V, followed by an additional 4 h at 1000 V, using 0.3 mg of plasminogen applied to each half of the gel bed. The flat apparatus used for focusing was the TLE Double Chamber 12 20 00 made by Desaga, in conjunction with the LKB Ampholine electrophoresing kit. A pH 3.5 to 10.0-range LKB gel bed was used; the support was LKB's 2117-510 Ultrodex, a specially purified Sephadex G-75.

**Zymography—** This method, used for the detection of focused zones of activable plasminogen on the gel bed, was done according to Heberlein and Barnhart (22), but using Whatman No. 1 chromatography paper instead of cellulose acetate strips, and Coomassie brilliant blue, rather than Ponceau S. For the direct visualization of focused proteins, chromatography paper was placed on the gel bed and the imprinted protein was subsequently stained with Coomassie blue.

**Activator Assays—** Activator assays were carried out as described earlier (20).

**Binding of e-Aminocaproic Acid—** Binding of this acid was measured as in the preceding paper (15).

RESULTS AND DISCUSSION

Comparison of Some Physical and Chemical Properties—

FIG. 1 shows the results of NH$_2$-terminal end group analysis

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; SDS, sodium dodecyl sulfate.

of plasminogen A and B, originating as described above, from the same plasma pool. It is clear that the predominant end group in preparation A is glutamic acid, while in B, it is lysine. Traces of other end groups can be seen in both plates; in B, some glutamic acid is still present; and valine and possibly some methionine are also detectable.

The sedimentation coefficients of A and B were 5.44 S and 4.60 S, respectively, in good agreement with the findings of Violand et al. (11).

Relative differences in the size of the peptide chain of the plasminogen molecules were measured by mobilities of the principle plasminogen bands in SDS gels, and are listed in Table I. These differences are small, but highly reproducible, and correlate very well with other measured properties. (The profiles of preparation A and B can be seen in gels C of Fig. 5. The two satellite bands appearing below the main plasminogen band in preparation B are discussed in connection with the activation pattern.) Based on measurements of their mobilities, the molecular weight of modified plasminogen (B) is 84,000, in good agreement with the expectation of the loss of a 7,000 fragment from the 91,000 native plasminogen.

The results of isoelectric focusing are shown in Fig. 2. The close correspondence between the Coomassie blue-stained bands and the activity zones in the zymograms indicates that all directly stainable bands are plasminogen. There are at least 13 bands distinguishable in the native preparation and about as many in the modified one. These patterns are highly reproducible; another native plasminogen gave a pattern which was indistinguishable from preparation A. The finding of Summaria et al. (23) that most of the isoelectric forms of the Glu-plasminogen group are distinctly more acidic than those of the Lys-plasminogen family, is confirmed here also. Siefring and Castellino (24) showed that part of this heterogeneity was due to differences in sialic acid content. They found, however, that even after neuraminidase treatment the
TABLE I
Comparison of some properties of native and modified plasminogen preparations

<table>
<thead>
<tr>
<th>Preparation*</th>
<th>Specific Activity in CTA units</th>
<th>NH₂ terminus</th>
<th>Relative mobility in SDS gel electrophoresis</th>
<th>Activa-bility ratio</th>
<th>Lim ric as r → 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38A*</td>
<td>38.3</td>
<td>Glu</td>
<td>0.317</td>
<td>1.84</td>
<td>104</td>
</tr>
<tr>
<td>35</td>
<td>37.0</td>
<td>Glu</td>
<td>0.319</td>
<td>1.63</td>
<td>76</td>
</tr>
<tr>
<td>33</td>
<td>33.1</td>
<td>Glu</td>
<td>0.317</td>
<td>1.75</td>
<td>84</td>
</tr>
<tr>
<td>38B</td>
<td>36.0</td>
<td>Lys ⇒ Met, Val, Glu</td>
<td>0.329</td>
<td>0.78</td>
<td>32</td>
</tr>
<tr>
<td>32</td>
<td>34.7</td>
<td>Lys ⇒ Ser(?), Val</td>
<td>0.359</td>
<td>0.87</td>
<td>20</td>
</tr>
<tr>
<td>37</td>
<td>36.2</td>
<td>Glu = Lys = Met</td>
<td>0.324</td>
<td>1.04</td>
<td>29</td>
</tr>
</tbody>
</table>

* Preparations 38A and 38B were made of the same plasma pool (see "Materials and Methods").

