The Mechanism of the Inhibition of Plasmin Activity by \( \epsilon \)-Aminocaproic Acid*

(Received for publication, December 21, 1970)

William J. Brockway and Francis J. Castellino‡

From The Department of Chemistry, Program in Biochemistry and Biophysics, The University of Notre Dame, Notre Dame, Indiana 46556

SUMMARY

The streptokinase-induced conversion of human plasminogen to plasmin is inhibited by concentrations of \( \epsilon \)-aminocaproic acid which stimulates the esterolytic activity of plasmin on the synthetic substrate, tosyl-L-arginine methyl ester. The inhibitory effect of \( \epsilon \)-aminocaproic acid decreases as its concentration is decreased and is eliminated upon incubation of plasminogen with streptokinase, regardless of the presence of \( \epsilon \)-aminocaproic acid. At increased levels, \( \epsilon \)-aminocaproic acid further functions as a competitive inhibitor of plasmin activity on tosyl-L-arginine methyl ester with a \( K_I \) of 0.32 \( \text{m} \). In the presence of concentrations of \( \epsilon \)-aminocaproic acid (0.05 \( \text{m} \)) sufficient to nearly saturate its inhibitory effect on the plasminogen to plasmin conversion, the sedimentation coefficient (\( S_{20,w} \)) of plasminogen decreases from a native value of 5.0 ± 0.1 S to 3.8 ± 0.1 S without a decrease in molecular weight suggesting a gross conformational change in plasminogen induced by \( \epsilon \)-aminocaproic acid. This conformational alteration is also evidenced in circular dichroism measurements. The effect of \( \epsilon \)-aminocaproic acid on the conformation of plasminogen is readily reversible and restoration of the native structure is apparent after dialysis.

A mechanism for the inhibition of the plasminogen to plasmin conversion by \( \epsilon \)-aminocaproic acid is postulated involving the formation of a plasminogen-\( \epsilon \)-aminocaproic acid complex, which due to the altered conformation of plasminogen, is not acted upon by streptokinase. This inactive complex is rapidly reversible, yielding a fully streptokinase-reactive plasminogen.

Plasmin (EC 3.4.4.14), a proteolytic enzyme which hydrolyzes fibrin clots is formed from plasminogen, its inactive plasma protein precursor. Specific activators isolated from several sources are known to induce plasmin formation from plasminogen. These activators can be isolated from either bacterial sources (streptokinase), or human origin (urokinase and plasma activator), and are also present in a variety of animal tissues (pig heart activator).

Robbins and coworkers have demonstrated that the activation of human plasminogen to plasmin by urokinase or trace amounts of streptokinase takes place by the urokinase- or streptokinase-induced cleavage of a single arginyl-valine bond in the plasminogen molecule (1, 2). Human plasminogen consists of a single polypeptide chain and activation of the molecule to plasmin results in a two chain structure stabilized by disulfide bridges (1, 2). The two chains have molecular weights of 25,700 and 57,200 daltons and are called the light and heavy chains, respectively (3).

Several investigators have noted that compounds such as \( \epsilon \)-aminocaproic acid and \( \beta \)-aminomethylbenzoic acid are potent antifibrinolytic agents. Although there are numerous papers published on these compounds, the mechanism of their antifibrinolytic activity is not clear. Alkjaersig, Fletcher, and Sherry (4) reported that \( \epsilon \)Acp\(^2 \) acts as an inhibitor of the plasminogen-plasmin conversion, thus manifesting its antifibrinolytic activity. Other theories have been presented such as \( \epsilon \)Acp acting as an antiplasmin (5), and papers reporting that \( \epsilon \)Acp was antifibrinolytic due to its inducing a conformational alteration in the structure of the substrate, fibrin, have been published (6, 7). More recently, it has been demonstrated that \( \epsilon \)Acp has no effect on the activation of human plasminogen (8).

