Hydrodynamic Studies on the Streptokinase Complexes of Human Plasminogen, Val_{442}-Plasminogen, Plasmin, and the Plasmin-Derived Light (B) Chain†

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ABSTRACT: Sedimentation velocity and sedimentation equilibrium studies have been carried out on the Glu- and Lys-plasminogen–streptokinase complexes as well as on the complexes formed by Val_{442}-plasmin and the light (B) chain of plasmin. Sedimentation equilibrium molecular weights are consistent with a 1 to 1 molar complex in all cases and give values consistent with the differences in size of the plasminogen moieties. Sedimentation velocity determinations in the presence of protease inhibitors give values consistent with the conformational differences already reported for the Glu- and Lys-plasminogen molecules. However, unlike Glu-plasminogen, the addition of ε-aminocaproic acid or lysine does not alter the conformation of the Glu-plasminogen complex. The values of the sedimentation coefficient and the molecular weight of the plasmin and the Val_{442}-plasmin–streptokinase complexes increase to those of a dimer when determined in the absence of active-site inhibitors but return to monomer values when these inhibitors are added. Thus, dimer formation requires the presence of an available active site in at least one of the two molecules involved and is reversible.

The development of enzymatic activity by streptokinase requires the formation of a 1 to 1 molar complex with plasminogen which induces a conformational change in the plasminogen molecule with the concomitant development of an active site (McClintock & Bell, 1971; Reddy & Markus, 1972). This complex can then react with other plasminogen molecules to cleave a single arginyl–valyl bond and form the active protease plasmin (Summaria et al., 1967). While initially the activity is due entirely to the complex with plasminogen, later it will be due to the complex with plasmin (Reddy & Markus, 1972). At the same time that the plasminogen is being modified, there is also a specific fragmentation of the streptokinase moiety into at least five major identifiable fragments, (Siefring & Castellino, 1976). While the hydrodynamic properties of the complex have been reported by several investigators (Davies et al., 1961; DeRenzo et al., 1963; Ling et al., 1966; Summaria et al., 1970), these results did not define the form being investigated which was most likely the complex with plasmin. The discovery that plasminogen can exist with the N-terminal amino acid being either glutamine or lysine (Wiman & Wallen, 1973) and that the complex can form with Val_{442}-plasminogen (Wohl et al., 1980) and with the light (B) chain of plasmin (Summaria & Robbins, 1976) suggested a reexamination of the hydrodynamic properties of the entire family of complexes. This reexamination should place special emphasis on the molecular form of the plasminogen and the streptokinase present in the complex both before and after the centrifugal analysis.

Experimental Procedures

Materials. Streptokinase was a gift from Dr. R. Lundên (AB Kabi, Stockholm, Sweden) and urokinase a gift from Abbott Laboratories (North Chicago, IL). *p*-Nitrophenyl *p*-guanidinobenzoate (NPGB) and elastase were purchased from Sigma (St. Louis, MO). Leupeptin was purchased from the Peptide Research Institute (Osaka, Japan) and the aprotinin from FBA Pharmaceuticals (New York, NY). ε-Aminocaproic acid (EACA) was purchased from Aldrich Chemicals (Milwaukee, WI).

Preparation of Plasmin(ogen). Human Glu- and Lys-plasminogens were prepared from Cohn fraction III and fraction $III_{2,3}$ by a modified affinity chromatography method using L-lysine–Sepharose (Summaria et al., 1972).

Human Lys-plasmin was prepared by activating Lys-plasminogen with urokinase in a 25% glycerol buffer by a procedure previously described (Robbins et al., 1967).

Val_{442}-plasminogen was prepared by digestion of Lys-plasminogen with elastase as described by Sottrup-Jensen et al. (1978).

Streptokinase Complexes. The Glu- and Lys-plasminogen–streptokinase complexes were prepared in the presence of 0.001 M NPGB by the addition of equimolar amounts of streptokinase and either Glu- or Lys-plasminogen. The mixture was incubated at 0 °C for 2 h after the NPGB concentration was brought to 0.002 M. The complex was precipitated with ammonium sulfate, centrifuged, and redissolved. The plasmin–streptokinase complex was prepared by the same procedure as the Lys-plasminogen complex except in the absence of NPGB. In some experiments, NPGB was added after the plasmin complex was redissolved.

