Steady State Kinetics of Activation of Human and Bovine Plasminogens by Streptokinase and Its Equimolar Complexes with Various Activated Forms of Human Plasminogen*

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The steady state kinetic parameters of activation of human Glu- and Lys-plasminogens and bovine plasminogen by streptokinase and its equimolar complexes with Glu- and Lys-plasminogen, the plasmin, and the plasmin-derived light(B) chain, as well as urokinase, were determined. Activation rates were measured at pH 6.0 and 30° by determining the initial velocity of Nα-Chz-L-lysine-p-nitrophenyl ester hydrolysis by the plasmin generated. The dissociation constant, Kplg, for activation of human plasminogen, by all the activators studied was comparable (about 1 to 3 μM) while the catalytic rate constant, kcat, ranged from about 3 to 52 s⁻¹ and the kcat/Kplg values varied from about 1 to 41 μM⁻¹ s⁻¹, with the highest value observed for the preformed light(B) chain streptokinase complex. Glu-plasminogen activation by streptokinase (Glu-plasminogen streptokinase) and the light(B) chain streptokinase complex proceeded at one-third the rate of that of Lys-plasminogen activation, with a kcat for Glu-plasminogen of about 7 and 19 s⁻¹ compared to a kcat for Lys-plasminogen of about 27 and 52 s⁻¹, respectively. Lys-plasminogen activation by Glu- and Lys-plasminogen-streptokinase and Lys-plasmin-streptokinase complexes gave kcat/Kplg values of about 15 to 22 μM⁻¹ s⁻¹. Lys-plasminogen activation by urokinase was much slower, with a kcat/Kplg value of about 1 μM⁻¹ s⁻¹. Activation of bovine plasminogen by the streptokinase complexes showed decreased catalytic rates, with kcat/Kplg values between about 0.5 and 3 μM⁻¹ s⁻¹, the lowest value was for the Lys-plasminogen-streptokinase complex.

The stoichiometry of the interaction of the light(B) chain with streptokinase was confirmed using both esterase and activator parameters; the equimolar complex formed at pH 6.0 and 30° at concentrations of 3 × 10⁻⁹ M, indicating a very low dissociation constant and probably an even lower one for streptokinase binding to both plasminogen and plasmin. Incubation of solutions of the light(B) chain-streptokinase complex at 30°, up to 60 min, showed no change in esterase activity but some loss in activator activity occurred after 15 min; significant fragmentation of streptokinase began after 5 min (to SK1) and continued after 15 min (to SK2 and SK3). When Glu-plasminogen, or Lys-plasminogen, or Lys-plasmin was preincubated with equimolar amounts of streptokinase for 1 min at 30°, the Kplg increased significantly while the kcat remained the same, with a significant lowering in the kcat/Kplg value to about 7 μM⁻¹ s⁻¹, indicating a decrease in activator efficiency. This decrease was accompanied by the appearance of SK1 as the major streptokinase component and SK2 as the minor component of the complex. These results also indicate the necessity for intact, or early degradation forms, of streptokinase in an activator complex, for maximum activator efficiency.

In these studies, we have continued our work on the definition of the functional properties of the streptokinase-induced activator species with various activated forms of human plasminogen. We have found lower dissociation constants for activation (KpLg) than for esterase activity (KpLg) toward Nα-Chz-L-lysine-p-nitrophenyl ester, with the catalytic rate constants being in the same range. The specificity and enzymatic efficiency values (kcat/Kplg and kcat/KpLg) were 10- to 20-fold greater for the hydrolysis of the specific Arg-Arg-Val activation bond than for the ester substrate.

