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Interaction of Streptokinase and Rabbit Plasminogen†

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ABSTRACT: The ability of rabbit plasminogen and plasmin to form complexes with streptokinase has been studied and compared to the human system. At 1:1 or 10:1 ratios of streptokinase to rabbit plasminogen, no complex was observed by sucrose density centrifugation; however, almost all the rabbit plasminogen was converted to plasmin. Under identical conditions with human plasminogen, a complex was formed which consisted of altered streptokinase and human plasmin. When the acylating agent *p*-nitrophenyl *p'*-guanidinobenzoate was added to either human or rabbit plasminogen prior to addition of a molar equivalent of streptokinase, reactive complexes were formed in each case which consisted of human or rabbit plasminogen and native streptokinase. Upon treatment of human plasmin with a molar equivalent of streptokinase, a complex is formed which consists of altered streptokinase and human plasmin. This complex possesses the ability to activate bovine plasminogen. Human plasmin, inactivated with diisopropyl fluorophosphate, retains the capability of complexing with streptokinase but this complex does not activate bovine plasminogen. Upon treatment of rabbit plasmin or diisopropyl fluorophosphate inactivated rabbit plasmin with streptokinase no complexes or bovine plasminogen activator activity results. These results coupled with kinetic observations suggest that only rabbit plasminogen can serve as a plasminogen proactivator with streptokinase, whereas either human plasminogen or human plasmin can serve as a plasminogen proactivator with streptokinase. It is further observed that the reason for the increased streptokinase sensitivity of human plasminogen compared to rabbit plasminogen can be explained by the relative stability of streptokinase in the human plasminogen activator complex compared to the rabbit plasminogen activator complex.

Plasminogen is a single-chain protein of mol wt 81,000–88,000, depending on the species (Barlow *et al.*, 1969; Sodetz *et al.*, 1972), and plasmin is a two-chain molecule of approximately the same molecular weight, stabilized by disulfide bond(s) (Barlow *et al.*, 1969; Sodetz *et al.*, 1972). Many agents mediate the conversion of plasminogen to plasmin, among which is the bacterial endotoxin streptokinase. The mechanism of activation of human plasminogen by streptokinase has been a widely studied subject. The problem resolves itself into the manner in which the nonproteolytic protein, streptokinase, catalyzes the proteolytic cleavage required for conversion of plasminogen into plasmin. Further, the fact that not all species of plasminogen are capable of activation by streptokinase raises other interesting questions.

It has been demonstrated that human plasmin and streptokinase can form a 1:1 complex (Zylber *et al.*, 1959; Kline and Fishman, 1961; Markus and Werkheiser, 1964; Ling *et al.*, 1965; Hummel *et al.*, 1965). This complex possesses the ability to convert any species of plasminogen into plasmin (Blatt *et al.*, 1964; Wulf and Mertz, 1969) and is called "plasminogen activator." It was also found that "activator" can form whether human plasminogen or plasmin is used as the starting material (McClintock and Bell, 1971; Reddy and Markus, 1972). A possible mechanism of activation of human plasminogen by streptokinase, employing all the above considerations, has been nicely formulated by Reddy and Markus (1972).

Recently, some details of the mechanism of the streptokinase-mediated activation of human plasminogen proposed by Reddy and Markus (1972) have been challenged by Taylor and Beisswenger (1973). The latter authors propose that streptokinase can only react with the human plasmin, which usually contaminates human plasminogen preparations. This interaction leads to formation of a modified streptokinase capable of directly activating human plasminogen. However,

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several authors have shown conclusively that preformed human plasmin was not a requirement for the generation of the activator active site (Mounter and Shipley, 1958; Summaria *et al.*, 1969; McClintock and Bell, 1971; Reddy and Markus, 1972). This fact was not discussed in the mechanism proposed by Taylor and Beisswenger (1973).

We have previously shown (Sodetz *et al.*, 1972) that rabbit plasminogen is sensitive to activation by streptokinase. However, human plasminogen possesses a greater degree of sensitivity to streptokinase than does rabbit plasminogen. Therefore, we wished to determine the ability of streptokinase to complex with rabbit plasminogen and plasmin in order to detect whether the decreased sensitivity of rabbit plasminogen toward streptokinase activation could be explained at the level of interaction between the two proteins. Further, we wished to compare some aspects of the streptokinase-mediated activation of rabbit plasminogen and human plasminogen.

Materials and Methods

Proteins. Human plasminogen was prepared by the method of Deutsch and Mertz (1970), as modified by Brockway and Castellino (1972). Rabbit plasminogen was prepared as we have described in an earlier manuscript (Sodetz *et al.*, 1972) and bovine plasminogen was prepared from fresh bovine plasma in the same manner.

High-purity streptokinase was prepared from kabikinase, generously donated by Dr. Hugo Nihlén, by a combination of salt fractionations and isoelectric focusing (W. J. Brockway and F. J. Castellino, manuscript in preparation). This material produced a single band on polyacrylamide gels at pH 9.5 (Davis, 1964) and only a very small impurity on sodium dodecyl sulfate gels (Weber and Osborn, 1969). Isoelectric focusing analysis performed as previously described (Sodetz *et al.*, 1972), utilizing pH 3–6 carrier ampholytes, demonstrated a single peak for this material.