† Measured by azocaseinolysis at 37° after 25 min of activation at 25° with 62 CTA units of urokinase per 100 μl of activation mixture. CTA units of plasmin activity calculated by comparison with a standard solution from the National Red Cross.

‡ Calculated as described in legend to Fig. 6.

§ Ratio of caseinolytic activities after 15 min of urokinase activation in 3 mM, to that in 0.1 mM L-aminocaproic acid (see section on "Activability of Two Plasminogens").

The ordinate intercept of a Scatchard plot, related to the strength of binding of the first L-aminocaproic acid molecule.

preparation still contained at least three major, and a number of minor bands. It is noteworthy that, while in the modified plasminogen, all bands seem to have shifted to higher isoelectric points, (loss of the highly acidic NH₂-terminal peptide), the degree of heterogeneity has not changed. Since the end groups in the two preparations are over 90% glutamic acid, or lysine, respectively, the observed microheterogeneity must be due, beside the differences in sialic acid content, to genuine sequence differences or, perhaps, to tight binding of charged small molecular material.

Activability of Two Plasminogens — That the rate by which urokinase activates modified plasminogen is considerably greater than the rate of native plasminogen activation was described by Claeys and Vermylen (9), and the phenomenon was studied in detail by Thorsen et al. (13, 25). Both of these groups used a method of visualization of these effects which appear very useful to us: the measurement of the rate of urokinase activation as a function of L-aminocaproic acid concentration. Fig. 3 (top) is a repeat of their work and it fully confirms their interesting findings. In essence, this type of experiment shows that: (a) at low inhibitor concentration, the rate of urokinase activation of both kinds of plasminogen is weakly inhibited (the caseinolytic activities plotted on the y axis correspond to amounts of plasmin formed in 15 min); (b) the rate of activation of native plasminogen is lower than that of the modified one; (c) between 0.1 and 1 mM inhibitor the native plasminogen becomes as easily activable as its modified counterpart; and (d) at even higher concentrations, the activation of both plasminogens is strongly inhibited. The measurements over the entire L-aminocaproic acid concentration range are reproduced here in order to explain our use of the "activability ratios". This is the ratio of caseinolytic activities measured at the lowest and highest activity points of the curve for the native plasminogen, i.e. it is the absorbance obtained at 3 mM L-aminocaproic acid, divided by that measured at 0.1 mM concentration of the same. This ratio, which varies between about 1.8 for the native, and 0.8 for the completely modified plasminogen, is a highly reliable, sensitive, and reproducible index of the conformational state of this protein. We now routinely measure this ratio for every new preparation and find that it correlates very well with other conformational criteria (Table 1). It is important, of
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The lower panel of Fig. 3 shows a similar experiment, but this time, instead of urokinase, we used streptokinase in catalytic amounts as the activator. It is evident that the response of the two plasminogens to these two activators is very similar at the low e-aminoacproic acid concentrations, but is strikingly different above 1 mM. The curves show that the inhibitor is about 10 times as effective in inhibiting activation by streptokinase than by urokinase. This is the reason why the biphasic response of the native plasminogen, so pronounced in urokinase activation, is only rudimentary in streptokinase activation: inhibition of activation begins before a substantial degree of facilitation is achieved. Finally, it is noted that, on the basis of the binding data for the inhibitor presented in the preceding paper (15), we are now in the position to assign the initial phase of these curves, i.e. the mild inhibition of activation, to the binding of the first inhibitor molecule to the "strong" site, and the region of facilitation of activation to the incipient saturation of the "weak" binding sites by the inhibitor.