There have been numerous studies dealing with the kinetics of activation of human plasminogen to plasmin by either urokinase, or streptokinase, or equimolar complexes of plasminogen and plasmin with streptokinase or staphylokinase (1-9). In all cases the activation proceeded with hyperbolic kinetics, which indicated that only one kinetically important event was occurring during the assays. Most of the studies dealing with kinetics of activation did not produce relevant dissociation and rate constants. Christensen and Möllertz (7), and Christensen (9) obtained kinetic constants for the activation of human Glu- and Lys-plasminogens by urokinase using coupled assays at pH 7.8 with Nα-benzoyl-L-arginine ethyl ester as the substrate. Buck and Boggiano (4) obtained some kinetic data on the activation of human Lys-plasminogen by the human plasmin-streptokinase complex using coupled as-
The purpose of this study was to establish the steady state kinetic parameters of the activation process of human Glu- and Lys-plasminogen and bovine plasminogen, by streptokinase, by a number of equimolar streptokinase complexes with various activated forms of human plasminogen, and by urokinase. In this study attempts were made to compare the functional properties of each activator enzyme species with the structural differences deriving from the original plasminogen moiety of the complex, or from the streptokinase moiety. In this respect, the relationship of activator activity to streptokinase fragmentation has been recently described (10). In addition, we have attempted to evaluate the steady state functional parameters that distinguish a protease species from an activator enzyme species (8), and we have attempted to determine those functional parameters which differentiate between the active site of an enzyme specific for activation of plasminogen, i.e. urokinase, and the active site of a protease forced into a new structure which has a similar activator specificity, i.e. the equimolar streptokinase complexes with various activated forms of human plasminogen.

**EXPERIMENTAL PROCEDURES**

**Human and Bovine Plasminogens and Plasmins, Human Plasmin-derived Light(B) Chain, Human Light(B) Chain-Streptokinase Complexes, and General Methods**

Glu-plasminogen was prepared from human serum and plasma Fraction III whereas Lys-plasminogen was prepared from plasma Fraction III, by an affinity chromatography method with L-lysinesubstituted Sepharose (11, 12). Both plasminogen preparations had specific activities of 28 to 30 I.U./mg of protein. Glu-plasminogen was passed through a column (0.9 x 25 cm) of Bio-Gel P-2 which was equilibrated with a 0.1 M potassium phosphate, 0.1 M NaCl, 0.01 M lysine buffer, pH 6.0. Glu-plasminogen affinity chromatography forms were prepared as previously reported (13). Lys-plasmin, specific activity of 30 I.U./mg of protein, was prepared from Lys-plasminogen by activation with catalytic amounts of urokinase (40,000 I.U./mg, a gift from G. H. Barlow, Abbott Laboratories) in 25% glycerol for 20 h by methods previously described (molar ratio of plasminogen to urokinase of 1600:1) (14). Bovine plasminogen was purified from fresh frozen bovine plasma by the same affinity chromatography methods as used for human plasminogen; bovine plasmin was prepared in the same manner as described for human plasmin. Both bovine plasminogen and plasmin had specific activities of 20 to 30 I.U./mg of protein. All zymogen and enzyme preparations were purified from fresh frozen bovine plasma by the same affinity chromatography methods as used for human plasminogen; bovine plasmin was prepared in the same manner as described for human plasmin.