Human and rabbit plasmins were prepared by urokinase activation of the corresponding plasminogens (Sodetz *et al.*, 1972).

Azocasein was prepared from α-casein (Worthington Biochemical Corp.) by the method of Charney and Tomarelli (1947).

Urokinase was purchased from Calbiochem.

Sucrose density centrifugation experiments were based on the method of Martin and Ames (1961). The plasminogen or plasmin in 0.05 M Tris-HCl–0.01 M L-lysine (pH 8.0) was mixed with the required ratio of streptokinase in the same solvent. The final concentration of plasminogen or plasmin was 8–10 mg/ml. This solution (0.100 ml) was carefully layered on 4.6 ml of a linear gradient consisting of 5–20% sucrose in 0.05 M Tris-HCl–0.01 M L-lysine (pH 8.0). The gradient was formed with aid of a Lucite block as described by Martin and Ames (1961). Each tube was then inserted into a SW-39 swinging-bucket rotor and centrifuged in a Model L centrifuge at 38,000 rpm for 15 hr at 4°. At the conclusion of the run the tubes were rigidly mounted and drained into a fraction collector after first inserting a draining device through the bottom of the tube. Fractions containing 0.15 ml were collected. Each fraction was diluted with 0.2 ml of water and the optical densities were determined at 280 nm. The appropriate peaks were pooled, dialyzed against water, and lyophilized. The material was redissolved in sodium dodecyl sulfate buffer and applied to either 5 or 7% polyacrylamide gels in sodium dodecyl sulfate for analysis of the components (Weber and Osborn, 1969).

Assays. Burst assays utilizing NphBzoGdn[1] were carried out in 0.05 M Veronal buffer (pH 8.2) as described by Chase and Shaw (1970). Tos-Arg-OMe assays of plasmin were performed as previously described (Brockway and Castellino, 1971) except that only a single level of Tos-Arg-OMe (0.01 M) was assayed. The effect of streptokinase on the Tos-Arg-OMe activity of plasmin was measured by adding the desired quantity of streptokinase prior to addition of Tos-Arg-OMe.

Azocasein assays of plasmin were performed at 25°. Plasmin (40 μg) was incubated with 0.5% azocasein in 0.2 ml of 0.05 M Tris-HCl (pH 8.0) for 15 min. The reaction was stopped by addition of 0.8 ml of 5% trichloroacetic acid. The mixture was centrifuged and 0.5 ml of the supernatant was added to 0.5 ml of 0.5 M NaOH. The absorbance was measured at 410 nm against an azocasein blank. In experiments designed to determine the effect of streptokinase on the azocasein activity of plasmin, the desired quantity of streptokinase was added to the plasmin solution 10 min prior to addition of azocasein. All other steps were as above.

Assays of bovine plasminogen activator activity of plasmin in the presence and absence of added streptokinase were also carried out at 25° with all reagents in 0.05 M Tris-HCl (pH 8.0). The procedure described is a slightly modified form of that described by Ling *et al.* (1965). Plasmin (0.8–1.5 mg/ml) was incubated with the desired quantity of streptokinase for 5 min at 25°. An aliquot of this mixture equivalent to 4 μg of plasmin was added to 40 μg of bovine plasminogen in a volume of 0.225 ml. After 5-min incubation, 0.025 ml of 0.1 M Tos-Arg-OMe was added to the mixture and the reaction was allowed to proceed for 10 min. The remaining Tos-Arg-OMe was analyzed as previously described (Brockway and Castellino, 1971).

Results

Figure 1A shows the sucrose density centrifugation pattern which results when human plasminogen, pretreated with NphBzoGdn, is added to a molar equivalent of streptokinase. Clearly a complex is formed. Analysis of the components in this complex by sodium dodecyl sulfate polyacrylamide gel electrophoresis in Figure 5 demonstrates that approximately equal molar quantities of plasminogen and native streptokinase are present. NphBzoGdn assays demonstrate that this complex contains one active site per mole of plasminogen originally added. Figure 1B shows the sucrose density centrifugation pattern of human plasminogen mixed with an equal molar quantity of streptokinase in the absence of NphBzoGdn. Again, a complex is formed. Analysis of this complex by sodium dodecyl sulfate gel electrophoresis in Figure 5 demonstrates that approximately equal molar quantities of altered streptokinase[2] and human plasmin are present. Again NphBzoGdn assays demonstrate one active site per mole of plasminogen originally added. Figure 2 presents sucrose density centrifugation patterns of human plasmin (2A) and Dip-F-treated human plasmin (2B) when each was mixed with equal molar quantities of streptokinase. In each case complexes

[1] Abbreviations used are: NphBzoGdn, *p*-nitrophenyl *p'*-guanidinobenzoate; Tos-Arg-OMe, α-*N*-tosyl-L-arginine methyl ester; Dip-F, diisopropyl fluorophosphate.

[2] When streptokinase exists in a complex with human plasmin, there is a peptide(s) of total mol wt 7000–8000 digested from the amino terminus. This is our functional definition of "altered streptokinase." A manuscript detailing these observations is currently in preparation.