Due to these apparent inconsistencies, we have undertaken a study of the mechanism of the inhibition of plasmin activity by \( \epsilon \)Acp. Our results conclusively demonstrate that this inhibition is complex and the mechanism proposed involves both inhibition at the level of the conversion of plasminogen to plasmin and the inhibition of the proteolytic activity of plasmin.

EXPERIMENTAL PROCEDURE

Materials

Purification of Plasminogen Human plasminogen was prepared in one step from Cohn III, prepared from age outdated citrated human plasma, by an affinity chromatography technique utilizing L-lysine bound to Sepharose 4B (Pharmacia) to selec-

* The abbreviations used are: \( \epsilon \)Acp, \( \epsilon \)-aminocaproic acid; (tosyl)-\( \text{AMe}, N\)-\( \alpha \)-tosyl-L-arginine methyl ester.
of conditions they are described in appropriate sections of the manuscript. All assay components were prepared in 0.1 M phosphate buffer, pH 7.5. Approximately 50 ml of Cohn III extract were applied and the column was eluted with the same buffer. When no further absorbance at 280 nm was obtained, 0.1 M phosphate buffer-0.2 M \( \epsilon \)-ACA, pH 7.5, was added. The peak obtained after this addition was pooled and found to contain human plasminogen. \( \epsilon \)-ACA, \( \epsilon \)-aminocaproic acid, tively retard the plasminogen (9). Our Sepharose-lysine columns were prepared by suspending 100 ml of Sepharose 4B in water and adding 10 g of cyanogen bromide (Eastman). The pH was adjusted to and maintained at pH 11 by repeated additions of 4 N sodium hydroxide. After approximately 20 min of reaction the activated Sepharose 4B was washed with 2 liters of cold 0.1 M sodium bicarbonate on a Buchner funnel. The resin was then suspended in 0.1 M phosphate buffer, pH 9.0, for the coupling reaction.

In order to couple L-lysine to the activated Sepharose, 100 ml of a solution containing 15 mmole of L-lysine in 0.1 M phosphate buffer, pH 9.0, were added to 100 ml of the cyanogen bromide-activated Sepharose 4B. The solution was allowed to react overnight at 4° with gentle stirring. The solution was then filtered and the gel suspended in a buffer of 0.1 M phosphate, pH 7.5. Under these conditions approximately 25 mmole of L-lysine were coupled per ml of gel.

In order to purify plasminogen from human plasma using this technique, 5 ml of Sepharose-lysine were packed into an 11-mm (diameter) column and equilibrated with 0.3 M phosphate buffer, pH 7.5. Approximately 50 ml of Cohn III extract were passed through the column and eluted with 0.3 M phosphate buffer, pH 7.5, until a steady base-line, indicating no further absorbance, was obtained. At this point, a solution of 0.1 M phosphate buffer-0.2 M \( \epsilon \)-ACA, pH 7.5, was percolated through the column and a sharp peak was immediately obtained. The yield of plasminogen is 85% under these conditions. No detectable plasmin activity was found in this plasminogen preparation. Fig. 1 shows a typical elution profile.

Other Proteins—Streptokinase (Varidase) was obtained from Lederle Laboratories through a local drug outlet in vials containing 20,000 units of activity.

Reagents—\( \epsilon \)-ACA was purchased from Calbiochem and tosyl-AMe was purchased from Cyclo Chemical Company. All other reagents were the best commercially available.

Methods

Plasmin Assays—Since these assays were done under a variety of conditions they are described in appropriate sections of the manuscript. All assay components were prepared in 0.1 M Tris-hydrochloride, pH 8.0, and all assays were performed at 30°.

