Rotational Diffusion Analysis of the Conformational Alterations Produced in Plasminogen by Certain Antifibrinolytic Amino Acids†

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ABSTRACT: The effect of ε-aminocaproic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid on the rotational diffusion of human and rabbit plasminogen has been studied. Polarization of fluorescence measurements on each plasminogen, lightly labeled with 5-dimethylaminonaphthalene-1-sulfonyl chloride, indicates that the rotational relaxation time for human plasminogen is 262 ± 10 nsec, whereas the value for rabbit plasminogen is 284 ± 10 nsec. These data suggest a rigid structure for each plasminogen which significantly deviates from an anhydrous sphere of the same molar volume as plasminogen. Saturation of the single binding site on plasminogen for the fibrinolytic inhibitors ε-aminocaproic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid, with these inhibitors, leads to a gross reduction of the rotational relaxation times of the proteins. Values of 158 ± 8 and 174 ± 8 nsec are obtained for human and rabbit plasminogen, indicating that complete binding of these inhibitors causes a conformational shift in plasminogen to a less rigid structure. These data reinforce our previous studies on certain inhibitor-induced conformational alterations of various plasminogens. Titration of the reduction of the initial polarization accompanying binding of these inhibitors to each plasminogen allows estimates of the inhibitor dissociation constants to be made. The values obtained are in agreement with the accepted dissociation constants of these same inhibitors to human plasminogen.

There have been many reports through the years concerning synthetic inhibitors of fibrinolysis. Okamoto and colleagues have demonstrated that various aromatic sulfhydryl compounds as well as various homologs of ε-aminocaproic acid, *p*-aminomethylbenzoic acid, and 4-aminomethylcyclohexane-1-carboxylic acid were antifibrinolytic. These investigators have rated the effectiveness of these compounds as fibrinolytic inhibitors and found that ε-aminocaproic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid were among the most potent (for a review see Okamoto *et al.*, 1968). More recently, Loeffler *et al.* (1970) investigated many bridged bicyclic and polycyclic amino acids as potential fibrinolytic inhibitors and found several to be much more inhibitory than the classical ε-aminocaproic acid.

The mechanism of action of these synthetic inhibitors has been speculated. Alkjaersig *et al.* (1959) demonstrated that ε-aminocaproic acid inhibits the conversion of plasminogen to plasmin, thus manifesting its antifibrinolytic activity. Maxwell and Allen (1966) and Maxwell *et al.* (1968) have shown that ε-aminocaproic acid alters the structure of fibrin, leading to a diminished capacity for digestion by plasmin. Also, it has been postulated that ε-aminocaproic acid is an antiplasmin (Okamoto, 1959). Our previous studies (Brockway and Castellino, 1971, 1972b) suggest that ε-aminocaproic acid and several related compounds inhibit the interaction of streptokinase and human plasminogen leading to inhibition of the formation of plasminogen activator. All the above observations are consistent with the loss of the observed fibrinolytic activity of plasma in the presence of compounds such as ε-aminocaproic acid.

Alkjaersig *et al.* (1959) initially demonstrated that binding of ε-aminocaproic acid to human plasminogen caused a conformational alteration in the protein. This conformational alteration of human plasminogen is also induced by *trans*-4-aminomethylcyclohexane-1-carboxylic acid and is correlated with the formation of a 1:1 complex of these compounds with plasminogen (Abiko *et al.*, 1969). We have demonstrated by sedimentation velocity analysis and circular dichroism measurements that this conformational alteration in plasminogen is a general property of several species of plasminogen and is induced by a wide variety of aliphatic and aromatic amino acid analogs of ε-aminocaproic acid. We wished to further investigate this important conformational alteration of plasminogen and to obtain additional information on the gross conformation of native and inhibitor-bound plasminogen. In order to gain some insight into these questions we have chosen to study the fluorescence polarization of free and inhibitor-bound plasminogen. This article represents a report of these studies.