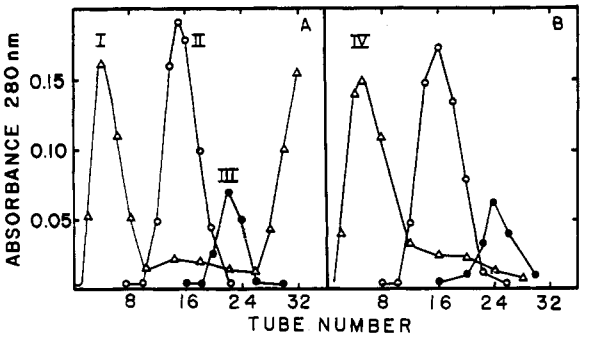

FIGURE 1: Sucrose density gradient analysis of the interaction between human plasminogen and streptokinase. Three cells were run simultaneously: (A) ○, contained only human plasminogen; ●, contained only streptokinase; △, human plasminogen was mixed with a 10-fold excess of NphBzoGdn prior to addition of a 1 molar equiv of streptokinase; (B) ○ and ● are as in A; △, human plasminogen and streptokinase (1:1) were incubated at 25° for 10 min prior to centrifugation. The conditions of the centrifugation were 38,000 rpm for 15 hr at 4°. I, II, III, and IV refer to the sodium dodecyl sulfate gel number shown in Figure 5. Tube 1 represents the cell bottom.

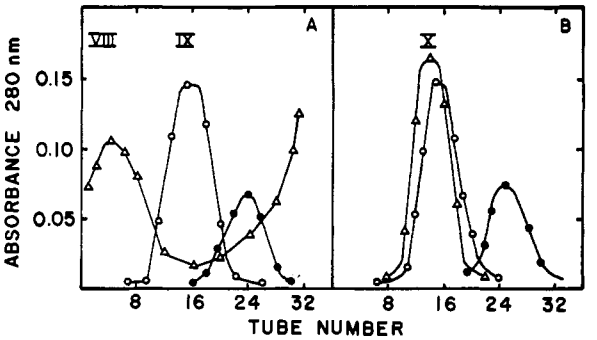

FIGURE 3: Sucrose density gradient analysis of the interaction between rabbit plasminogen and streptokinase. Three cells were run simultaneously: (A) ○, contained only rabbit plasminogen; ●, contained only streptokinase; △, rabbit plasminogen was mixed with a 10-fold excess of NphBzoGdn prior to addition of 1 molar equiv of streptokinase; (B) ○ and ● are as in part A; △, rabbit plasminogen and streptokinase (1:1) were incubated at 25° for 10 min prior to centrifugation. The conditions of the centrifugation were 38,000 rpm for 15 hours at 4°. VIII, IX, and X refer to the sodium dodecyl sulfate gel number shown in Figure 6. Tube 1 represents the cell bottom.

were formed, which when analyzed by sodium dodecyl sulfate electrophoresis in Figure 5 consisted of human plasmin and altered streptokinase or human Dip-F plasmin and native streptokinase.

Figure 3A shows the sucrose density centrifugation pattern resulting from the incubation of streptokinase and rabbit plasminogen, pretreated with NphBzoGdn. Clearly, a complex is formed. Analysis of the components of this complex on sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 6) shows the presence of native streptokinase and rabbit plasminogen. However, from observations on the intensity of the bands, it appears that under these initial conditions less than 1 mol of streptokinase is bound per mol of rabbit plasminogen. NphBzoGdn "burst" assays indicate that only 0.2–0.4 mol of active site is produced when a 1:1 mixture of streptokinase and rabbit plasminogen are mixed in the presence of a 10-fold excess of NphBzoGdn. The number of moles of active sites per mole of plasminogen increases as the ratio of streptokinase to rabbit plasminogen is raised and appears to approach 1:1 at a 10–15:1 ratio of the two proteins. Figure 3B shows the sucrose density centrifugation patterns resulting when equimolar amounts of streptokinase and rabbit plasminogen are mixed in the absence of NphBzoGdn. Here, no stable complex results; but as can be observed from Figure 6 all the rabbit plasminogen is converted to rabbit plasmin. Further, no native or altered streptokinase can be observed. The same results are obtained regardless of the ratios of streptokinase to rabbit plasminogen up to at least 10:1. These results suggested that streptokinase and rabbit plasmin formed an unstable complex. This was verified by the observations presented in Figure 4A,B. In this case, no complex resulted when equimolar quantities of streptokinase and rabbit plasmin (4A) or Dip-F-treated rabbit plasmin were mixed. Increasing the amount of streptokinase up to 10:1 had no effect on these results.