Activator Activity of Equimolar Streptokinase Complexes—The activator activities of the streptokinase complexes of plasminogen and of plasmin are of clinical interest (see Ref. 28 and references therein), and their biochemistry was studied in detail recently (20). Earlier work has shown (27) that the activator activity of the initial streptokinase-plasminogen complex, "preserved" in acetyllysine methylester, was considerably higher than that of the modified streptokinase-plasmin complex, but the dependence of this activity on the streptokinase/plasminogen ratio has not been demonstrated. In a recent paper (20), we showed that the activator activity of the complex between modified plasminogen and streptokinase was greater than that of the complex involving the native plasminogen, but we could not be entirely certain that this was due only to conformational differences between the preparations. We now present streptokinase titrations of the activator activities of native and modified plasminogen, as well as the streptokinase dependence of the resulting plasmin, using the twin preparations A and B. Fig. 4 shows that the complex between intact streptokinase and native plasminogen is twice as powerful an activator of bovine plasminogen as the complex between modified streptokinase and plasmin, but the intact streptokinase-modified-plasminogen complex is 3 times as strong. As expected, there is no difference in the activator activities of the modified streptokinase-plasminogen complexes originating from the two preparations since, under conditions of full plasminogen activation, the NH₂-terminal peptide is completely cleaved off both from the native plasminogen and from the glu heavy chain (28) (see below). It should be noted that the activities of the plasminogen complexes are higher than were reported in the paper quoted above (20). In that study, care had to be taken to avoid streptokinase excess, as this would have interfered with the interpretation of the results, and therefore, streptokinase quantities somewhat below the molar equivalence were used. The relative activity of 3.2 for the complex involving the modified plasminogen now agrees well with 3.5, reported originally by Reddy and Markus (27) for a plasminogen now known to have been fully modified.

Activation of Equimolar Streptokinase-Plasminogen Complex—In this experiment, the twin preparations A and B were activated with an equimolar amount of streptokinase in the absence of lysine in the medium. Fig. 5 shows the SDS gel patterns obtained from aliquots withdrawn from the activation mixtures at the indicated times. Recorder tracings of the plasminogen and heavy chain regions of the gels, along with an indication of the relative mobilities of the various peaks, are shown in Fig. 6. The following observations can be made: (a) activation of the native plasminogen (A) is slower than that of the modified zymogen, as can be easily seen by following the accumulation of light chain in the two groups of patterns. We now know that there are two reasons for this, the modified plasminogen is more easily activable (see above), as others have demonstrated, and the initial activator, i.e. the streptokinase-modified-plasminogen complex, is a better activator than the corresponding complex with the native zymogen, as shown in this study. (b) The pathway of activation of the native plasminogen follows the pattern described by McClintock et al. (28) for the equimolar streptokinase complex; native plasminogen first yields a high molecular weight heavy chain which is gradually converted to a low molecular weight heavy chain. That activation of Glu-plasminogen by equimolar streptokinase initially yields a heavy chain of larger molecular weight than the one derived from similarly activated Lys-plasminogen was also described by Summaria et al. (29). In the present case, the process is somewhat more complex in that three bands can be distinguished for both the native plasminogen and the resulting heavy chain (see the 10-min gel in Fig. 5). It is clear, however, that the largest heavy chain, of mobility 0.41, practically disappears by 10 min, and the intensity of the third band (0.45) gradually increases. An even smaller heavy chain, of 0.49 mobility, also increases in quantity. Whether the same modification, i.e. loss of the NH₂-terminal peptide from plasminogen directly, also occurs at the same time is difficult to tell; at 10 min, the most intense band (0.34) is the middle one. Whether this is due to conversion of the 0.30 to 0.34 band, or the result of differential rates of conversion to plasmin, is uncertain. (c) The modified plasminogen (B) lacks, as expected, the slowest moving plasminogen band (0.30), but has two additional bands of mobilities intermediate between plasminogen and the heavy chain (0.37 and 0.40) which are completely absent from the gels of the native.
plasminogen. These two bands with a molecular weight of 78,000 and 75,000, respectively, diminish in intensity with time. Whether this means that these two bands represent additional plasminogen variants, or are unrelated proteins which are gradually digested by the plasmin formed during activation, is uncertain. We are presently attempting to clear up this problem. The heavy chains of the modified plasminogen have all the bands of the native one with the exception of the largest one (0.41) which, therefore, must come directly from the heaviest plasminogen (0.3) when that is present. (d) The breakdown pattern of the streptokinase is very similar in the two complexes and shows, beside the native form, all five partially proteolyzed products described earlier (20).