**Activation Rates**

The procedure made use of a coupled assay (5, 7, 9) in which Na+-Cbz-L-lysine-p-nitrophenyl ester was kept constant at 2.2 x 10⁻⁴ M, while the acetone concentration was 0.5% (v/v). Inhibition by substrate was observed above 2.5 x 10⁻⁴ M substrate concentration. The plasminogen concentration was varied from 0.25 to 2.5 μM. The assay was performed by addition of plasminogen (1 to 10 μM, 0.25 to 2.5 x 10⁻⁴ M) and N'-Cbz-L-lysine-p-nitrophenyl ester (12 μM, 1.9 x 10⁻⁴ M to the assay cuvette containing 1.0 ml of 0.1 M potassium phosphate, 0.1 M NaCl, 0.01 M lysine buffer, pH 6.0, 30°C, immediately prior to addition of the activator (3 x 10⁻¹² mol in 10 μl) as described by Kosow (5). Streptokinase, or the performed light(B) chain-streptokinase complex, was added directly to the assay cuvette, while the plasminogen, plasmin, and light(B) chain-streptokinase complexes were prepared individually before each assay. After mixing, the absorbance of the assay mixture was monitored at 340 nm using a Beckman model 25 Spectrophotometer; ΔAA values were calculated between 0.35 to 1.5 min after start of the reaction, except at very high reaction velocities, where smaller time intervals had to be used. The amount of plasminogen converted to plasmin during this time did not exceed 2% while a 10 to 15% maximum hydrolysis of ester substrate could be allowed since the error introduced to the velocity calculation by such a change is not more than 5%. The data obtained from the activation curves were plotted as ΔAA/t against t with the slope of the straight line being equal to b/2 (as shown below). The kcat values and their standard errors, were obtained from the concentration and velocity values with the help of a program on the statistical analysis of Michaelis-Menten kinetics described by G. N. Wilkinson (19), using a Hewlett-Packard 67 calculator. The molecular weights and absorbance constants used for all calculations were the same as found in Table 1 of Ref. 8.

**Kinetics of Activation of Human and Bovine Plasminogens**

The activation rate was deduced from the initial rate of hydrolysis of N'-Cbz-L-lysine-p-nitrophenyl ester (811, 12) as described by Kosow (5). Streptokinase, or the performed light(B) chain-streptokinase complex, was added directly to the assay cuvette, while the plasminogen, plasmin, and light(B) chain-streptokinase complexes were prepared individually before each assay. After mixing, the absorbance of the assay mixture was monitored at 340 nm using a Beckman model 25 Spectrophotometer; ΔAA values were calculated between 0.35 to 1.5 min after start of the reaction, except at very high reaction velocities, where smaller time intervals had to be used. The amount of plasminogen converted to plasmin during this time did not exceed 2% while a 10 to 15% maximum hydrolysis of ester substrate could be allowed since the error introduced to the velocity calculation by such a change is not more than 5%. The data obtained from the activation curves were plotted as ΔAA/t against t with the slope of the straight line being equal to b/2 (as shown below). The kcat values and their standard errors, were obtained from the concentration and velocity values with the help of a program on the statistical analysis of Michaelis-Menten kinetics described by G. N. Wilkinson (19), using a Hewlett-Packard 67 calculator. The molecular weights and absorbance constants used for all calculations were the same as found in Table 1 of Ref. 8.

**Equations for Determining kcat and km of Activation**

Equations for determining kcat and km of activation:

- kcat = Vmax / [E] (1)
- km = [S] / kcat (2)
- V = kcat [E] / (1 + Km) (3)

where kcat is the catalytic constant, and km is the Michaelis constant.

**Development of Kinetic Equations**

The steady state kinetic parameters were determined using a Steady-State Kinetic Analysis Program on a statistical analysis as described by G. N. Wilkinson (19).

Activation Rates

The procedure made use of a coupled assay (5, 7, 9) in which N'-Cbz-L-lysine-p-nitrophenyl ester (Bachem Chemical Co.) hydrolysis was monitored at pH 6.0 and 30°C, as previously described (8, 10). The assay mixture had a total volume of 1.027 ml, the concentration of N'-Cbz-L-lysine-p-nitrophenyl ester was kept constant at 2.2 x 10⁻⁴ M, while the acetone concentration was 0.5% (v/v). Inhibition by substrate was observed above 2.5 x 10⁻⁴ M substrate concentration.
and substituting this value of $E$ into Equation 1 yields:

$$v = \frac{dP}{dt} = v_n + k_{\text{cat}} \left( A_0 + \frac{v_{\text{max}} [P]_0}{[P]_0 + K_{P}} \right) t$$  \hspace{1cm} (4)