The Val_{442}-plasmin–streptokinase complex was prepared by reacting equimolar quantities of Val_{442}-plasminogen and streptokinase for 10 min at 0 °C. One aliquot of the formed complex was immediately incubated with 0.001 M NPGB.

The equimolar light B chain–streptokinase complex was prepared from the plasmin–streptokinase complex by partial reduction and alkylation as previously described (Summaria & Robbins, 1976).

Proteolytic and Plasminogen Activator Activities. Proteolytic and plasminogen activator activities were determined on a casein substrate as previously described (Summaria & Robbins, 1976). The plasminogen activator activity assay used Glu-plasminogen as the substrate. Glu- and Lys-plasminogen had activities of 28 IU/mg of protein; the specific activity of Val_{442}-plasminogen was 61 IU/mg of protein. The plasminogen activator activities were 72000, 48000, and 160000 IU/mg of protein for the Lys-plasmin–, Val_{442}-plasmin–, and light B chain–streptokinase complexes, respectively.

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Table I: Hydrodynamic Properties of Complex Components (Inhibited)[a]

sample	$s^0_{20,w}$ (S)	$\bar{v}$ (mL/g)	mol wt	Stokes radius (Å)	frictional ratio, f/f_0
SK	3.11	0.719	52 700	41.4	1.47
Glu-Plg[b]	5.87	0.715	87 900	37.6	1.28
Glu-Plg[c]	4.40		87 500	49.9	1.70
Lys-Plg[b]	4.30	0.715	82 500	48.3	1.68
Lys-Plg[c]	4.30				
Lys-Pln[b]	4.10	0.715	82 900	50.8	1.76
Lys-Pln[c]	4.10				
Val_{442}-Plg[b]	2.70	0.720	37 800	34.5	1.55
Pln B chain[b]	2.32	0.720	25 100	26.7	1.37

[a] Abbreviations: SK, streptokinase; Plg, plasminogen, Pln, plasmin. [b] Analyzed in 0.1 M phosphate buffer, pH 6.0, containing 0.1 M NaCl, 0.001 M EACA, and 0.001 M NPGB. [c] Same as in footnote *b* with the addition of 0.1 M EACA.

Polyacrylamide Gel Electrophoresis (PAGE). Both sodium dodecyl sulfate (SDS)– and non-SDS–PAGE were performed on the samples recovered from the ultracentrifuge cell to ensure that their integrity was not altered. The method was as previously described (Summaria & Robbins, 1976).

Sedimentation Analysis. All experiments were made in a Spinco Model E analytical ultracentrifuge equipped with both schlieren and photoelectric scanning optics. Sedimentation velocity experiments were all at 56 000 rpm while sedimentation equilibrium experiments were at speeds ranging from 8000 to 14 000 rpm depending on molecular size. All runs were at, or near, room temperature. Low concentration runs using photoelectric scanning optics were scanned at 280 nm. Calculations of sedimentation coefficients and sedimentation equilibrium molecular weights were made by using equations described by Schachman (1957). The frictional ratio, f/f_0, and the Stokes radius, R_s, were calculated from the sedimentation coefficient and the equilibrium molecular weight by using equations derived by Svedberg & Peterson (1959) an Mann et al. (1981), respectively.

Partial specific volumes, $\bar{v}$, were determined on a precision densimeter (H. Paar, Graz, Austria) by using the mechanical oscillator technique (Kratky et al., 1973). The values are presented in Tables I and II.

Results

The hydrodynamic properties of the starting components for the complexes are shown in Table I. All were analyzed in the presence of 0.001 M NPGB. In all cases, the molecular form was confirmed by removing the material from the ultracentrifuge at the conclusion of the sedimentation run and subjecting the solution to both SDS– and non-SDS–PAGE. The addition of 0.1 M EACA to the buffer confirmed the results of others (Castellino & Violand, 1979; Markus et al., 1979) by changing only the sedimentation coefficient and the conformation of the Glu form of plasminogen (Table I).

Table II shows the hydrodynamic properties of the complexes of plasminogen and its derivatives with streptokinase. All samples are in buffer containing 0.001 M NPGB. As with the starting components, the integrity of the molecular form was confirmed by PAGE at the conclusion of the experiment. Addition of 0.1 M EACA had no effect on the measured sedimentation coefficient of any of the molecular forms (Table II).