The data in Figure 7A illustrate the effect of streptokinase on the Tos-Arg-OMe, casein, and bovine plasminogen activator activities of human plasmin. Streptokinase–plasmin

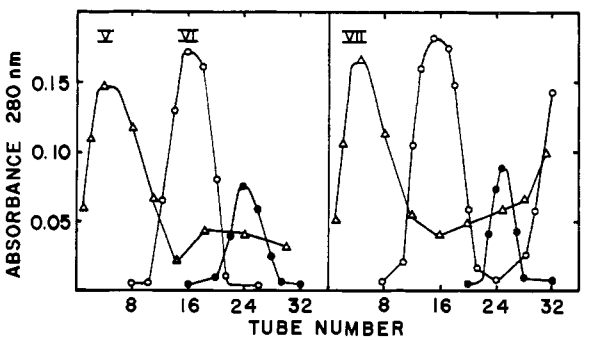

FIGURE 2: Sucrose density gradient analysis of the interaction between human plasmin and streptokinase. Three cells were run simultaneously: (A) ○, contained only human plasmin; ●, contained only streptokinase; △, human plasmin was mixed with 1 molar equiv of streptokinase; (B) ○, contained human plasmin previously inactivated with diisopropyl fluorophosphate; ●, contained streptokinase; △, human plasmin was inactivated with diisopropyl fluorophosphate prior to addition of 1 molar equiv of streptokinase. The conditions of the centrifugation were 38,000 rpm for 15 hr at 4°. V, VI, and VII refer to the sodium dodecyl sulfate gel number shown in Figure 5. Tube 1 represents the cell bottom.

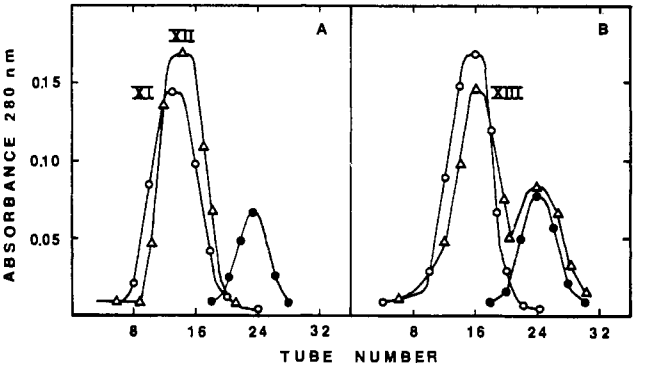

FIGURE 4: Sucrose density gradient analysis of the interaction between rabbit plasmin and streptokinase. Three cells were run simultaneously: (A) ○, contained only rabbit plasmin; ●, contained only streptokinase, △, rabbit plasmin was mixed with a molar equivalent of streptokinase; (B) ○, contained rabbit plasmin previously inactivated with diisopropyl fluorophosphate; ●, contained streptokinase; △, rabbit plasmin was inactivated with diisopropyl fluorophosphate prior to addition of 1 molar equiv of streptokinase. The conditions of the centrifugation were 38,000 rpm for 15 hr at 4°. XI, XII, and XIII refer to the sodium dodecyl sulfate gel number shown in Figure 6. Tube 1 represents the cell bottom.

complexes catalyze the hydrolysis of the ester substrate at the same efficiency as does plasmin alone. However, the proteolytic activity of human plasmin, as measured on an azocasein substrate, decreases as a function of the amount of plasmin bound to streptokinase and completely ceases at a 1:1 stoichiometry of streptokinase:human plasmin. These results are identical to those found previously (Markus and Werkheiser, 1964; Ling *et al.*, 1965). This loss of plasmin activity is not due to streptokinase masking the active site of plasmin since the above studies with Tos-Arg-OMe show full plasmin activity. The activity loss of streptokinase–plasmin complexes toward azocasein is likely due to a decrease in accessibility of the active site toward large substrates. Although the general proteolytic activity of plasmin is lost as a direct consequence of complexing with streptokinase, there is a parallel increase in activity of this complex toward bovine plasminogen activation. It has been proposed that this is due to streptokinase directing bovine plasminogen into the active site of the complex (Buck and Boggiano, 1971). Again, maximal activity is found at a 1:1 stoichiometry of streptokinase and human plasmin.

Figure 7B shows the same experiments for rabbit plasmin. Here, mixtures of rabbit plasmin and streptokinase do not lead to losses in the ability of rabbit plasmin to hydrolyze azocasein nor lead to gains in bovine plasminogen activator activity. These results suggest that streptokinase and rabbit plasmin do not form a stable complex, in agreement with the data in Figure 4. Figure 8 shows the results of some kinetic experiments designed to test the ability of streptokinase and rabbit plasminogen to activate bovine plasminogen. Here, when rabbit plasminogen and streptokinase are mixed prior to addition of bovine plasminogen, bovine activator activity is noted at short preincubation times of rabbit plasminogen and streptokinase. This activator activity decreases with time and is completely restored upon readdition of fresh streptokinase. These results indicated that the activator activity of rabbit plasminogen and streptokinase is lost due to proteolysis of the streptokinase upon prolonged incubation times. Parallel analysis of sodium dodecyl sulfate of rabbit plasminogen and streptokinase with time, in Figure 9, shows that considerable proteolysis of streptokinase occurs but the rabbit plasminogen is not converted to plasmin during this period at the initial concentrations of plasminogen used in the activation mixtures. Thus, upon restoration of plasminogen activator activity by readdition of fresh streptokinase, rabbit plasminogen must serve as the proactivator.