Rewriting this equation in order to separate the variables yields:

$$dP = \left[ v_n + k_{\text{cat}} \left( A_0 + \frac{v_{\text{max}} [P]_0}{[P]_0 + K_{P}} \right) \right] dt$$  \hspace{1cm} (5)

Using the boundary condition $P = P_0$ for $t = 0$ and substituting $A_{340}/c$ for $P$, the integration of Equation 5 gives:

$$A_{340} - A_{3400} = c (v_n + k_{\text{cat}} A_0) t + \frac{c v_{\text{max}} [P]_0}{[P]_0 + K_{P}} \frac{t^2}{2}$$

which is of the form:

$$\Delta A_{340} = a t + \frac{b t^2}{2}$$ \hspace{1cm} (7)

where $a = c (v_n + k_{\text{cat}} A_0)$ that is, the initial velocity resulting from the spontaneous hydrolysis of the ester and the hydrolysis by the activator. A plot of $\Delta A_{340}$ versus $t$ results in a straight line with an intercept of $a$ on the ordinate axis and a slope of $b/2$. The parameter which can be calculated from the slope, $b/2$, is proportional to the initial rate of activation of plasminogen, since:

$$b = \frac{k_{\text{cat}} A_0}{2}$$

From the values of $a$, $v_n$, $A_0$, and $a$, one can calculate $k_{\text{cat}}$. Dividing the value of $b$ by this factor yields the initial rate of activation of plasminogen. If $v_n$ is negligible and $A_0$ is small enough such that $k_{\text{cat}} A_0$ is negligible then the first term of Equation 6 drops out.

### RESULTS

**Interaction of Human Plasmin-derived Light(B) Chain with Streptokinase**—In a previous report (15), it was shown that a functionally active light(B) chain could be isolated which forms an equimolar light(B) chain • streptokinase complex with activator activity. In the present study, the titration of the light(B) chain with streptokinase was monitored by measuring both esterase and activator activities (initial velocities) after addition of increasing amounts of streptokinase to a fixed amount of light(B) chain up to and in excess of the equimolar complex (Fig. 1). Both esterase and activator activities increased linearly with increasing molar ratios of streptokinase to light(B) chain and reached maximum activities at a 1:1 molar ratio (within ±15%). The zero point for esterase activity is not at zero velocity since the light(B) chain does have some esterase activity in the absence of streptokinase.

The formation of the active light(B) chain • streptokinase complex is much slower than the formation of the active plasmin • plasminogen • streptokinase complexes, since at similar concentrations of 1 to 5 × 10^{-7} M it takes between 5 to 7 min to develop maximum esterase activity, whereas the plasmin • plasminogen • streptokinase complex has maximum esterase activity on mixing, and the Lyso-plasminogen • streptokinase complex has maximum esterase activity in 1 min. Increasing the molar concentrations of light(B) chain and streptokinase decreases the reaction time, since at 4 × 10^{-5} M concentrations, maximum esterase activity is developed in 2 min. Although the dissociation constant of streptokinase with the light(B) chain, or with plasminogen, has not been determined, we have used 3 × 10^{-8} M concentrations for preparation of the various activator solutions. Since we have obviously achieved equimolar complex formation at close to 1:1 molar ratios (i.e. stoichiometric binding), it is clear that the dissociation constant has to be orders of magnitude lower.

Equimolar complexes of light(B) chain and streptokinase were incubated at 30° for various times, and measurements of esterase and activator activities and streptokinase fragmentation distributions were made (Table I). It is evident that the esterase activity remained stable for 60 min, i.e. no active sites were lost, but a loss of activator activity as well as fragmentation of streptokinase occurred between 15 and 30 min. Some loss in activator activity followed with continuous fragmentation of streptokinase to its SK2 and SK3 forms. These results, relating loss of activator activity to streptokinase fragmentation, are consistent with those obtained by Markus et al. (10) with human Glu- and Lys-plasminogen-and plasmin-streptokinase complexes.

