The Formation of an Enzymically Active Complex Between DFP-Inhibited Plasmin and Streptokinase[1] (37267)

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Streptokinase (SK), an extracellular product of Group A and C streptococci, combines with human plasminogen and/or plasmin to form a molecular complex (activator) which can convert bovine plasminogen to plasmin (1–5). At least two enzymic activities develop in mixtures of streptokinase and plasminogen: (a) plasmin, which hydrolyzes casein and (b) activator, which converts bovine plasminogen to plasmin (1–5). Although the active center of human plasmin has been described (6), the biochemical nature of the activator complex, its structure and its enzymatic active site are poorly understood. Thus, we undertook to determine if the same diisopropylfluorophosphate (DFP)-inhibitable center were responsible for both activities.

Materials and Methods. Chemical reagents. Diisopropylfluorophosphate (DFP), (Sigma Chemical Co., St. Louis, MO) was diluted to 10^{-1} *M* with 2-propanol and stored in 1.0 ml aliquots in vacuum desiccator. Tritiated diisopropylfluorophosphate (New England Nuclear, Boston, MA), 880 mCi/mmole was also brought up in 2-propanol to a concentration of 1.0 mCi/0.36 ml.

Protein reagents. Streptokinase was prepared as previously described with a DEAE-cellulose column (Whatmann DE-52, England) (7), after ammonium sulfate precipitation of crude material (Varidase, Lederle, Pearl River, NY). The purified product contained 85,000–120,000 units/mg protein and less than 10% contaminating proteins as estimated from polyacrylamide gel discontinuous electrophoresis. Purified streptokinase was stored in 2.5–3.0 ml aliquots of about 0.5 mg/ml concentration at —60°.

Plasminogen was purified as previously described (1) by double column chromatography on carboxy methyl cellulose (Schleicher and Schuell, Keene, NH) of a pseudoglobulin fraction of plasma (Cutter, Berkeley, CA). The purified protein contained 23–32 Remmert and Cohen caseinolytic units/mg protein and less than 10% contaminating proteins as estimated from discontinuous electrophoresis. Purified plasminogen was stored at —60° in 1.0 ml aliquots of about 0.5 mg/ml. Plasmin was prepared from either purified plasminogen or the pseudoglobulin fraction by incubating plasminogen and streptokinase in about 100:1 ratio by weight for 30 min, 23° at pH 7.5. An equal volume of glycerol was added, and the solution was left 16–18 hr at the same temperature. Optimal caseinolytic activity was achieved in this manner. Subsequent experimental results using either the pure or the cruder preparation were comparable. Plasmin was stored in 1 mg/ml aliquots at —20°.

Enzymic assays. Plasmin was assayed by caseinolysis. One milliliter of 2% casein (Fisher, King of Prussia, PA) was incubated with 1 ml sample containing approximately 100 μg plasmin for 30 min at 37°. The reaction was then stopped with 3 ml of 1 *M* perchloric acid. After at least 10 min at 4°, the mixture was filtered through Whatmann No. 40 filter paper and absorbance at 280 nm determined. Controls of buffer

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plus casein were processed as above and the results subtracted from the test samples. Standard curves were established experimentally with a plasmin in glycerol preparation obtained from the Division of Biologic Standards, NIH.

Bovine plasminogen activator activity was assayed on bovine fibrin-agar plates (8). These plates contained 0.0625% bovine fibrinogen (Bovine Fraction I, Armour, Chicago, IL), 1% Noble agar (Difco, Detroit, MI), and 2 units bovine thrombin (Parke-Davis Co., Detroit, MI). Samples to be tested were inserted in 20 μl wells cut into the agar. The plates remained at 23° for 48 hr with results being recorded at 18 and 48 hr. Results were expressed as areas of lysis per sample. Standard curves relating area of lysis to micrograms per milliliter of plasminogen in the activator were established.