When the NPGB concentration was reduced in half to 0.0005 M, an increase in heterogeneity of the sample was noted in the schlieren pattern, and the peak became broad and appeared to be a partial separation of two components. PAGE, on the two recovered samples, indicated a partial transformation to the Lys-plasmin complex in the sample at the lower NPGB level. However, the sedimentation coefficient had increased rather than decreased as would be expected for a Glu–Lys transformation in the complex (Table II).

When a sample of Lys-plasmin–streptokinase complex was analyzed with and without the presence of 0.001 M NPGB, the sedimentation coefficient increased from 4.8 to 10.1 S when NPGB was absent. At the conclusion of the sedimentation run, the sample without NPGB was brought to 0.001 M NPGB, and on recentrifugation, the sedimentation coefficient reverted to 4.8, indicating that this was a reversible process. The sedimentation equilibrium molecular weight was found to be 307 000 in the absence of NPGB, indicating a dimerization of the molecule. The same type of results was obtained with the Val_{442}-plasmin complex with and without NPGB. The sedimentation coefficient increased from 3.86 to 7.30 S and the molecular weight from 100 000 to 174 000 in the absence of NPGB and once again was reversible. Similar experiments with Lys-plasmin, light B chain, and the light B chain–streptokinase complex did not show this dimerization when NPGB was absent. The hydrodynamic properties of the complexes without NPGB are summarized in Table III.

On SDS–PAGE, the uninhibited samples differed from the inhibited samples in that the streptokinase was no longer intact but was in the form of fragment 2 (Siefring & Castellino, 1976). There was no evidence of dimer.

Figure 1 shows the concentration dependence of the sedimentation coefficient for the Lys-plasmin–streptokinase complex with and without 0.1 M EACA but in the absence of NPGB. In the absence of EACA, the dissociation of dimer to monomer becomes evident at about 2 mg/mL, while in the presence of EACA the dissociation becomes apparent at a higher concentration, about 5 mg/mL. The Val_{442}-plasmin–streptokinase complex behaved in a similar manner, with the

Table II: Hydrodynamic Properties of Plasminogen–Streptokinase Complexes (Inhibited)

sample	$s^0_{20,w}$ (S)	$\bar{v}$ (mL/g)	mol wt	Stokes radius (Å)	frictional ratio, f/f_0
Glu-Plg–SK[a]	5.90	0.715	156 000	66.4	1.81
Glu-Plg–SK[b]	5.85				
Lys-Plg–SK[a]	4.80	0.721	139 700	71.5	2.13
Lys-Plg–SK[b]	4.80				
Lys-Pln–SK[a]	4.70	0.721	139 600	73.0	2.11
Lys-Pln–SK[b]	4.70				
Val_{442}-Pln–SK[a]	3.86	0.720	100 000	63.9	2.07
Val_{442}-Pln–SK[b]	3.90				
Pln B chain–SK[a]	3.79	0.730	73 900	46.4	1.66
Pln B chain–SK[b]	3.84				

[a] Analyzed in 0.1 M phosphate buffer, pH 6.0, containing 0.1 M NaCl, 0.001 M EACA, and 0.001 M NPGB. [b] Same as in footnote *a* with the addition of 0.1 M EACA.

Table III: Hydrodynamic Properties of Plasmin–Streptokinase Complexes (Uninhibited)

sample[a]	$s^0_{20,w}$ (S)	mol wt	Stokes radius (Å)	frictional ratio, f/f_0
Lys-Pln-SK	10.1	307 000	74.7	1.67
Val_{442}-Pln-SK	7.3	174 000	58.8	1.59
Pln B chain-SK	3.8	74 200	46.4	1.66

[a] All samples were at a concentration of approximately 2.5 mg/mL in 0.1 M phosphate buffer, pH 6.0, containing 0.1 M NaCl and 0.001 M EACA.