**Activation of Human Glu-Plasminogen**—The data on the steady state kinetic parameters of Glu-plasminogen activation are summarized in Table II.\textsuperscript{2} The apparent dissociation constants, $K_{D}$ of Glu-plasminogen with streptokinase (Glu-plasminogen • streptokinase) and with the light(B) chain • streptokinase complex, are very similar, 1.73 and 2.76 μM, respectively, and about 10-fold lower than for the Na-Cbz-L-lysine-p-nitrophenyl ester (8). The catalytic rate constant, $k_{cat}$ for streptokinase, 6.71 s^{-1}, was about one-third that of the light(B) chain • streptokinase complex, 19.00 s^{-1}; the latter rate constant was similar to the esterase catalytic rate constant (8) for this enzyme species. Kinetic data on the activation of the Glu-1- and Glu-2-plasminogen affinity chromatography forms by streptokinase and
Comparison of functional properties and streptokinase fragment distribution during incubation of the light(B) chain-streptokinase complex

The complex was mixed at 1 mg/ml of streptokinase (SK) at equimolar concentration, and incubated in a 30°C bath: aliquots were taken separately for each incubation time and purpose.

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>ΔAAmax/min⁻¹</th>
<th>ΔΔAAmin/min⁻²</th>
<th>Percent of SK and SK fragments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.193</td>
<td>0.144</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.215</td>
<td>0.148</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>0.224</td>
<td>0.164</td>
<td>46</td>
</tr>
<tr>
<td>9</td>
<td>0.220</td>
<td>0.165</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>0.222</td>
<td>0.160</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>0.225</td>
<td>0.090</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>0.220</td>
<td>0.078</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0.224</td>
<td>0.076</td>
<td>0</td>
</tr>
</tbody>
</table>

* An error of ±10% in the esterase and activator velocities was calculated.

The relative distributions of the streptokinase fragments were calculated from integrated acrylamide/dodecyl sulfate gel scans in duplicates. The streptokinase fragment notation SK, SK1, and SK2 corresponds to the Markus et al. (10) notation of SKα, SKβ, and SKγ, respectively.

The apparent dissociation constants, Kdiss, for all of the streptokinase activator species used in this study. The overall second order rate constants, kcat/Km, for Glu-plasminogen activation by these activators are very high, 3.9 and 6.9 μM⁻¹ s⁻¹, respectively, indicating a high degree of enzymatic specificity and efficiency (20).

**Activation of Human Lys-plasminogen** - The data on the steady state kinetic parameters of Lys-plasminogen activation are summarized in Table II. The integrity of the Glu- and Lys-plasminogens, and Lys-plasmin, as well as that of streptokinase, was assured in all of the activation assays by mixing the components of the complexes at 3 x 10⁻⁵ M, at 0°C. Acrylamide gel/dodecyl sulfate gel electrophoresis of samples prepared at a concentration of 4 x 10⁻⁵ M showed intact streptokinase and plasminogen.

The apparent dissociation constants, Kdiss, for all of the streptokinase activator species used are similar, 1.03 to 1.65 μM, with the urokinase value being slightly higher, 2.81 μM, so that the Kdiss is not a distinguishing parameter among these activators. The catalytic rate constants, kcat, for streptokinase, and the Glu- and Lys-plasminogen-streptokinase complexes are similar and identical with the esterase catalytic rates of these enzyme species (8).