Materials and Methods

Proteins. Human plasminogen was prepared from Cohn

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fraction III, donated by Cutter Laboratories, by gradient elution from Sepharose 4B–L-lysine affinity chromatography columns, as previously described (Brockway and Castellino, 1972a). Rabbit plasminogen was prepared from rabbit plasma as described earlier (Sodetz *et al.*, 1972). In each case plasminogen fraction 2 from the affinity columns was used as the source of plasminogen for these experiments.

Conjugates of 5-dimethylaminonaphthalene-1-sulfonyl chloride[1] and each plasminogen were prepared by dissolving the proteins in 0.1 M phosphate buffer, pH 7.5, containing 5% acetone. The protein concentration was usually between 4 and 7 mg/ml. A small aliquot of a stock solution of 20% DnsCl in acetone was then added such that the molar ratio of DnsCl to protein in solution was 10:1 to 3:1. The solution was stirred overnight at 4° and dialyzed against several changes of 0.05 M NH_4HCO_3. In order to remove traces of free dye, the dialysate was percolated through a column of Dowex 2 (HCO_3^-) resin equilibrated with 0.05 M NH_4HCO_3. The column eluate was then concentrated by pressure ultrafiltration to a small volume and dialyzed against 0.1 M phosphate buffer, pH 7.5, for polarization measurements. The degree of labeling of each plasminogen was determined from optical density measurements at 280 and 345 nm. The extinction coefficients were: 4.3×10^3 M^{-1} cm^{-1} for the bound Dns (Weber, 1952), 1.39×10^5 M^{-1} cm^{-1} for human plasminogen (Robbins *et al.*, 1968), and 1.5×10^5 M^{-1} cm^{-1} for rabbit plasminogen.

Flourescence polarization measurements were carried out on an Aminco-Bowman spectrophotofluorometer. Interference and cut-off filters were interposed in the excitation and emission channels to eliminate light from second-order diffraction and scattering. Excitation was carried out at a wavelength of 346 nm and emission was filtered through Corning CS-3-72 filters and monitored at 515 nm. For polarization of the light, Polacoat 105 polarizers with an effective range of 200–800 nm were used. The polarization of fluorescence (P) for each sample was measured at crossed (I_{EB}, I_{BE}) and parallel (I_{EE}, I_{BB}) positions of the polarizers. The value of P can be obtained from eq 1 (Price *et al.*, 1962). The subscripts denote the orientation of the electrical vector of the light which passes the excitation and emission channels. E is the vertical and B is the horizontal orientation.

$$P = \frac{I_{EE} - I_{EB}(I_{BE}/I_{BB})}{I_{EE} + I_{EB}(I_{BE}/I_{BB})} \quad (1)$$

Rotational relaxation times (ρ_h) of each plasminogen in the presence and absence of 0.1 M ε-aminocaproic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid were obtained from the equation of Perrin (1926) where P_0 is the limiting polarization, τ is the lifetime of the fluorescent state, V is the molar volume of the protein, and T and η are the temperature and viscosity of the solvent. Plots of $[(1/P) - (1/3)]$ *vs.* T/η yield the molar volume of the protein with prior knowledge of τ. The value of ρ_h can then be calculated from

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right) + \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(\frac{\tau R}{V}\right)\left(\frac{T}{\eta}\right) \quad (2)$$

$$\rho_h = \frac{3\eta V}{RT} \quad (3)$$

[1] Abbreviation used is: DnsCl, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

In the experiments reported here we have varied the value of T/η by additions of glycerol or powdered sucrose to the solutions of interest. The temperature of all experiments was 25°. The solvent in all cases was 0.1 M phosphate, pH 7.5, plus the desired concentration of inhibitor.

Fluorescence lifetimes of the labeled plasminogens were determined by pulsed excitation of the fluorescent material followed by monophoton counting of the emission. The apparatus (Amata and Ludwig, 1967) as well as the excitation source (D'Alessio and Ludwig, 1964) were as described. The excitation wavelength was isolated by a Bausch and Lomb monochromator. Excitation and emission wavelengths were identical with those used for fluorescence polarization measurements.