Table IV: Effect of Different Inhibitors on Dimerization of the Lys-Plasmin-Streptokinase Complex

inhibitor	concn (M)	$s_{20,w}$ (S)
none		10.2
leupeptin	6×10^{-5}	10.0
	6×10^{-3}	7.2
	1×10^{-2}	6.5
aprotinin	3.2×10^{-5}	6.4
	1×10^{-4}	6.0
DFP	1×10^{-2}	5.5

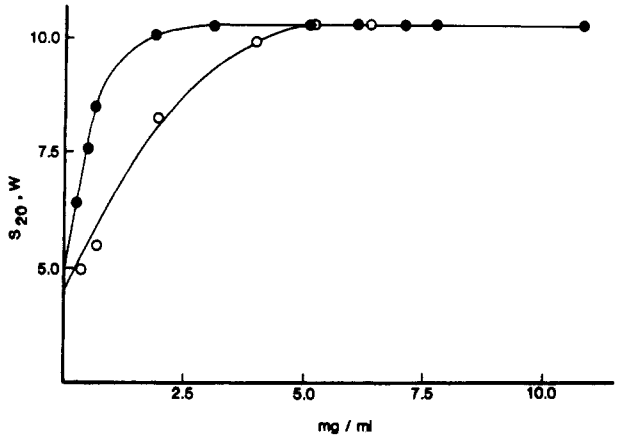

FIGURE 1: Concentration dependence of the sedimentation coefficient of the Lys-plasmin–streptokinase complex in the absence of active-site inhibitors; (●) no EACA; (○) in the presence of 0.1 M EACA.

dissociation starting at about the same concentrations.

The use of other inhibitors such as leupeptin, aprotinin, or diisopropyl fluorophosphate (DFP) had the same effect as seen with NPGB. The concentration of the different inhibitors required to inhibit dimer formation was dependent on its efficiency in blocking the active site (Table IV).

Discussion

The Glu- and Lys-plasminogen–streptokinase complexes show a significant difference in their $s_{20,w}$ value. This is due to a difference in conformation of the two complexes similar to the difference already described for the two plasminogens by others (Castellino & Violand, 1979; Markus et al., 1979) and is reflected in the calculated Stokes radii and the f/f_0 for the two forms (Table II). However, there is a change in the size, shape, and perhaps conformation of all the complexes; in all cases, the molecules increase in both their Stokes radius and their frictional ratio. This increase appears to be primarily associated with the streptokinase moiety since it approximates the same value for all the complexes. The Glu-plasminogen–streptokinase complex differs from Glu-plasminogen in the effect of EACA or lysine on the conformation. Unlike the conformational change of Glu-Plasminogen brought about by addition of EACA or lysine (Castellino & Violand, 1979; Markus et al., (1979), there is no effect on the Glu complex (Table III). Therefore, in the form of an activator rather than a substrate, the conformation becomes more rigid. It is possible that that the streptokinase prevents EACA binding to a site necessary for the conformational change or that the streptokinase attaches to the plasminogen in such a manner that even with EACA binding the conformational change cannot occur. The molecular weights of all the complexes are consistent with 1:1 molar complexes, and the differences in molecular weight are consistent with the differences in the size of the plasminogen moieties.

The Lys-plasmin–streptokinase complex behaves in an interesting manner. When the active site is blocked with active-site titrants such as NPGB or DFP or with inhibitors such as aprotinin and leupeptin, its behavior is identical with that of the Lys-plasminogen–streptokinase complex (Table II). However, when this complex is analyzed in the absence of such reagents so that the active site is available, the hydrodynamic properties change. The sedimentation coefficient increases from 4.7 to 10.1 S, and the sedimentation equilibrium molecular weight increases to 300 000, indicating that the molecule is a dimer. This dimerization is dependent on the presence of the active site and can be reversed by the addition of active-site blockers or by very high dilution (Figure 1). The difference in the dissociation curve in the presence of EACA reflects the fact that EACA is an inhibitor for plasmin but is not nearly as effective as NPGB and the other inhibitors cited previously.

The heterogeneity observed in the Glu-plasminogen complex on lowering the NPGB concentration can be attributed to the presence of a mixture of the Glu-plasminogen complex with the Lys-plasmin complex in the dimer f orm, the latter being generated either by the dissociation of NPGB from some of the active sites or by the presence of an NPGB concentration insufficient to saturate all the available sites. This would explain the increase in the sedimentation coefficient since the mixture would involve molecules of $s = 5$ S and $s = 10$ S the value obtained being dependent on the ratio of the two forms in the mixture. The dimer form is not seen in SDS–PAGE since it dissociates under the high charge of the SDS so that the observation of dimerization would be missed if that were the only method employed.