Discontinuous polyacrylamide gel electrophoresis. Discontinuous electrophoresis was run in the cold at pH 8.5–9.0 with a 7% running gel (9). Both upper and lower gels contained 0.3 *M* epsilon aminocaproic acid. Gels were stained with Coomasie blue dye.

Determination of radioactivity. Radioactivity emitted by tritium was determined by liquid scintillation in a Beckman Model LS-133. Gels were run in duplicate, one being fixed and stained, the second being sliced every 2–3 mm along its horizontal axis. These slices were then eluted 16–18 hr with 0.2–0.3 ml distilled water. The eluates were then applied to glass fiber discs (Whatmann) in scintillation vials and dried. Scintillation fluid containing 15.1 g PPO and 1.14 g POPOP in 3.78 liter toluene (Packard, Downers Grove, IL) was added and the vials were counted (10).

Experimental procedure and results. Plasmin inhibition by DFP. Plasmin in glycerol was dialyzed against 0.001 *N* HCl for 1 hr at 4° before the addition of 0.1 *M* lysine was added to the solution which then contained approximately 0.5 mg/ml plasmin. A thousandfold excess of DFP was added (final concentration about 10^{-2} *M* DFP) to the test sample while a control sample was untreated. Both samples were adjusted to pH 7–8 with 0.1 *N* NaOH. After 18–22 hr at 23°, the samples were individually dialyzed for 4 hr against 2 changes of phosphate–lysine buffer (pH 7.4 0.1 *M* lysine, 0.3 *M* phosphate). Both the DFP-treated and the control samples were assayed in duplicate on casein. Inhibition of 89–94% of the caseinolytic activity present in the control and 99–100% of the activity present before processing was achieved in this manner. Plasmin inhibited by DFP had no discernible activity on fibrin plates.

DIP-plasmin plus streptokinase. Fifty micrograms each of plasmin and diisopropylphosphoryl-plasmin (DIP-plasmin) prepared above were next incubated with 10 μg purified SK (5:1 by wt; about 2–3:1 molar) for 2 min at 23° to form the activator complex. These were then diluted 1:10, 1:50, 1:100 and 1:500 with the same phosphate buffer described previously and assayed in fibrin-agar plates. No difference in activator activity between plasmin-SK and (DIP-plasmin)-SK could be detected by this method (Fig. 1).

(Plasmin-SK) plus DFP. Plasmin prepared as described in Methods was dialyzed against 0.001 *N* HCl and 0.1 *M* lysine. Approximately 0.3 mg purified SK was incubated with 1.5 mg plasmin for 2 min at 23°. The plasmin-SK complex was divided into two samples. The first of these was untreated while to the second DFP was added to a final concentration of 10^{-2} *M* (10^3 excess). Both samples were adjusted to pH 7–8 with 0.1 *N* NaOH and left 18–22 hr at 23°. They were then dialyzed for 4 hr against 3 changes of 1.0 liter each 0.1 *M* lysine phosphate buffer (pH 7.4), at 4°. The samples were adjusted to a protein concentration of approximately 50 μg/ml with phosphate buffer and diluted 1:10, 1:50, 1:100 and 1:500 with the same buffer. These dilutions were then assayed for activator activity on fibrin-agar plates. Complete inhibition of the activator activity by DFP was demonstrated by this technique (Fig. 1).

Radioactive studies. Trace amounts of ^{3}H-DFP were added to the DFP used above. Samples were then subjected to discontinuous electrophoresis. Radioactivity was determined on duplicate gels as described in Methods. The area of radioactivity from tritium