Sedimentation velocity analyses of both labeled plasminogens in the presence and absence of both fibrinolytic inhibitors were performed at 20° on the Beckman Model E analytical ultracentrifuge. In order that low protein concentrations could be employed, scanner optics were used at 280 nm. The solvents used were identical with those for fluorescence polarization measurements. All sedimentation coefficients were corrected to the density and viscosity of water at 20°. Calculations of dissociation constants of the inhibitors to each plasminogen from sedimentation velocity analyses were performed as previously described (Brockway and Castellino, 1972a).

Chemicals. ε-Aminocaproic acid was purchased from Aldrich Chemical Co. *trans*-4-Aminomethylcyclohexane-1-carboxylic acid was prepared by the procedure of Naito *et al.* (1968). DnsCl was a product of Pierce Chemical Co. All other reagents were the best commercially available.

Results

Isolation and analysis of the dansylated plasminogens indicated that human plasminogen incorporated 0.6 mol of Dns/mol of plasminogen and rabbit plasminogen incorporated 0.52 mol of Dns/mol of plasminogen. This low level of modification did not alter the ability of each plasminogen to become activated to plasmin by streptokinase or urokinase. Values of the $s^0_{20,w}$ of each plasminogen as well as the effect of ε-aminocaproic acid on the $s^0_{20,w}$ of each plasminogen were not altered by dansylation. The dissociation constant of ε-aminocaproic acid for Dns human and rabbit plasminogen was measured by ultracentrifugal analysis (Brockway and Castellino, 1972a) and found to be very similar to unlabeled plasminogen. A summary of these values can be found in Table I.

Figure 1 illustrates the isothermal fluorescence polarization data of human plasminogen in the absence of ε-aminocaproic acid and in the presence of sufficient quantities of this inhibitor required for saturation of the single inhibitor binding site on plasminogen. Figure 2 illustrates the same data for rabbit plasminogen. The plots for *trans*-4-aminomethylcyclohexane-1-carboxylic acid were purposely omitted from presentation since they were practically identical with those for ε-aminocaproic acid-saturated plasminogen. A summary of the mean harmonic rotational relaxation times (ρ_h) for each case is presented in Table II.

In Figure 3 we have plotted the decrease in the initial polarization of human plasminogen as a function of added ε-aminocaproic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid. Figure 4 presents the same data for rabbit plasminogen. At the midpoint of these plots the relationship between the inhibitor dissociation constants (K_I) and the total

TABLE I: A Comparison of Some Structural Parameters for Native and Lightly Dansylated Plasminogens.

Species	Degree of labeling (mol/mol)	$s^0_{20,w}$ (S) N[a]	$s^0_{20,w}$ (S) I[b]	$K_I \times 10^3$ (M) ε-Aminocaproic Acid	τ (nsec)
Native rabbit plasminogen		5.48 ± 0.06	4.60 ± 0.05	3.12 ± 0.2	
Dansylated rabbit plasminogen	0.52	5.53 ± 0.07	4.67 ± 0.05	2.48 ± 0.2	12.9 ± 0.4[c] 12.9 ± 0.4[d] 12.7 ± 0.4[e]
Native human plasminogen		5.12 ± 0.05	4.10 ± 0.05	0.45 ± 0.08	
Dansylated human plasminogen	0.60	5.08 ± 0.06	4.13 ± 0.05	0.40 ± 0.09	12.8 ± 0.4[c] 12.9 ± 0.4[d] 12.6 ± 0.4[e]