Discussion

The results obtained in this study confirm and extend the mechanism proposed by Reddy and Markus (1972) for the activation of human plasminogen by streptokinase. A unified mechanism can be expressed as

$$\mathrm{SK} + \mathrm{HPg} \rightleftharpoons \mathrm{SK{\cdot}HPg} \longrightarrow \{\mathrm{SK{\cdot}HPg'}\} \longrightarrow \{\mathrm{SK{\cdot}HPm}\} \longleftarrow \mathrm{HPm} + \mathrm{SK} \quad (1)$$

$$\mathrm{HPg} + \begin{Bmatrix}\mathrm{SK{\cdot}HPg'}\\ \text{or}\\ \mathrm{SK{\cdot}HPm}\end{Bmatrix} \longrightarrow \mathrm{HPm} \quad (2)$$

where streptokinase (SK) and human plasminogen (HPg) interact to form a physical complex (SK·HPg). This complex rearranges to form an altered {SK·HPg′} which now possesses an active site. According to McClintock and Bell (1972) this complex is of limited stability and the plasminogen moiety in the complex is cleaved to give a streptokinase–human plasmin {SK·HPm} complex. This complex can also form in reaction 1 from SK and HPm. The complexes {SK·HPg′} and {SK·HPm} are termed plasminogen activators since they possess the ability to convert human plasminogen to human plasmin, as depicted in step 2. In fact, any species of plasminogen will be converted to plasmin by the human plasminogen activators. This mechanism also explains the well-known fact that if stoichiometric amounts of human plasminogen and streptokinase are mixed, plasminogen activator activity predominates whereas if catalytic amounts of streptokinase and human plasminogen are mixed, human plasmin activity predominates (Kline and Fishman, 1961). This is because in the former case reaction 1 will dominate whereas in the latter case reaction 2 will dominate. The results presented in the study reported here systematically analyze the complexes which occur under various conditions of incubation and are entirely consistent with the mechanism outlined above.

In Figures 1 and 5, it can be seen that if a 1:1 mixture of human plasminogen and streptokinase is subjected to sucrose density ultracentrifugation, a stoichiometric complex is formed which consists of human plasmin and altered streptokinase. The results shown in Figures 2 and 5 demonstrate that if a 1:1 mixture of human plasmin and streptokinase is subjected to sucrose density centrifugation exactly equivalent results are obtained. These data confirm reaction 1 as above outlined. It can also be seen from Figures 2 and 5 that when diisopropyl fluorophosphate inhibited plasmin and streptokinase are mixed, a complex will still form. This result conflicts with previously reported observations which claim that streptokinase and Dip-plasmin will not interact (DeRenzo *et al.*, 1967). Upon analysis of the "activator" activity of the Dip-inhibited plasmin–streptokinase complex, we find that this complex will not activate bovine plasminogen. These results seem to suggest that the active site of human plasmin is somehow necessary for the activation of bovine plasminogen. Whether the plasmin active site is necessary for direct activation of bovine plasminogen or whether the plasmin active site is necessary in order to first induce an alteration in streptokinase prior to activation of bovine plasminogen is open to further analysis. The final point required for evaluation of reaction 1 is the direct demonstration of the interaction between human plasminogen and streptokinase. This interaction can only be shown if the active site which develops in the complex can be immediately inactivated, in order that conversion of plasminogen to plasmin within the complex can be arrested. The results shown in Figures 1 and 5 demonstrate that if NphBzoGdn is added to plasminogen prior to addition of streptokinase, the active site which develops in plasminogen after addition of streptokinase is immediately acylated and further reactions cease. Although Reddy and Markus (1972) have come to similar conclusions concerning the interaction of human plasminogen and streptokinase in the presence of NphBzoGdn, they have never demonstrated that streptokinase and human plasminogen can in fact exist in a complex. These results on the human system are a valuable addition to their data.

Kinetic analyses of the human plasmin–streptokinase complex, as shown in Figure 7A, are in agreement with previously reported observations that this complex does not lose activity toward small ester substrates (Tos-Arg-OMe), but pro-

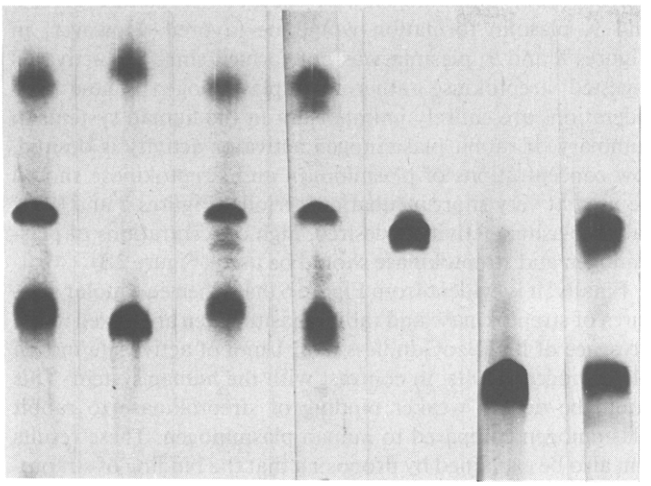

FIGURE 5: Sodium dodecyl sulfate gel electrophoretograms of selected pools from sucrose density gradient experiments of human plasminogen or plasmin and streptokinase. Refer to Figure 1 and 2 for gel designations. Left to right, I–VII. These gels were run at different periods of time and small mobility variations between gels are not meaningful.