This same phenomenon is seen with the Val_{442}-plasmin–streptokinase complex; the sedimentation coefficient increases to 7.3 S and the equilibrium molecular weight to 174 000. Powell & Castellino (1970), studying this same complex in a sucrose density gradient centrifugation experiment, noted that this complex was an aggregating species but failed to recognize that the requirement for aggregation was the presence of an active site. The plasmin B light chain–streptokinase complex does not show this same behavior when the active site is available; this is most likely caused by perturbations near the active site when the molecular is reduced and alkylated. Since this observation is not made with Lys-plasmin, with Val_{442}-plasmin, or with the isolated light B chain of plasmin, it appears that while one of the prerequisites is the active site, a second is the presence of the streptokinase moiety. It would seem that the dimer is formed between the active site of one molecule with a site on the streptokinase portion of the second molecule or that the streptokinase confers a conformation which allows for interaciton between two active sites. Experimentally, it has not been possible to differentiate among the several possibilities.

These results, when compared to previously published data, indicate that all of the previous results were on the Lys-

plasmin(ogen) form of the complex and that our results constitute the first characterization of the Glu-plasminogen–streptokinase complex. Our results also explain the literature discrepancy on the value of the sedimentation coefficient. The value of s = 10 S reported by Ling et al. (1966) and Davies et al. (1961) was on a Lys-plasmin–streptokinase complex with an available active site, while the s value near 5 S reported by De Renzo et al. (1963) and Summaria et al. (1970) was on the Lys-plasmin–streptokinase complex in which the active site was blocked.

Registry No. Plasminogen, 9001-91-6.

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Calcium Ion Binding to Pancreatic Phospholipase A_2 and Its Zymogen: A ^{43}Ca NMR Study†

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ABSTRACT: Calcium ion binding to phospholipase A_2 and its zymogen has been studied by ^{43}Ca NMR. The temperature dependence of the band shape of the calcium-43 NMR signal has been used to calculate the calcium ion exchange rate. The on-rate was calculated to be 5×10^6 M^{-1} s^{-1}, which is 2 orders of magnitude less than the diffusion limit of the hydrated Ca^{2+} ion in water. The ^{43}Ca quadrupole coupling constant for calcium ions bound to phospholipase, χ = 1.4 MHz, is significantly larger than those found for EF-hand proteins, indicating a less symmetric site. For prophospholipase A_2, we found χ = 0.8 MHz, indicating a calcium binding site, which is somewhat more symmetric than the EF-hand sites. The dependence of the ^{43}Ca NMR band shape on the calcium ion concentration showed that there are two cation binding sites on the phospholipase A_2 molecule: $K_1 = 4 \times 10^3$ M^{-1} and K_2 = 20 M^{-1}. The strong site was found to be affected by a pK_a = 6.5 and the weak site by pK_a = 4.5.

Phospholipase A_2 (PLA_2)[1] specifically catalyses the hydrolysis of the 2-acyl linkage of all phospholipids (van Deenen & de Haas, 1964: de Haas et al., 1968). The enzyme has been isolated from several different sources including snake venom (Wells & Hanahan, 1969; Joubert & van der Walt, 1975) and bee venom (Shipolini et al., 1971), as well as from mammalian pancreas (de Haas et al., 1968; Dutilh et al., 1975). Indications of the presence of a similar intracellular activity in other mammalian tissues have been obtained (Brockerhoff & Jensen, 1974). Phospholipase is secreted as a zymogen by the pancreas. The amino acid sequences of bovine, porcine and equine pancreatic phospholipases A_2 as well as that of the zymogen have been determined (Puyk et al., 1977; Fleer et al., 1978; Evenberg et al., 1977). The zymogens are converted to the active enzyme via a specific tryptic cleavage of the Arg–Ala bond, which removes an N-terminal heptapeptide from the proenzyme (Abita et al., 1972).

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[1] Abbreviations: NMR, nuclear magnetic resonance; PLA_2, phospholipase A_2; $PPLA_2$, prophospholipase A_2; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DPPC, diphenylcarbamyl chloride; Tris, tris(hydroxymethyl)aminomethane.

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