In general, the assays consisted of converting plasminogen to plasmin with streptokinase and following the action of plasmin on tosyl-AMe. Analysis of the amounts of tosyl-AMe cleaved by plasmin were performed essentially as described by Hestrin (10) with the following minor modifications. After a given time of reaction a 0.2-ml aliquot of the reaction mixture was added to a solution containing 0.2 ml of 4 N sodium hydroxide and 0.2 ml of 2 N hydroxylamine hydrochloride. These conditions were sufficient to immediately stop the enzymatic reaction. The reaction was allowed to proceed for 30 min, and 0.2 ml of a solution of 4 N hydrochloric acid containing 6 g of trichloroacetic acid was added following by addition of 0.2 ml of water. Following this, 4 ml of a solution containing 0.11 m ferric chloride in 0.004 M hydrochloric acid were added and the absorbances of these solutions were determined on a Gifford model 240 spectrophotometer at 525 nm against an appropriate blank. This procedure allowed us to determine the final concentration of tosyl-AMe from a standard curve. Initial concentrations of tosyl-AMe were obtained in the same fashion by preparing incubation mixtures in the absence of any enzymes. The rate of reaction of plasmin with tosyl-AMe was shown to be linear at the times of incubation used in these studies.

Ultracentrifuge Studies—Sedimentation coefficients of plasminogen in 0.1 M phosphate, pH 7.5, and plasminogen in 0.1 M phosphate-0.05 M \( \epsilon \)-ACA, pH 7.5, were measured in a Spinco model E analytical ultracentrifuge using absorption optics at 280 nm. Protein concentrations were approximately 0.2 mg per ml. Sedimentation coefficients were calculated in the usual manner and corrected to the density and viscosity of water at 20° (11).

Circular Dichroism Studies—These were performed with a Cary 60 spectropolarimeter circular dichroism apparatus using 1-, 5-, and 10-mm cells. These cells were interchanged during a run so that the optical density of the protein did not exceed 1.0 at any wave length. The ellipticity \( [\theta] \) values were recorded directly from the instrument and converted to molecular ellipticity \( [\theta] \) expressed in degree cm² per dmole of amino acid according to the relationship

\[
[\theta] = \frac{M\bar{\theta}}{100C}
\]

where \( M \) is the mean residue weight of the protein, \( \bar{\theta} \) the path length in the sample solution in centimeters, and \( C \) is the protein concentration in grams per ml.

Polyacrylamide Gel Electrophoresis—These experiments were performed at pH 9.5 (12), pH 4.3 (13), pH 3.2 in 6.25 M urea (13), and in sodium dodecyl sulfate (14).

RESULTS

Characterization of Plasminogen—The plasminogen isolated by the affinity chromatography technique indicated multiple bands when examined by polyacrylamide gel electrophoresis at pH 9.5, 4.3, or 3.2 in 0.25 M urea. However, all bands exhibited plasmin activity upon addition of streptokinase as demonstrated in gel slicing experiments. These results are consistent with the observations that human plasminogen consists of multiple molecular forms (4, 9, 15). In agreement with these facts, plasminogen gave only one band when examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The molecular weight of
plasminogen calculated by this technique was 82,000, a value in agreement with previously published values (1). Moreover, plasminogen exhibited a boundary indicating that a single protein was present when examined by sedimentation velocity in the ultracentrifuge. Thus, there is no evident heterogeneity in size of the plasminogen preparations, and the heterogeneity in charge obtained on gel electrophoresis can be explained by the multiple molecular form hypothesis.

Fig. 2 presents Lineweaver-Burke plots from which the $K_m$ and $V_{max}$ values for plasmin, obtained from streptokinase activation of plasminogen, on tosyl-AMe were determined. The $K_m$ was found to be 0.0065 M and the $V_{max}$ was 12.1 micromoles of tosyl-AMe cleaved per min per mg. This is the highest specific activity reported for plasmin to this time and reflects the high quality of the plasminogen preparation.