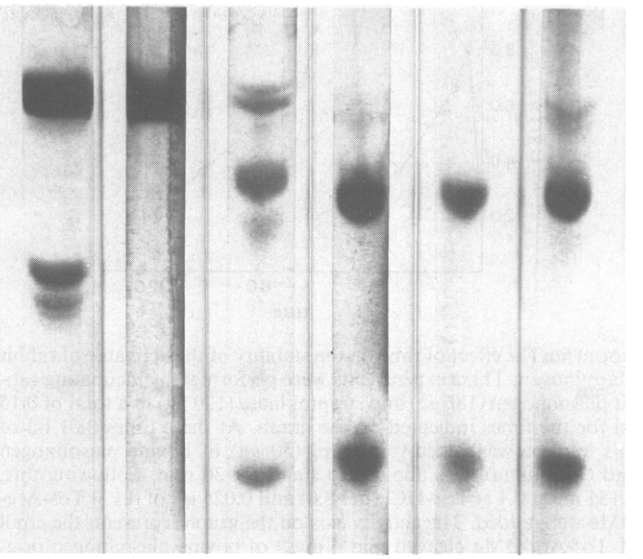

FIGURE 6: Sodium dodecyl sulfate gel electrophoretograms of selected pools from sucrose density gradient experiments of rabbit plasminogen or plasmin and streptokinase. Refer to Figure 3 and 4 for gel designations. Left to right, VIII–XIII. These gels were run at different periods of time and small mobility variations between gels are not meaningful.

tein substrates (azocasein) are not accessible to this active site (Reddy and Markus, 1972). Bovine plasminogen, however, is directed to the active site in the activator complex, most likely due to specific interactions between bound streptokinase and bovine plasminogen (Buck and Boggiano, 1971).

The data reported for the interaction between streptokinase and rabbit plasminogen and plasmin appear to suggest differences between the rabbit and human systems regarding activation by streptokinase.

The following mechanism can be proposed for the rabbit system

$$\mathrm{SK} + \mathrm{RPg} \rightleftharpoons \mathrm{SK{\cdot}RPg} \longrightarrow \{\mathrm{SK{\cdot}RPg'}\} \longrightarrow \mathrm{SK^*} + \mathrm{RPm} \quad (3)$$

$$\mathrm{RPg} + \{\mathrm{SK{\cdot}RPg'}\} \longrightarrow \mathrm{RPm}$$

where all symbols are as above except that RPg refers to rabbit plasminogen, RPm refers to rabbit plasmin, and SK* refers to extensively degraded SK. The major differences between the rabbit and human systems are that streptokinase does not appear to interact as stably with rabbit plasmin (Figures 4 and 6) as with human plasmin (Figures 2 and 5) and that mixtures of streptokinase and rabbit plasmin do not lead to bovine plasminogen activator activity (Figure 7B). However, from Figures 3 and 6, it is obvious that streptokinase will complex with rabbit plasminogen only if NphBzoGdn is first added to rabbit plasminogen to arrest any further proteolysis and prevent RPm from forming. If NphBzoGdn is not added to rabbit plasminogen prior to streptokinase addition, only rabbit plasmin and proteolytically digested streptokinase can be observed in gels. What must be happening here is the $\{\mathrm{SK{\cdot}RPg'}\}$, which possesses an active site, converts the RPg in the complex to RPm. Unlike the human system, the SK·RPm complex is not stable and dissociates to RPm and SK (which is then digested by RPm, leading to SK*). These observations are consistent with the scheme as outlined above. We should make clear at this point that we cannot completely rule out the interaction of SK and native RPm. We are only stating that if these two proteins do interact, the complex is not stable. This is due to the digestion of streptokinase by plasmin. What we are stating is that we feel the interaction of rabbit plasmin and streptokinase (if any) is not significant in terms of plasminogen activator activity. The activator for the rabbit system is the $\{\mathrm{SK{\cdot}RPg'}\}$ complex. This is substantiated by the data presented in Figures 8 and 9. These figures show that maximal activator activity of mixtures of streptokinase and rabbit plasminogen occur when fresh streptokinase and rabbit plasminogen exist as components of the activator complex. However, unlike the human system, rabbit plasminogen activator loses its activity with time due to digestion of streptokinase by the activator. We should point out that Reddy and Markus (1972) proposed that the principal activator in the human plasminogen system is $\{\mathrm{SK{\cdot}HPg'}\}$ although $\{\mathrm{SK{\cdot}HPm}\}$ can also serve this function, when present. Although this has been more indirectly proven in the human system, it is obvious that in the rabbit system $\{\mathrm{SK{\cdot}RPg'}\}$ is the only activator of plasminogen.