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**Steady state kinetic constants for activation of human and bovine plasminogen**

<table>
<thead>
<tr>
<th>Activator preparations</th>
<th>Kcat</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Glu-plasminogen activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. SK</td>
<td>1.72 ± 0.27</td>
<td>6.71 ± 0.75</td>
</tr>
<tr>
<td>2. B -SK (preformed)</td>
<td>2.75 ± 0.26</td>
<td>18.00 ± 1.32</td>
</tr>
<tr>
<td>Human Lys-plasminogen activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. SK</td>
<td>1.27 ± 0.20</td>
<td>26.68 ± 3.50</td>
</tr>
<tr>
<td>4. Glu-Plg·SK</td>
<td>1.40 ± 0.25</td>
<td>21.80 ± 2.50</td>
</tr>
<tr>
<td>5. Lys-Plg·SK</td>
<td>1.03 ± 0.14</td>
<td>22.60 ± 1.50</td>
</tr>
<tr>
<td>6. Lys-Pln·SK</td>
<td>1.12 ± 0.24</td>
<td>16.80 ± 1.70</td>
</tr>
<tr>
<td>7. B -SK (preformed)</td>
<td>1.23 ± 0.11</td>
<td>52.00 ± 2.64</td>
</tr>
<tr>
<td>8. B -SK (formed)</td>
<td>1.65 ± 0.16</td>
<td>43.24 ± 5.42</td>
</tr>
<tr>
<td>9. UK</td>
<td>2.81 ± 0.27</td>
<td>5.52 ± 0.17</td>
</tr>
<tr>
<td>Bovine plasminogen activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. SK</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11. Lys-Plg·SK</td>
<td>6.39 ± 1.23</td>
<td>3.42 ± 0.47</td>
</tr>
<tr>
<td>12. Lys-Pln·SK</td>
<td>1.49 ± 0.12</td>
<td>4.23 ± 0.15</td>
</tr>
<tr>
<td>13. B -SK (preformed)</td>
<td>3.50 ± 0.50</td>
<td>8.85 ± 0.65</td>
</tr>
</tbody>
</table>

* The abbreviations used in the table are: Plg, plasminogen; Pln, plasmin; SK, streptokinase; UK, urokinase; B -SK (preformed), (Lys-Plg + SK (mol:mol)-derived (B) chain·SK); B -SK (formed), (Lys-Pln-derived (B) chain + SK (mol:mol)). The molecular weights used for all calculations were: Glu-plasminogen, 83,800; Lys-plasminogen, 82,400; Lys-plasmin, 76,600; light(B) chain, 28,700; streptokinase, 44,000; urokinase, 31,700.

The preformed light(B) chain-streptokinase complex had about 80% of its streptokinase in the SK2 form and 10% in each of the SK1 and SK3 forms, while the formed complex, after 7 min of incubation, had 50% intact streptokinase and 50% in the SK1 form.

**Activation of Bovine Plasminogen** - The data on the steady state kinetic parameters of bovine plasminogen activation are also summarized in Table II. The steady state kinetic data for the hydrolysis of N'-Cbz-t-lysine-p-nitrophenyl ester by bovine plasmin at pH 6.0 and 30°C are: kcat = 35.2 ± 0.35 μM⁻¹ s⁻¹ and kcat/Km = 13.35 ± 0.35 s⁻¹. The activation rate constants for bovine plasminogen by the Lys-plasminogen·, Lys-plasmin·, and light(B) chain-streptokinase complexes, are very similar to the data obtained for the human plasminogen species except that the kcat/Km values for these three activators are much lower, and in the case of the Lys-plasminogen-streptokinase complex, the Kdiss is significantly higher, 6.39 μM; consequently, the kcat/Km value of this activator is appreciably lower, 0.5 μM⁻¹ s⁻¹. Thus, the bovine plasminogen substrate must require conversion of this activator species to the plasmin-streptokinase form for maximum enzymatic efficiency.