**Inhibition of Conversion of Plasminogen to Plasmin by εAcp in Absence of Prior Incubation of Plasminogen with Streptokinase—** Fig. 3 shows the inhibitory effect of εAcp on the activation of plasminogen to plasmin. In these experiments prior incubation of plasminogen with streptokinase was omitted. The data for the curve in the absence of εAcp in Fig. 3 was obtained by incubating the assay components in the following order: 0.1 ml of plasminogen (26 μg), 0.025 ml of tosyl-AMe (100 μmoles per ml), 0.115 ml of 0.1 M Tris-HCl, pH 8.0. The reaction was initiated by the addition of 0.01 ml of streptokinase containing 10 to 400 units/ml. The reaction was allowed to proceed for 10 min and the concentration of tosyl-AMe cleaved (10 to 400 units/ml) was determined as described under “Methods.” The data for the curve in the presence of 0.02 M εAcp in Fig. 3 was obtained by incubating the assay components in the following order of addition: 0.1 ml of plasminogen (26 μg), 0.01 ml of 0.5 M εAcp, 0.025 ml of tosyl-AMe (100 μmoles per ml), 0.105 ml of 0.1 M Tris-HCl, pH 8.0. The reaction was initiated and the tosyl-AMe concentration determined as described above.

**Effect of εAcp on Activity of Plasmin—** Fig. 4 shows the direct effect of εAcp on the activity of plasmin. These experiments were conducted as follows. Plasminogen, 0.1 ml, was incubated with 0.010 ml (200 units) of streptokinase for 10 min. Under these conditions plasminogen is completely converted to plasmin. Following this 0.1 ml of εAcp containing 0.005 to 0.1 mmole was added followed by 0.015 ml of 0.1 M Tris-HCl, pH 8.0. The reaction was initiated by the addition of 0.025 ml of tosyl-AMe (100 μmoles per ml) and incubated for 10 min. The concentration of tosyl-AMe was determined as described under “Methods.”

The results from Fig. 4 show that εAcp has a complex effect directly on the activity of plasmin. It is quite evident from Fig. 4 that at concentrations of εAcp approximately 0.1 M a direct stimulation of plasmin occurs and at increased levels a direct inhibition of plasmin occurs.

Since, as Fig. 4 indicates, high concentrations of εAcp appeared to directly inhibit the activity of plasmin, it was of interest to determine the type of inhibition exhibited by εAcp on plasmin esterolytic activity to tosyl-AMe. These experiments were performed as shown above except that the εAcp concentration was held constant at 0.2 M or 0.4 M and the concentration of tosyl-AMe varied. Parallel experiments were performed in the absence of εAcp in order to determine $K_m$ and $V_{max}$ for plasmin. These results are shown in Fig. 2. Clearly, competitive inhibition is obtained and the $K_i$ for εAcp on plasmin is 0.32 M. There was no significant effect of the high εAcp concentrations on the colorimetric assay of tosyl-AMe and control experiments demonstrated that this inhibitory effect of εAcp was specific and not due to increased ionic strength.

**Effect of Concentration of εAcp on Inhibition of Conversion of Plasminogen to Plasmin—** Since the data of Fig. 4 indicated that εAcp had both a stimulatory and an inhibitory effect directly on plasmin, these effects required a consideration when attempting to isolate the effects of εAcp on the conversion of plasminogen to plasmin. Therefore, we performed studies to measure the effect...
were carried out with additions in the order given: 0.1 ml of plasminogen was added to the reaction mixture, followed by 0.01 ml of 0.5 M Tris buffer, and 0.01 ml of e-aminocaproic acid (eAcp) containing 0.05 to 25 μmoles. The reaction was initiated with 0.01 ml of tosyl-AMe (100 μmoles per ml), 0.105 ml of 0.1 M Tris·HCl, pH 8.0. The reaction was allowed to proceed for 10 min. The amount of tosyl-AMe cleaved was analyzed as described under “Methods.”

The results obtained are presented in Fig. 5. Concentrations of 0.05 M eAcp represent the minimum concentration at which maximum inhibition occurs.

**Figure 4.** The effect of eAcp (e-ACA) on the activity of plasmin. In these experiments plasminogen was fully converted to plasmin by 80 units per ml of streptokinase prior to addition of the indicated concentrations of eAcp. Therefore, the activation or inhibition observed is due to the direct effect of eAcp on plasmin. The initial concentration of tosyl-AMe was 0.010 M.