At this point, it might seem that the results of Figure 3B

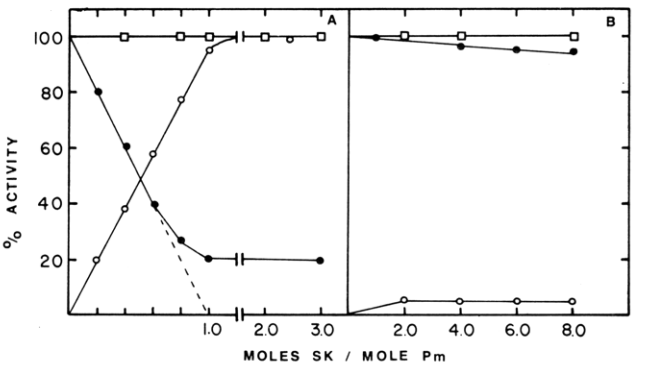

FIGURE 7: Activity studies. (A) Of human plasmin as a function of added streptokinase toward: □, Tos-Arg-OMe; ●, azocasein; and ○, bovine plasminogen; (B) activity of rabbit plasmin as a function of added streptokinase toward: □, Tos-Arg-OMe; ●, azocasein; ○, bovine plasminogen. Assays were performed as described in Methods.

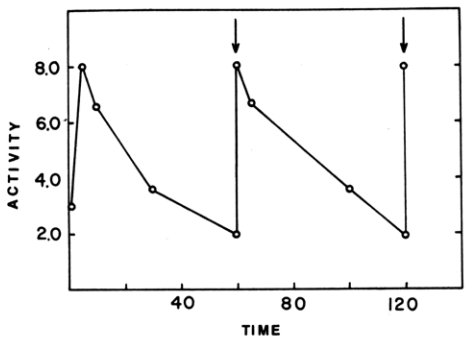

FIGURE 8: The effect of time on the stability of the activator of rabbit plasminogen. These experiments were performed by incubating rabbit plasminogen (186 μg) and streptokinase (120 μg) in a total of 0.15 ml for the times indicated on the graph. At these times 0.01 ml of this solution was added to 0.06 ml (50 μg) of bovine plasminogen and this mixture was allowed to stand for 30 min. Following this, 0.155 ml of 0.1 M Tris-HCl (pH 8.0) and 0.025 ml of 0.1 M Tos-Arg-OMe were added. The activity axis on the graph represents the μmol of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of bovine plasminogen originally added. The arrows in the graph represent the times at which a 0.01 ml (40 μg) aliquot of fresh streptokinase was added to the rabbit plasminogen–streptokinase solution prior to addition to bovine plasminogen.

are in opposition with those of Figures 8 and 9. Although similar ratios of rabbit plasminogen and streptokinase are used in each experiment, the rabbit plasminogen is converted to rabbit plasmin in Figure 3B, whereas no conversion of rabbit plasminogen to plasmin occurred in Figures 8 and 9. We have found in the course of our studies that the ratio of rabbit plasminogen and streptokinase is not the only important consideration in the activation of rabbit plasminogen. The initial concentration of rabbit plasminogen is an equally important factor. The higher the initial concentration of rabbit plasminogen, at a certain ratio of rabbit plasminogen to streptokinase, the more favorable the situation for plasmin formation. Since in Figure 3B much higher initial concentrations of rabbit plasminogen were used, compared to Figures 8 and 9, plasmin formation would be favored. However, in Figures 8 and 9, plasmin was not formed since the activator digested streptokinse rather than plasminogen. These considerations are entirely unimportant in the human system. In summary, if rabbit plasminogen activator activity is desired, low concentrations of plasminogen and streptokinase should be used at very short incubation periods (Figures 8 and 9). If rabbit plasmin activity is desired, high concentrations of plasminogen and streptokinase should be used (Figure 3B).

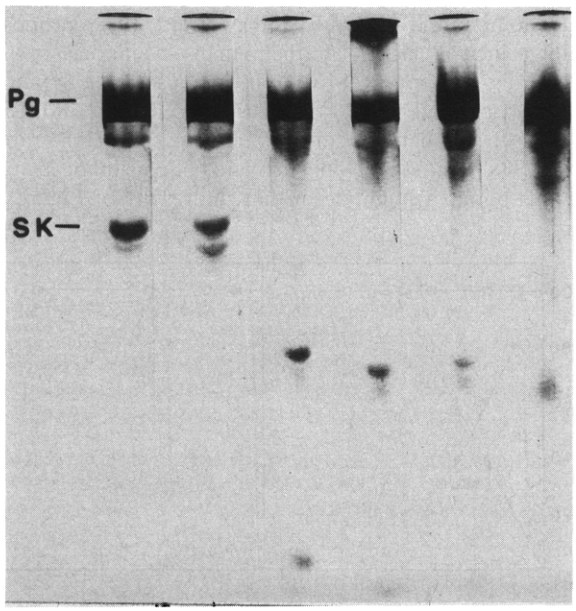

FIGURE 9: Sodium dodecyl sulfate–mercaptoethanol polyacrylamide gel analysis of the stability of the activator of rabbit plasminogen. Concomitant with the withdrawal of samples from the rabbit plasminogen–streptokinase solution for analysis of their activator activity, these samples were also placed in gels. The gels in this figure represent, left to right, times 0-, 5-, 10-, 15-, 30-, and 60-min incubation of rabbit plasminogen and streptokinase.

Finally, it is evident from Figure 6 that when equimolar mixtures of streptokinase and rabbit plasminogen are mixed in the presence of NphBzoGdn, less than 1 mol of active site/mol of plasminogen results, in contrast with the human system. This could be due to weaker binding of streptokinase to rabbit plasminogen compared to human plasminogen. These results can also be explained by proposing that the binding of streptokinase might be the same to both species but the incomplete formation of an active site may be due to a refractory conformational alteration in rabbit plasminogen compared to human plasminogen. In any event, the net result is that it is apparently much more difficult to form and stabilize rabbit plasminogen activator compared to human plasminogen activator. This is likely the basis of the higher observed degree of sensitivity of human plasminogen toward streptokinase compared to the rabbit system.