[a] Value for plasminogen in 0.1 M phosphate, pH 7.5. [b] Value for plasminogen in 0.1 M phosphate–0.1 M ε-aminocaproic acid, pH 7.5. [c] Value for Dns–plasminogen in 0.1 M phosphate, pH 7.5. This value is independent of the glycerol concentration. [d] Value for Dns–plasminogen in 0.1 M phosphate–0.1 M ε-aminocaproic acid, pH 7.5. This value is independent of the glycerol concentration. [e] Value for Dns–plasminogen in 0.1 M phosphate–0.1 M *trans*-4-aminomethylcyclohexane-1-carboxylic acid, pH 7.5. This value is independent of the glycerol concentration.

inhibitor concentration ($[I_T]$) is (Brockway and Castellino, 1972a)

$$K_I = [I_T] - [P_g \cdot I]$$

where $[P_g \cdot I]$ is the concentration of the plasminogen–inhibitor complex and is calculated with prior knowledge of the plasminogen concentration. We estimate a dissociation constant for ε-aminocaproic acid of 5×10^{-4} M to human plasminogen and 3.2×10^{-3} M to rabbit plasminogen. The K_I values for *trans*-4-aminomethylcyclohexane-1-carboxylic acid are 7.2×10^{-5} M to human plasminogen and 4.1×10^{-4} M to rabbit plasminogen.

Discussion

The data presented in Table II demonstrate that native human and rabbit plasminogen possess hydrodynamic properties which significantly deviate from those expected for an anhydrous sphere. The translational frictional ratios (f/f_{min}) in each case are significantly higher than unity. The same conclusion is obvious from the observed larger rotational relaxation times of each plasminogen compared to an anhydrous sphere of the same molar volume as plasminogen. This observed deviation from anhydrous symmetry can be explained by hydration or conformational factors, or by a combination of these factors. Hydration will be an important factor but any reasonable assumption as to the extent of hydration of native plasminogen still requires a very significant contribution from conformational considerations.

More importantly, it can be seen from Table II that addition of the fibrinolytic inhibitors, ε-aminocaproic acid and *trans*-4-aminomethylcyclohexane-1-carboxylic acid, to either plasminogen leads to decreases in the rotational diffusion properties of these proteins, as revealed by the decreases in the rotational relaxation times. However, increases in the translational frictional ratios of each plasminogen are observed in the presence of these compounds. At first glance, this would appear to be a contradiction since it would be expected that changes in the rotational relaxation times of plasminogen should parallel the changes in the translational frictional ratios. The observed effects can be reconciled by citing as a precedent studies on the effect of acid on the structure of bovine serum albumin. In this case, addition of acid caused a decrease in the $s^0_{20,w}$ and a concomitant decrease in the rotational relaxation times of the protein, exactly as observed in the studies reported in this article. This effect was found to be consistent with an acid-induced loss in internal

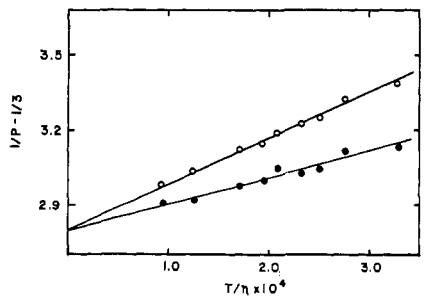

FIGURE 1: Fluorescence polarization plots of Dns–human plasminogen: (○) human plasminogen in 0.1 M phosphate, pH 7.5; (●) human plasminogen in 0.1 M phosphate–0.1 M ε-aminocaproic acid, pH 7.5. In each case the protein concentration was 9.5×10^{-6} M and the temperature was 25°.

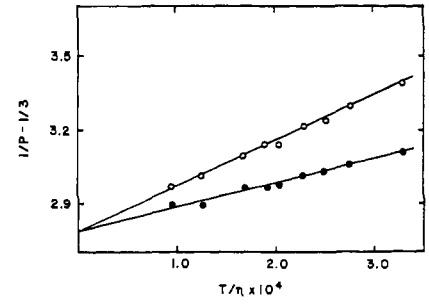

FIGURE 2: Fluorescence polarization plots of Dns–rabbit plasminogen: (○) rabbit plasminogen in 0.1 M phosphate, pH 7.5; (●) rabbit plasminogen in 0.1 M phosphate–0.1 M ε-aminocaproic acid, pH 7.5. In each case the protein concentration was 9.8×10^{-6} M and the temperature was 25°.