The percentage of inhibition was obtained by dividing the initial velocity obtained in the absence of eAcp by the initial velocity obtained in the presence of eAcp on the same concentration of tosyl-AMe and subtracting the result from 100%. There was no preliminary incubation of plasminogen with streptokinase in these experiments. The order of addition of reagents is described in the text. The data of Fig. 4 were added to the experimental data presented in Fig. 5 (△). This curve now represents the inhibition of the conversion of plasminogen to plasmin by eAcp after correcting for the stimulation of eAcp on plasmin.

**Figure 5.** The inhibition of the conversion of plasminogen to plasmin by various concentrations of eAcp (e-ACA). △, the percentage of inhibition was obtained by dividing the initial velocity of plasmin on 0.010 M tosyl-AMe in the presence of a given amount of eAcp by the initial velocity obtained in the absence of eAcp on the same concentration of tosyl-AMe and subtracting the result from 100%. There was no preliminary incubation of plasminogen with streptokinase in these experiments. The order of addition of reagents is described in the text. The data of Fig. 4 were added to the experimental data presented in Fig. 5 (△). This curve now represents the inhibition of the conversion of plasminogen to plasmin by eAcp after correcting for the stimulation of eAcp on plasmin.

**Table I.**

| Assay condition | Initial velocity |
|-----------------|-----------------|
| Experiment 1    | 7.2             |
| No eAcp, 200 units of streptokinase per ml; 10 min of preincubation |         |
| Experiment 2    | 7.3             |
| 0.02 M eAcp, 200 units of streptokinase per ml; 10 min of preincubation |         |
| Experiment 3    | 7.5             |
| No eAcp, 800 units of streptokinase per ml; 10 min of preincubation |         |
| Experiment 4    | 7.5             |
| 0.02 M eAcp, 800 units of streptokinase per ml; 10 min of preincubation |         |

* The concentrations referred to are final assay concentrations. Units of micromoles per min per mg of plasminogen initially added. The initial tosyl-AMe concentration was 8.0 μmoles per ml and the velocity is not maximal under these conditions.

**Effect of Prior Incubation of Plasminogen with Streptokinase on Inhibitory Properties of eAcp**—The effect of prior incubation of plasminogen and streptokinase in the presence of 0.02 M eAcp has been examined at two levels of streptokinase concentration. These experiments were carried out as follows in the indicated orders of addition: 0.1 ml of plasminogen (17 μg), 0.01 ml of eAcp containing 0.05 to 25 μmoles, 0.025 ml of tosyl-AMe (100 μmoles per ml), 0.105 ml of 0.1 M Tris·HCl, pH 8.0. The reaction was initiated with 0.01 ml of streptokinase (225 units) and allowed to incubate for 10 min. The concentration of tosyl-AMe in a 0.2 ml aliquot of the reaction mixture was determined as described under “Methods.” The results obtained are presented in Table I. Clearly, the prior incubation abolishes the inhibitory effect of eAcp at the streptokinase concentrations indicated.

**Table I.**

| Assay condition | Initial velocity |
|-----------------|-----------------|
| Experiment 1    | 7.2             |
| No eAcp, 200 units of streptokinase per ml; 10 min of preincubation |         |
| Experiment 2    | 7.3             |
| 0.02 M eAcp, 200 units of streptokinase per ml; 10 min of preincubation |         |
| Experiment 3    | 7.5             |
| No eAcp, 800 units of streptokinase per ml; 10 min of preincubation |         |
| Experiment 4    | 7.5             |
| 0.02 M eAcp, 800 units of streptokinase per ml; 10 min of preincubation |         |