**Activation of Lys-plasminogen by Incubated Activators** - Analysis of Streptokinase Fragmentation - In an attempt to correlate streptokinase fragmentation with activation parameters, equimolar complexes were made with the activators at relatively high concentrations (4 x 10⁻⁵ M). Lys-plasminogen
was activated, in these experiments, in the same manner as described in the experiments detailed in Table II except that the activator species, Glu- and Lys-plasminogen·streptokinase complexes, were incubated for 1 min at 30°C prior to addition to the activation assay mixture. Samples were analyzed in the acrylamide gel/dodecyl sulfate electrophoretic system for evaluation and quantitation of streptokinase fragmentation in these activator species, as previously reported (8). These analyses showed that fragmentation of the streptokinase component in the Glu-plasminogen·streptokinase(1), Lys-plasminogen·streptokinase(2), and Lys-plasmin·streptokinase(3) activator complexes occurred in the following manner; streptokinase: (1) 15%, (2) 10%, (3) 0%; SK1 form: (1) 50%, (2) 60%, (3) 80%; SK2 form: (1) 35%, (2) 30%, (3) 20%; respectively. The catalytic rate constants, \( k_{\text{cat}} \), of these complexes were 22 to 24 s\(^{-1}\) which were similar to the same complexes with intact streptokinase, while the apparent dissociation constants, \( K_{\text{diss}} \), increased 3- to 4-fold for all of these activator species with an average \( k_{\text{cat}}/K_{\text{diss}} \) value of 6.5 \( \mu\text{M}^{-1} \text{s}^{-1} \). This \( k_{\text{cat}}/K_{\text{diss}} \) value is one-third of the value obtained with the species that had intact streptokinase in the complex.

**DISCUSSION**

In a previous report (15) it was shown that the human plasmin-derived light(B) chain forms a stoichiometric complex with streptokinase, and in this study (Fig. 1) it was demonstrated that both esterase and activator activities increase linearly to a level which corresponds to the stoichiometric complex. Stoichiometric activator complexes of plasminogen and plasmin with streptokinase had been previously demonstrated (23, 24). The fact that the activator solutions in this study were prepared at concentrations of 3 \( \times \) \( 10^{-8} \) M suggests that the dissociation constant for the light(B) chain-streptokinase interaction is orders of magnitude lower than \( 10^{-6} \) M, and that the dissociation constant for plasmin and plasminogen is even lower since these proteins react appreciably faster with streptokinase.

As noted in a previous publication (8), and also in this study (Table I), the esterase activity of the light(B) chain-streptokinase complex does not change with streptokinase fragmentation. But, in this study, when the activator activity drops, increased fragmentation beyond SK1 occurs (Table I). It is thus obvious that the degradation of streptokinase to SK2 does not influence the structure of the catalytic site and the primary binding site, but it probably does influence binding subsites. The most important portion of the specificity pocket formed by the complex is not in the immediate vicinity of the catalytic site, because the esterase activity does not change. Such behavior is not unique to the streptokinase complex species with various activated forms of human plasminogen since other examples of this concept were pointed out by Wolfenden (20).

As is evident from Table II, the \( K_{\text{diss}} \) of any of the streptokinase activator enzyme species for any of the plasminogens is approximately the same, and so is that of urokinase for Lys-plasminogen. On the other hand, the \( k_{\text{cat}} \) values differ appreciably and these differences are manifested in the \( k_{\text{cat}}/K_{\text{diss}} \) values. A comparison of the catalytic rates of the human Glu- and Lys-plasminogens shows at least 3-fold higher rates for the Lys-form than for the Glu-form. Such relative activation rates have been suggested from data on urokinase activation of these two plasminogen forms (9, 21, 22). Christensen’s (9) analysis of the kinetic data favored the reaction scheme where only one peptide bond, Arg-104-Val, is cleaved during the conversion of human Glu-plasminogen to plasmin. This mechanism of activation had been previously postulated on the basis of nonkinetic arguments (17, 25). As expected, streptokinase activation of Lys-plasminogen proceeds at the same rate as the preformed Lys-plasminogen·streptokinase complex does, since the streptokinase must bind to plasminogen instantaneously, under the conditions of these experiments, to form an activator complex. Since the plasminogen· and plasmin·streptokinase activator species, and streptokinase (Table II), preparations 3 to 6) contain intact streptokinase (shown in acrylamide gel/dodecyl sulfate electrophoresis), it is not unexpected that they all have approximately the same \( k_{\text{cat}} \) and \( K_{\text{diss}} \). Our results with these plasminogen· and plasmin·streptokinase activator species incubated for 1 min at 30°C prior to addition to the zymogen substrate showed that activator specificity and efficiency decreases with increasing degradation of streptokinase beyond the SK1 form. Presumably, increasing proteolytic degradation of bound streptokinase, and consequent rearrangements of the active site would affect more the remote specificity subsites and gradually the catalytic rate-determining subsites closer to the scissile bond. The most efficient activator species is the light(B) chain-streptokinase complex (Table II, preparations 7 and 8), which could be a result of the extra deformability lent to the light(B) chain by absence of the heavy chain, and a facilitated reshaping by streptokinase.