TABLE II: Hydrodynamic Parameters of Human and Rabbit Plasminogens Obtained from Fluorescence Polarization Measurements.

Species	ρ_h (nsec), 25°	ρ_0[c] (nsec), 25°	ρ_h/ρ_0	f/f (min)[d]
Human plasminogen	262 ± 10	71	3.69	1.41
Human plasminogen[a] + ε-aminocaproic acid	156 ± 8	71	2.20	1.75
Human plasminogen[b] + *trans*-4-aminomethylcyclohexane-1-carboxylic acid	160 ± 8	71	2.25	1.75
Rabbit plasminogen	284 ± 10	76.2	3.73	1.38
Rabbit plasminogen[a] + ε-aminocaproic acid	174 ± 8	76.2	2.28	1.64
Rabbit plasminogen[b] + *trans*-4-aminomethylcyclohexane-1-carboxylic acid	174 ± 8	76.2	2.28	1.64

[a] Refers to human or rabbit plasminogen in 0.1 M phosphate–0.1 M ε-aminocaproic acid. [b] Refers to human or rabbit plasminogen in 0.1 M phosphate–0.1 M *trans*-4-aminomethylcyclohexane-1-carboxylic acid. [c] Calculated from $\rho_0 = 3\eta V/RT$, where ρ_0 is the mean harmonic rotational relaxation time of an equivalent sphere of the same apparent molar volume of plasminogen. The value of V for human plasminogen was calculated to be 58,630 cm³/mol based on a mol wt of 82,000 and $\bar{v}$ of 0.715 cm³/g. The value of V for rabbit plasminogen was calculated to be 62,920 cm³/mol based on a mol wt of 88,000 and a $\bar{v}$ of 0.715 cm³/g. [d] Calculated from $f/f_{min} = D_{max}{}^0/D_0$. $D_{max}{}^0$ was calculated as found in Tanford (1967). D^0 was calculated based on the molecular weight and $s^0_{20,w}$ for each plasminogen under the conditions of interest.

rigidity in the molecule (Harrington *et al.*, 1956; Weber and Young, 1964). Using these studies as a model, we can conclude that binding of the fibrinolytic inhibitors to plasminogen induces a loosening of the internal structure of the protein and leads to the adaptation of a more flexible conformation in plasminogen. This less rigid conformation allows additional degrees of rotational freedom in plasminogen which are not allowed by the native conformation. The increase in the value of f/f_{min} upon binding of these amino acids indicates that this more flexible conformation is also more asymmetrical than the conformation of native plasminogen. A model such as this is consistent with the observed rotational and translational properties of the protein. Although we are proposing a reasonably specific model based on our results, it should be remembered that there are many factors potentially capable of influencing fluorescence depolarization measurements. Therefore, this model would only be strictly correct in the absence of complicating and unmeasurable external contributions to the system.

In considering the conformation adapted by plasminogen upon inhibitor binding we have omitted consideration of possible changes in hydration of plasminogen accompanying binding. The primary reason for this is that unreasonable assumptions would have to be made to explain the observed hydrodynamic differences solely on the basis of hydration. Also, consideration of the translational *vs.* the rotational changes in plasminogen accompanying binding of inhibitors only in terms of hydration differences would be inconsistent to the data obtained. For example, if we were to conclude that the observed increase in f/f_{min} of plasminogen following inhibitor binding were due to an increase in hydration of the protein then the value of ρ_h would also be required to increase. This is clearly not the case here. Although we are not ruling out small contributions for differences in hydration of inhibitor-bound *vs.* free plasminogen, these contributions clearly are not of primary importance and lead only to small alterations in the value of the molar volume. A further complication to these results can arise if the action of the inhibitor on plasminogen is affected by the concentration of glycerol. The fact that the plots of $[(1/P) - (1/3)]$ *vs.* T/η for Dns–plasminogen in the presence of the inhibitors are linear