* The concentrations referred to are final assay concentrations. Units of micromoles per min per mg of plasminogen initially added. The initial tosyl-AMe concentration was 8.0 μmoles per ml and the velocity is not maximal under these conditions.
return to the native value is evident upon dialysis against buffers. Decrease in the $s_{20,w}$ of plasminogen upon addition of ε-Acp and a gross unfolding of the molecule. Since the absence of ε-Acp, the effect of 0.05 M ε-Acp on the sedimentation coefficient ($s_{20,w}$) of plasminogen is given in Table III. There is a decrease in the $s_{20,w}$ value of plasminogen as the streptokinase concentration is increased there is a more rapid conversion of plasminogen into plasmin and thus a higher rate of reaction of plasmin with tosyl-AMe. Consistent with this hypothesis, when plasminogen is incubated with different concentrations of streptokinase for various times prior to addition of tosyl-AMe, there is no dependence of plasmin activity on streptokinase concentration. This effect can best be explained by considering plasminogen (Pg) is activated by streptokinase (SK) to form plasmin (Pm). The plasmin possesses esterase activity on tosyl-AMe (8). In Fig. 2, in the absence of ε-Acp and without prior incubation of plasminogen with streptokinase, the enzymatic activity of plasmin increases as the streptokinase concentration is increased. This effect is in accord with the data presented in this manuscript.

**DISCUSSION**

The studies presented here show that the mechanism of the inhibition of plasmin activity by ε-Acp is complex and involves both inhibition of the conversion of plasminogen to plasmin and stimulation of plasmin at low ε-Acp concentrations and direct inhibition of plasmin activity at high ε-Acp concentrations.

A schematic mechanism for these effects can be illustrated which is in accord with the data presented in this manuscript. This scheme is as follows:

```
P_g + SK → P_m + S → P_m + P
```

In this diagrammatic representation, under normal conditions, plasminogen ($P_g$) is activated by streptokinase (SK) to form plasmin ($P_m$). The plasmin possesses esterase activity on tosyl-AMe ($S$). In Fig. 2, in the absence of ε-Acp and without prior incubation of plasminogen with streptokinase, the enzymatic activity of plasmin increases as the streptokinase concentration is increased. This effect can best be explained by considering plasminogen as the substrate for streptokinase action. Then, as the streptokinase concentration is increased there is a more rapid conversion of plasminogen into plasmin and thus a higher rate of reaction of plasmin with tosyl-AMe. Consistent with this hypothesis, when plasminogen is incubated with different concentrations of streptokinase for various times prior to addition of tosyl-AMe, there is no dependence of plasmin activity on streptokinase concentration given a sufficient time of incubation. This occurs since all the plasminogen present will eventually be converted into plasmin. We have shown this to be the case during the course of these studies and others have published confirmatory data (16). In considering the inhibition data in Fig. 3 at 0.02 M ε-Acp and no prior incubation of plasminogen with streptokinase, all one
can conclude at this point is that plasmin activity is competitively inhibited by eAcp. This inhibition can occur at the level of the conversion of plasminogen to plasmin or eAcp can be a direct inhibitor of plasmin. This question is resolved by coupling the data of Fig. 3 with that of Fig. 4. In Fig. 4 we conclusively demonstrate that eAcp concentrations up to 0.1 M have a stimulatory effect on the activity of plasmin. Thus, the inhibition of the plasminogen to plasmin conversion should in fact be greater than what is observed by performing experiments as in Fig. 3. We have made a rough correction for this stimulatory effect as illustrated in Fig. 5. Clearly, the net inhibition of plasminogen activity which occurs at eAcp concentrations to 0.1 M is due only to the inhibition of the plasminogen to plasmin conversion by eAcp. These effects are illustrated in the scheme presented above. It can be seen that plasminogen can react with eAcp and form a plasminogen-eAcp complex. This complex is refractive to activation by streptokinase. We feel that this complex can be rapidly converted to plasminogen and thus be activated by streptokinase for the following reason. As shown in Table I, the inhibitory effect of 0.02 M eAcp on the conversion of plasminogen to plasmin can be abolished by incubation of plasminogen, 0.02 M eAcp, and streptokinase prior to addition of tosyl-AMC. What must be happening in this case is that streptokinase, in an irreversible manner, reacts with the free plasminogen in the equilibrium, plasminogen + eAcp → plasminogen-eAcp, to form plasmin. Thus, the equilibrium is pulled toward free plasminogen. Given enough time of incubation before tosyl-AMC addition, the plasminogen-eAcp complex will completely dissociate into free plasminogen which will react with the streptokinase present to produce plasmin. Since, under these conditions all the plasminogen present will be converted into plasmin, no inhibition occurs when tosyl-AMC in the presence of eAcp is added, regardless of the presence of eAcp. On the other hand, inhibition at 0.02 M eAcp is only seen when all components of the assay mixture are added together in a definite order (Fig. 3—referred to as “no preincubation”). In this case the rate of plasmin reactivity with tosyl-AMC will be slower in the presence of eAcp than in its absence. All the data collected in this study are consistent with these views.