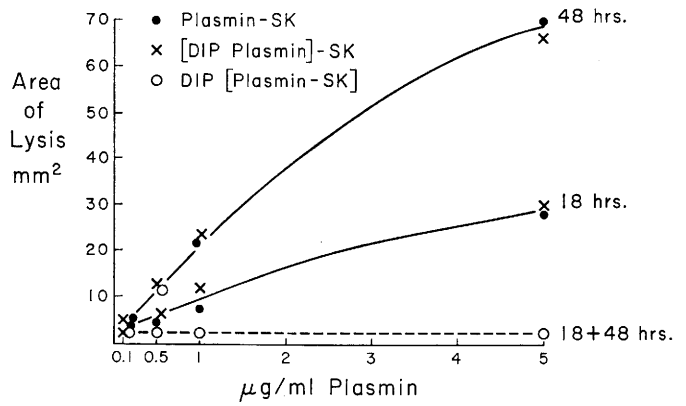

FIG. 1. Effect of DFP on activator activity. DIP-plasmin shown to be 89–94% inhibited on casein, was incubated with SK (5:1 wt ratio) and assayed in serial dilutions on unheated bovine fibrin agar plates. Similar quantities of uninhibited plasmin-SK were assayed at the same time. No difference in areas of lysis was noted after either 18 or 48 hr incubation. Furthermore, an aliquot of the uninhibited plasmin-SK activator complex treated with DFP and assayed simultaneously demonstrated no fibrinolytic activity at either 18 or 48 hr.

on the gel corresponded to the plasmin band on the ^{3}H-DIP-plasmin and to the activator region on the (^{3}H-DIP-plasmin)-SK complex and to the ^{3}H-DIP-(plasmin-SK) bands on the gel.

Discussion. Two enzymic activities are found in human plasmin-streptokinase mixtures. One, enriched in caseinolytic capacity, is attributed to plasmin; the second, enriched in esterase and bovine plasminogen activator activity, to the activator complex. Although DFP is known to be irreversibly bound to a serine in the active site of plasmin (6), little is known about the enzymic center of the activator. Others have demonstrated that activator once formed can be inhibited by DFP, but there is some controversy whether or not (DIP-plasmin) can form an activator complex with SK (4, 5). DeRenzo and colleagues (5) were unable to demonstrate by starch gel electrophoresis at pH 6.1 that DIP-plasmin and streptokinase formed a complex. Summaria and co-workers (4), on the other hand, demonstrated a (DIP-plasmin)-SK complex on starch gel electrophoresis at pH 6.0 after precipitating the complex in 0.8 *M* $(NH_4)_2SO_4$ from a mixture of purified plasmin and crude streptokinase (Varidase). This complex had neither proteolytic nor bovine plasminogen activator activities. Buck, Hummel and DeRenzo (11) did peptide mapping of reduced, alkylated and trypsinized DIP-^{32}P-plasmin and (DIP-^{32}P-plasmin)-SK. Because the distribution of the radiolabeled DFP appeared in similar peptides in both DIP-plasmin and (DIP-plasmin)-SK, these workers concluded that DFP was bound to the same site in both materials. They explained the appearance of radiolabel in multiple fragments by either incomplete trypsin digestion of the same peptide or the appearance of different peptides due to different isomeric forms of plasminogen. Another interpretation, of course, is that more than one peptide binds DFP, this would suggest that there were more than one DFP-inhibitable active site in plasmin and/or the activator complex. The present study confirms that the activator once formed is indeed inhibited by DFP and that (DIP-plasmin) can combine with SK to form an activator complex with as much bovine plasminogen activator activity as similar quantities of plasmin-SK. Thus, it seems probably that a new enzymic site involving different amino acids is formed by the interaction of SK with human plasminogen and/or plasmin. The identification of this active site, (i.e., from plasminogen or plasmin alone, SK alone. or plasminogen or plasmin and SK) of course remains to be determined.

Summary. Plasmin hydrolysis of casein was inhibited by DFP with the formation of DIP-plasmin. DIP-plasmin was able to react

with SK to form a bovine plasminogen activator complex which was equal in activity to the complex formed between similar amounts of non-DFP-treated plasmin and SK. Plasmin-SK once formed, however, was subsequently inhibited by DFP. Since DIP-plasmin retains bovine plasminogen activator potential, it would appear that a DFP-inhibitable site other than that in plasmin is responsible for this activator potential.

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