Bovine plasminogen is a much poorer substrate for the streptokinase activator complexes than are the Glu-plasminogen and Lys-plasminogen substrates. The fact that the \( k_{\text{cat}}/K_{\text{diss}} \) value for Lys-plasmin·streptokinase is severalfold lower than for Lys-plasmin·streptokinase on bovine plasminogen implies that this substrate can discriminate between the two complexes in favor of the plasmin complex, where the Lys-plasminogen substrate cannot.

The previous discussion, and the data in Table II, have a common denominator with the data reported on the activation of trypsinogen by enteroenzyme and trypsin (26). The latter data showed that while there was only a 5- to 6-fold difference in \( K_{\text{diss}} \) between the two activators, the \( k_{\text{cat}} \) for enteroenzyme activation was 2000-fold greater. Thus, the specificity of the activator enzyme species was manifest in the catalytic rate constant rather than in the dissociation constant. This conclusion leads to the same hypothesis as that postulated by Bauer et al. (27) for hydrolysis of amide bonds by Streptomyces griseus Protease 3: (a) the rate-limiting step in these reactions is acylation, and \( k_{\text{cat}} = k_{\text{diss}} \) (\( k_{\text{diss}} \) is acylation rate constant) while \( k_{\text{cat}}/K_{\text{diss}} \) is a true dissociation constant (28, 29) since nonproductive binding would be negligible at the low \( K_{\text{diss}} \) values shown in Table II; (b) the specificity of the streptokinase complex activator species, and urokinase, is related to contact sites several residues removed from the scissile bond. The inverse relationship between primary and secondary sites (30) and specificity is unimportant for activators, since they were intended to hydrolyze specific peptide bonds in proteins with a higher degree of specificity, and not small peptides. It is interesting to note that two different protease proenzyme activators like urokinase and enteroenzyme have identical enzymatic efficiencies; \( k_{\text{cat}}/K_{\text{diss}} \) = 6 to \( 7 \times 10^{4} \) \( \mu\text{M}^{-1} \text{s}^{-1} \) at pH 7.8 and pH 5.6, and 25°C, and on two different zymogens, plasminogen and trypsinogen, respectively (7, 26). Following this argument, strong binding specificity resulting from subsite interactions remote from the scissile bond would deter-
mine the exact binding of the substrate to be activated, and the catalytic efficiency of the primary subsites and the catalytic apparatus would be relatively much less important. The proteolytic properties inherent in an activator species like the human plasmin-streptokinase complex can be attributed to the rigid primary site specificity built in for plasmin’s role as a protease, and not as an activator. This hypothesis also allows for an easy explanation of the increased catalytic rate of the light(B) chain-streptokinase complexes in terms of the Thompson and Blout finding (31) that increasing length of peptide substrates increase &at much more than Km(app), since absence of the heavy(A) chain possibly allows the binding of more residues of the plasminogen substrate to the light(B) chain-streptokinase complex subsites. The number of the subsites is of course, undetermined at this time, but a comparison of data in the last two studies referred to (27, 30), and our data suggest a minimum of eight.

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