At this point we feel that we can perhaps explain some discrepancies which appear in the literature concerning the activation of plasminogen by streptokinase. For example, Muramatu et al. (16) proposed that there were two mechanisms of activation of plasminogen by streptokinase. The first mechanism, the details of which are not important for discussion here, occurred at low streptokinase concentrations and was based on the fact that when plasminogen was activated at low streptokinase concentrations (10 units), the activity of plasmin increased with time. In other words, incubation of streptokinase with plasminogen before substrate addition was necessary to obtain full plasmin activity. The second mechanism of activation of plasminogen by streptokinase, according to these authors, occurred at high streptokinase concentrations and was based on the fact that at high streptokinase concentrations (900 units) a much shorter incubation time of streptokinase with plasminogen was necessary before substrate addition to obtain full plasminogen activity. Although their mechanism is consistent with the data obtained, a more simple rate effect can also explain the data. According to our scheme, it is not necessary to propose two mechanisms of action. If one considers the action of streptokinase on plasminogen to be as stated above then at low streptokinase concentrations the rate of activation of plasminogen to plasmin will be slower than at high streptokinase concentrations.

In addition, Muramatu et al. feel that two mechanisms of action for streptokinase activation of plasminogen must exist since eAcp did not inhibit plasmin activity at high concentrations of streptokinase (800 units), whereas inhibition did occur at low concentrations of streptokinase (10 units). In these experiments incubation of plasminogen, eAcp, and streptokinase before substrate addition was performed. Again, we feel, based on our scheme, that it is not necessary to propose a complicated explanation. Clearly at 800 units of streptokinase, according to our scheme, all the plasminogen present is converted to plasmin regardless of the formation of a plasminogen-eAcp inactive complex. At these high streptokinase concentrations the rate of breakdown of this complex is very rapid. On the other hand, at 10 units of streptokinase, the rate of plasminogen-eAcp complex breakdown is sufficiently slow to require much larger prior incubation times. We have lengthened the prior incubation times with 10 units of streptokinase in an effort to demonstrate this point, but the rate of complex dissociation was so slow that inactivation of plasmin occurred, severely complicating our results. However, we have demonstrated that at several streptokinase concentrations above 100 units the inhibition by low eAcp can be abolished, supporting our contention.

Quite a different picture of the inhibition of plasmin activity occurs at high eAcp concentrations. Here, two effects are noted. (a) inhibition of plasmin activity at the level of the conversion of plasminogen to plasmin still occurs and (b) at high concentrations of eAcp is a competitive inhibitor of plasmin activity with a K₁ of 0.32 M. This effect is indicated in the scheme presented above. These results require no prolonged analysis since eAcp is a substrate analogue of L-lysine and L-lysine methyl ester is a substrate of plasmin. Therefore, eAcp probably binds at the substrate binding site of plasmin thereby producing competitive inhibition kinetics. The K₁ of eAcp toward plasmin is very high indicating that eAcp is not strongly bound to the enzyme. We are presently in the process of testing more substrate analogues as inhibitors of plasmin and a report on these studies will shortly appear.