**E-Aminocaproic Acid Binding to Native and Modified Plasminogen** — In the preceding paper, the binding of e-aminocaproic acid to native plasminogen was described (15). The data were satisfactorily fitted by a two-component binding isotherm with a single strong site ($k_1 = 112 \text{ mM}^{-1}$) and five much weaker sites ($k_2 = 0.2 \text{ mM}^{-1}$). Fig. 7 shows Scatchard plots for both the native and modified plasminogen as well as semilogarithmetic plots (“titration curves”) of the same data. It is apparent that the binding of the first ligand molecule to the modified plasminogen is approximately 3 times weaker than the corresponding step for the native one. This decrease in affinity, though not very large, is a consistent finding and holds for all modified preparations so far examined. (Some $\rho/c$ intercepts are listed in Table I.) The Scatchard plot for the modified protein is compatible with a total number of six binding sites, as was the native one (see inset to Fig. 7); its only remarkable feature is the crossing of the two curves beyond an $\rho$ of 1, found also in another pair of preparations. We were unable to fit this curve with a two-component binding function, the reason for which is obvious from the titration curve; it is apparent that following the first site there is in the modified plasminogen a binding site which is considerably stronger than the weak sites were in the native protein. The sites beyond this second one appear to be similar in both proteins. This curve was fitted with a three-component binding isotherm using the constants $k_1 = 28.6, k_2 = 3.8,$ and $k_3 = 0.1$ (all in $\text{mM}^{-1}$), the latter being the constant for the last four sites.

**Two States of an Equilibrium System: an Interpretation** — The studies of others quoted in the introduction to this and the preceding paper (15) have clearly established that: (a) cleavage of a peptide bond (or bonds) about 70 residues removed from the $\text{NH}_2$ terminus of plasminogen significantly alters the physicochemical, as well as the functional behavior of the molecule; (b) reversible binding of $\text{e}$-aminocaproic acid, l-lysine, or their structural analogues, also results in an altered conformational state; and (c) the two states so induced appear to be identical, or at least closely related. The last point is well illustrated by the circular dichroism studies of Sjöholm et al. (12) and by the finding of Violand et al. (11) that while the sedimentation constant of native and human plasminogen changed from 5.71 to 4.80 in 0.1 $\text{m}$ e-aminocaproic acid, the constant for modified plasminogen, 4.73, did not change significantly in the presence of the compound.

Ligand-stabilized conformations can be interpreted by assuming that the protein, even in the absence of ligand, oscillates between a number of related conformational states and ligands merely select the one which offers suitable binding sites for them (30). The stability of such a conformation is then reinforced by the free energy of ligand binding. In the simplest case, we may consider a protein which oscillates between states N and M, with N being the dominant time-average conformation, and assume that the constant $k_{NM}$ expresses the equilibrium position M/N. If we now consider that ligand L will bind to the protein only in the M conformation, and with a binding constant of $k_L = ML/(N)(L)$, then, for the overall reaction $N + L = ML$, we may write $k_{NM}k_L = ML/(N)(L)$. The free energy change corresponding to this new constant, expressing the binding of ligand to the protein initially in the N conformation, thus includes, in addition to the free energy change due to ligand binding itself, also that involved in the conformational change from N to M. The latter contribution can only be evaluated if the binding constant can be determined independently of the conformational transition. This possibility is rarely open, but in the
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In the present case, the availability of modified plasminogen, i.e., the protein already "fixed" in the B conformation, allows us, at least in principle, to evaluate the binding constant for the ligand, uncomplicated by the equilibrium constant for the transconformation. The binding constant so obtained can then be compared with the apparent binding constant for the native protein; their ratio should give the constant for the conformational transition, and, therefore, also the free energy change for the latter process. Thus, the free energy change involved in the saturation of modified plasminogen with ligand should be larger (more negative) than that involved in the binding to the native protein, because the latter process also includes the unfavorable energy contribution due to transferring the protein from an intrinsically more stable, to a less stable conformation.²

It is obvious that the above considerations will not hold for the assumed conformation binds significant amounts of ligand. While there is no concrete experimental evidence available to directly support this assumption, it appears reasonable; if the native conformation could also bind ligand, then at saturating concentrations a fraction of the total protein would be stabilized in that conformation. While from the physical parameters characterizing the protein in the modified conformation, we cannot tell whether a fraction of the molecules is in the native conformation, the observation that the "modified conformation" is the same (as judged by physical parameters) whether it is achieved by ligand stabilization, or by loss of the NH₂-terminal peptide, speaks against a mixture of conformations; it is unlikely that the proteolytic process would leave the same fraction of the protein in the native conformation as does ligand binding.