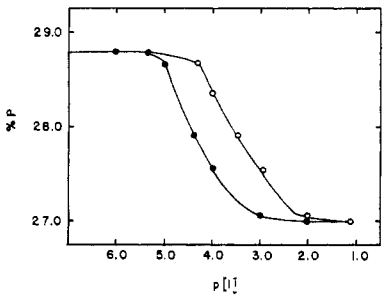

FIGURE 3: The effect of inhibitors on the initial polarization of human plasminogen: (●) *trans*-4-aminomethylcyclohexane-1-carboxylic acid; (○) ε-aminocaproic acid. p[I] represents the negative logarithm of the inhibitor concentration. The protein concentration was 8.7×10^{-6} M and the temperature was 25°.

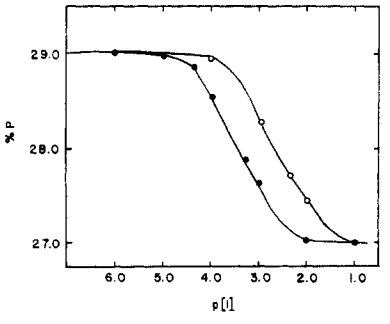

FIGURE 4: The effect of inhibitors on the initial polarization of rabbit plasminogen: (●) *trans*-4-aminomethylcyclohexane-1-carboxylic acid; (○) ε-aminocaproic acid. p[I] represents the negative logarithm of the inhibitor concentration. The protein concentration was 9.1×10^{-6} M and the temperature was 25°.

appears to rule out this possibility. The only manner in which this could be conclusively verified would be to perform inhibitor binding experiments on plasminogen at high levels of glycerol. However, many problems are apparent in this type of measurement and there is a good possibility that the data obtained in experiments of this type would lead to no refinement of the conclusions.

As a corollary to the primary intention of the measurements reported here, we have utilized the inhibitor-induced decrease in the initial polarization of plasminogen to measure the dissociation constants of these amino acids to plasminogen. Titration data on the decrease in initial polarization as a function of the amount of each inhibitor added to human and rabbit plasminogen are presented in Figures 3 and 4. The dissociation constants obtained from the midpoints of each curve are in complete agreement with the values we have obtained earlier by analyzing similar changes in the $s^0_{20,w}$ of plasminogens (Brockway and Castellino, 1972a). Plots of these data based on the principle of additivity of anisotropies (Weber, 1952), as outlined by Malencik and Anderson (1972), gave exactly the same dissociation constants as were obtained from Figures 3 and 4. Further, these dissociation constants are consistent with the values obtained by Abiko *et al.* (1969) for the same two amino acids to human plasminogen. The similarities in dissociation constants obtained in these two independent studies demonstrate that the inhibitor-induced loss of polarization of labeled plasminogen is a direct result of inhibitor binding.

In order to properly interpret the polarization experiments, it was necessary to demonstrate that the fluorescent-labeled plasminogens were conformationally similar to the native unlabeled plasminogen. Subsequent to labeling we have shown that the resulting plasminogens possessed molecular weights, $s^0_{20,w}$ values, immunological cross-reactivities, and activation properties very similar to unlabeled plasminogens. Further, labeling of each plasminogen did not lead to aggregation of the molecule. Therefore, the small amount of labeling most likely does not alter the extrapolation of the studies on labeled plasminogen to native plasminogen.

In conclusion, the studies reported here have further contributed to the analysis of the conformational alteration induced in plasminogen by certain antifibrinolytic amino acids. A model of the conformational change, based on studies of two plasminogens with two representative inhibitors, is consistent with an inhibitor-induced alteration of plasminogen to a less internally rigid, more asymmetric shape. This gross conformational change is likely the basis of the inhibition of the streptokinase-mediated activation of plasminogen in the presence of these agents.

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