Although the inhibitory effect of high concentrations of eAcp on the enzymatic activity of plasmin appears to be reasonably straightforward, the reason for the inhibitory effect of low concentrations of eAcp on the conversion of plasminogen to plasmin still requires explanation. We feel that this inhibition is due to eAcp causing a freely reversible conformational change in the plasminogen molecule. This conformational alteration produces a plasminogen which is not capable of being acted upon by streptokinase. This conformational alteration is clearly evidenced by analysis of the 0.26 values of plasminogen in the presence and absence of 0.05 M eAcp given in Table III. This conformational alteration is also evidenced in circular dichroism studies. Analysis of the data in Fig. 6 shows that plasminogen in the absence of eAcp possesses some, but not a great deal of, helical structure, as evidenced by the trough at 220 mm. This trough disappears upon addition of 0.05 M eAcp and there is a decrease of the molecular ellipticity. These conditions indicate that there is a considerable loss of structure of plasminogen upon addition of eAcp. The native structure reappears upon removing the eAcp by dialysis suggesting a freely reversible conformational transition.

With regard to the stimulatory effect of low concentrations of eAcp on the activity of plasmin, this is due to a direct effect on plasmin and not on the plasminogen to plasmin conversion. Our
data does not allow us to make a useful evaluation as to the mechanism of this stimulation at this point.

In conclusion, many factors require control when the inhibition of plasmin activity by eAcp is studied. We feel that we have conclusively explained the mechanism of the inhibition by eAcp as well as pointed out the reasons for some discrepancies which exist on this topic.

REFERENCES
1. ROBBINS, K. C., SUMMARIA, L., HSIEH, B., AND SHAN, R. J., J. Biol. Chem., 242, 2333 (1967).
2. SUMMARIA, L., HSIEH, B., AND ROBBINS, K. C., J. Biol. Chem., 242, 4270 (1967).
3. SUMMARIA, L., HSIEH, B., GROSKOPF, W. R., ROBBINS, K. C., AND BARLOW, G. H., J. Biol. Chem., 244, 359 (1969).
4. AILKAIERISCI, N., FLETCHER, A. F., AND SHEERY, S., J. Biol. Chem., 234, 832 (1959).
5. OKAMOTO, S., Keio J. Med., 8, 211 (1959).
6. EGOBLAD, K., Thromb. Diath. Haemorrh., 15, 137 (1966).
7. MAXWELL, R. E., AND ALLEN, D., Nature, 205, 211 (1966).
8. MAXWELL, R. E., AND ALLEN, D., Nature, 205, 211 (1966).
9. DEUTSCH, D., AND MEKTE, E. T., Fed. Proc., 29, 2253 (1970).
10. HESTRIN, S., J. Biol. Chem., 180, 249 (1949).
11. SCHULMAN, S., K., Ultracentrifugation in biochemistry, Academic Press, New York, 1969, p. 82.
12. ONSFILL, L., AND DAVIS, B. J., Disc electrophoresis, Distillation Products Industries, Rochester, New York, 1962.
13. PANYIM, S., AND CHALKELEY, R., Arch. Biochem. Biophys., 130, 327 (1969).
14. WEBER, K., AND OSBORNE, M. J., J. Biol. Chem., 244, 4406 (1969).
15. SUMMARIA, L., AND ROBBINS, K. C., Fed. Proc., 29, 408 (1970).
16. MURAGAI, M., HATAKANO, Y., ONISHI, T., SHIO, T., AND FUJI, S., J. Biochem. (Tokyo), 65, 329 (1969).
The Mechanism of the Inhibition of Plasmin Activity by ε-Aminocaproic Acid
William J. Brockway and Francis J. Castellino

J. Biol. Chem. 1971, 246:4641-4647.

Access the most updated version of this article at http://www.jbc.org/content/246/14/4641

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/14/4641.full.html#ref-list-1