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Multiple Forms of Glutamine Synthetase. Hybrid Formation by Association of Adenylylated and Unadenylylated Subunits†

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ABSTRACT: Fully adenylylated glutamine synthetase ($E_{\overline{12}}$) is rapidly inactivated in the presence of 4 M urea and 0.5 mM ADP, whereas unadenylylated enzyme ($E_{\overline{0.8}}$) is not. The kinetics of urea inactivation of a partially adenylylated enzyme preparation isolated directly from *Escherichia coli* extracts are significantly different from that of an *in vitro* mixture of $E_{\overline{0.8}}$ and $E_{\overline{12}}$ having the same average state of adenylylation. After 30-min exposure to inactivation conditions all adenylylated subunit activity is lost from mixtures of $E_{\overline{0.8}}$ and $E_{\overline{12}}$, but significant adenylylated activity remains in partially adenylylated native enzymes. It is concluded that the native enzyme preparations contain hybrid molecular forms composed of both adenylylated and unadenylylated subunits and that heterologous subunit interactions lead to stabilization of adenylylated subunit activity. Exposure of either $E_{\overline{0.8}}$ or $E_{\overline{12}}$ to 7 M urea at 0° leads to complete dissociation of subunits and complete loss of catalytic activity. Tenfold dilution of the dissociated subunit mixture with Tris·HCl buffer containing KCl, Mg^{2+}, Mn^{2+}, and 2-mercaptoethanol (pH 7.5) results in reassociation of the subunits to produce a 55–65% yield of catalytically active dodecameric aggregates that are indistinguishable from the original enzyme. The yield of active reconstituted enzyme is increased by the presence of ATP or ADP in the reassociation mixture and is diminished by the presence of other substrates and feedback inhibitors of glutamine synthetase including: glutamine, hydroxylamine, potassium arsenate, tryptophan, glycine, CTP, alanine, and AMP. Hybrid dodecameric aggregates produced by reversible dissociation of mixtures of $E_{\overline{12}}$ and $E_{\overline{0.8}}$ are similar to those present in partially adenylylated native enzyme preparations. $E_{\overline{0.8}}$ and $E_{\overline{12}}$ could not be separated from each other by electrophoresis. With succinylation of approximately 36 amino residues per mol wt 600,000, 75% of the catalytic activity is lost but the derivatized enzyme is readily separated from unmodified enzyme by electrophoresis. Hybrids produced by reversible dissociation of a mixture containing equal amounts of $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$ are readily separated from $E_{\overline{0.8}}$ and succinylated $E_{\overline{12}}$ by electrophoresis; these hybrids are a mixture of partially adenylylated molecules with from four to nine adenylylated subunits per dodecameric aggregate.

Previous studies have shown that glutamine synthetase from *Escherichia coli* has a mol wt of 600,000 and is composed of 12 apparently identical subunits arranged in two superimposed hexagonal rings (Woolfolk *et al.*, 1966; Shapiro and Ginsburg, 1968; Valentine *et al.*, 1968). The activity is regulated by the covalent attachment of one 5′-adenylyl group to a unique tyrosyl moiety of each subunit (Shapiro *et al.*, 1967; Kingdon *et al.*, 1967). Adenylylation is accompanied by changes in divalent ion specificity, in the pH optimum, and in susceptibility to inhibition by various products of glutamine metabolism and by other effectors (Kingdon *et al.*, 1967; Stadtman *et al.*, 1968). Since each one of the 12 subunits of glutamine synthetase can be adenylylated, the enzyme may exist in multiple molecular forms that differ from each other with respect to the number (0–12) and orientation of adenylylated subunits within single molecules. M. S. Raff and W. C. Blackwelder have calculated that 382 molecular forms of the enzyme are possible (personal communication). Other studies support the conclusion that hybrid forms of the enzyme (*i.e.*, enzyme molecules containing both adenylylated and unadenylylated subunits) do exist and that heterologous interactions between dissimilar subunits affect catalytic parameters (Ginsburg *et al.*, 1970; Denton and Ginsburg, 1970; Segal and Stadtman, 1972) and stability characteristics of the enzyme (Stadtman *et al.*, 1970).

When divalent cations are removed from glutamine synthetase (by treatment with EDTA), it undergoes transition to a "relaxed" state in which tryptophan, tyrosine, and sulfhydryl groups become exposed (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). On exposure to 1 M urea or pH 8.0 the "relaxed" enzyme is dissociated into mol wt 50,000 inactive subunits (Woolfolk and Stadtman, 1967b; Shapiro and Ginsburg, 1968). In earlier studies it was shown that upon adding divalent cations and decreasing the pH, these subunits reassociated to unstable aggregate forms that were similar but not identical in structure to the native enzyme (Woolfolk and Stadtman, 1967b; Valentine *et al.*, 1968). Reaggregation was accompanied by only transient restoration of catalytic activity.

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