Fig. 6. Densitometric tracings of the plasminogen and heavy chain bands shown in Fig. 5. The control patterns were recorded at a lower sensitivity setting than were the rest. The two patterns at the bottom are schematic drawings to show all occurring bands and their relative mobilities. The latter are calculated as fractions of the distance between the top of the separation gel and the M band (see legend to Fig. 5). SK bands are native and digested forms of streptokinase taken from the 1-min sample of series B in Fig. 5. Abbreviations as in Fig. 5.
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Fig. 7. Binding of e-aminocaproic acid (EACA) to native and modified plasminogen. Left panel, Scatchard plots; inset shows the "weak region" replotted. \( \beta \), molar binding ratio; \( c \), concentration of free ligand (mM). Right panel, semilogarithmic plot of the molar binding ratio against the free ligand concentration. The points are experimental and the lines are calculated using the constants given in the text. Binding was determined by ultrafiltration using e-amino[\(^1\)C]caproic acid (15).

the first binding site, since at that site we have shown that ligand is bound more strongly to the native than to the modified plasminogen. It may be that the first ligand molecule actually prefers the native state, or that it needs some residues of the NH₂-terminal sequence for its tight binding.\(^1\) While in the preceding paper (15), it was shown that binding to the first site in the native protein did contribute to the change in the sedimentation coefficient, the experiment shown in Fig. 3 of this paper suggests that saturation of the first site does not contribute to that aspect of the conformational change which leads to increased urokinase-activatability. This effect becomes apparent between 0.1 and 1 mM e-aminocaproic acid, \( i.e. \) in the range where saturation of the second binding site is beginning. It is in this region, \( i.e. \) where the two curves cross in Fig. 7, that the advantage of the modified over the native plasminogen is expressed. Using the constants listed in the text for the binding of the second ligand molecule\(^4\) to the two proteins, we obtain, at 20°C, for the native protein \( \Delta G^0 = -RT \ln 1000 = -4020 \) cal/mol, and for the modified plasminogen \( \Delta G^0 = -RT \ln 3800 = -4800 \) cal/mol. Thus, the free energy change for the binding of the second ligand molecule\(^5\) to the two proteins, we obtain, at 20°C, for the native protein lacks the characteristics of cooperative allosteric behavior, even though the two cardinal requirements of the latter, two conformational states and multiple binding sites (32), are present; allosteric behavior differs too little from Michaelis type behavior to be detected if more than 1% of the molecules are present initially in the conformation favored by the ligand (32).

References


\(^{1}\) As mentioned above, Wiman and Wallen (3) obtained evidence to show interaction between the NH₂-terminal peptide and the rest of the molecule, and proposed that the native conformation is stabilized by this interaction. Finding that e-aminocaproic acid caused the release of the peptide from its noncovalent attachment to previously modified plasminogen, they hypothesized that this ligand competed with the peptide for the same binding site on the protein molecule. In view of the multiplicity of binding sites for this ligand, described in this and the preceding paper (15), it would be desirable to repeat these observations as a function of the ligand concentration in order to identify the binding region responsible for the competitive phenomenon.

\(^{2}\) For this calculation, 3.6 mM⁻¹ was used for the modified, and 1.0 mM⁻¹ for the native plasminogen. The latter value corresponds to the first class constant for a group of five identical, noninteracting sites, and it equals \( (n + 1) JU(j) k_{\text{ass}} \), where \( n = 6, j = 1 \), and \( k_{\text{ass}} = 0.2 \) mM⁻¹ (see e.g. Ref. 31). (For the calculation of the free energies, the association constants were used in the \( m \)⁻¹, rather than mM⁻¹ dimension).
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