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PLASMINOGEN ACTIVATOR FROM CELLS TRANSFORMED BY AN ONCOGENIC VIRUS

INHIBITORS OF THE ACTIVATION REACTION

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Summary

This paper describes an assay for direct measurement of plasminogen activation and its application for determining the kinetic constants and for screening potential inhibitors of the reaction. The assay is based on the conversion of the single chain of ^{125}I-labelled plasminogen to the two chains of ^{125}I-labelled plasmin (EC 3.4.21.7), the latter then being separated from each other and from the plasminogen substrate by electrophoresis under reducing conditions in SDS-polyacrylamide gels.

The K_m of activator from transformed murine cells for human plasminogen was 180 nM.

A broad range of compounds was tested as potential inhibitors of plasminogen activation and of plasmin-catalyzed fibrinolysis respectively, and the two reactions differed qualitatively and quantitatively in their response to previous agents. The principal qualitative difference was in the susceptibility of the reactions to a spectrum of naturally-occurring macromolecular inhibitors: all of the macromolecular inhibitors that blocked the action of plasmin were without effect on murine activator or human urokinase (EC 3.4.99.26). A variety of small molecules inhibited both of the reactions tested, and showed significant quantitative differences; some of these were active at μM concentrations. The exacting specificity of plasminogen activators for macromolecules, both substrates and inhibitors, encourages the expectation that effective inhibitors of great specificity may be isolated from as yet undiscovered natural sources.

Introduction

A wide range of biological processes have recently been associated with cellular secretion of plasminogen activators. These include oncogenic transforma-

tion of fibroblasts by viruses [1—4], follicle rupture during ovulation in mammals [5], trophoblast implantation [6], migration of cultured cells [7], macrophages [8], and granulocytes [9], and the effects of tumor promoting substances [10]. The common denominators in all of these phenomena are localized extracellular proteolysis connected with tissue remodeling, cell migration, and, usually, hormonal control. Although plasminogen activators are serine proteases of the urokinase type, their hydrolytic specificity for macromolecular substrates is extremely limited, and they appear to induce significant proteolysis only by generating plasmin, the catalytically active product of plasminogen activation. The ultimate production of localized extracellular proteolysis is thus a two-step reaction: plasminogen activator converts inactive plasminogen to catalytically active plasmin, and plasmin degrades locally accessible proteins.

In view of the scope and complexity of phenomena related to plasminogen activator, specific inhibitors could be useful tools for the purification of this enzyme and for elucidating its role in various biological settings. That no such inhibitors are as yet available might be due, in part, to the fact that most studies of plasminogen activation have been performed using indirect fibrinolytic or caseinolytic assays combined with the measurement of plasmin formed during a preincubation of plasminogen and plasminogen activator [11—15]. These approaches are complicated by the autolytic action of plasmin, and by the degradative effect of plasmin on both plasminogen activator and plasminogen. Furthermore, such assays present the difficulty that activation of plasminogen is taking place simultaneously with plasmin-catalyzed fibrinolysis, and the possible inhibition of the two processes cannot easily be resolved into the separate individual components. In addition, there have been several investigations of inhibitors of plasminogen activator based on enzyme assays using synthetic substrates of low molecular weight [16,17].

To obtain unambiguous information about inhibitors of plasminogen activation, we have simplified the reaction conditions to permit direct quantitation of the amounts of plasmin formed. For this purpose we have used ^{125}I-labelled plasminogen as a substrate for plasminogen activator, and have separated any plasmin formed during the activation reaction by subsequent electrophoresis under reducing conditions in SDS-polyacrylamide gels [18—20].

To identify potential inhibitors, all substances selected for testing were first screened in an overall fibrinolysis assay. Any compound which inhibited the two-step reaction was then further tested for its effect on the two individual enzymes, plasminogen activator and plasmin. A preliminary report on part of this study was published previously [21].

Materials and Methods

Chemicals. Bovine pancreatic trypsin inhibitor (Kunitz inhibitor, Trasylol ®) and Germanin (Suramin) were gifts from Bayer AG, Wuppertal, G.F.R.; soybean trypsin inhibitor was obtained from Miles-Seravac Laboratories, Elkhart, Ind.; diisopropyl phosphorofluoridate from Sigma Chemical Co., St. Louis, Mo.; and lima bean trypsin inhibitor from P-L Biochemicals, Milwaukee, Wisc. *p*-Nitrophenyl-*p'*-guanidino benzoate was a gift from Dr. E. Shaw; trypsin

inhibitors prepared from leeches, sea anemones, cow colostrum, dog submandibular gland and boar seminal plasma were all gifts from Dr. Hans Fritz; leupeptin and antipain were gifts from Dr. Walter Troll; human placental urokinase inhibitor was a gift from Dr. Takehiko Kawano; urokinase was a gift from Leo Pharmaceutical Industries, Ballerup, Denmark. All other reagents were of the best commercially available grade.

Plasminogen activators. As described previously [4], serum-free culture fluid containing plasminogen activator was produced from cultures of mouse embryonal fibroblasts infected with mouse sarcoma virus. The culture fluid was concentrated 10-fold and this preparation contained 2000—3000 units (see ref. 4) plasminogen activator per ml.

When analyzed for protease and plasminogen activator activity after SDS-polyacrylamide gel electrophoresis, the preparations contained no significant protease other than plasminogen activator; the latter consisted predominantly of the form at approx. 48 000 molecular weight, which accounted for more than 90% of catalytic activity with plasminogen as substrate [4].

Plasminogen and plasmin. Plasminogen was prepared from outdated human plasma by the method of Deutsch and Mertz [22], using two cycles of affinity chromatography on lysine-Sepharose 4B. To suppress incidental proteolysis, a mixture of protease inhibitors was added to the plasma before processing. These included (at final plasma concentration): 30 μM *p*-nitrophenyl-*p'*-guanidino benzoate, 5 Kallikrein inhibitor units/ml bovine pancreatic trypsin inhibitor and 50 μg/ml lima bean trypsin inhibitor and the same concentrations of these inhibitors were maintained throughout the isolation procedure. The temperature was below 4°C during the entire procedure. The electrophoretic pattern of this plasminogen has been reported previously [21] *. The plasminogen was labeled with ^{125}I by the procedure of Helmkamp et al. [23], using a 4-fold molar excess of ICl relative to plasminogen. Plasmin was generated by incubation of plasminogen (7.5 mg/ml) with urokinase (100 Plough units/ml in 50% glycerol, 0.05 M Tris-HCl (pH 8.1) for 24 h (see Ref. 24 and Results).

Assays. A standard assay for fibrinolysis initiated by plasminogen activation was performed in plastic petri dishes coated with bovine ^{125}I-labelled fibrin. To monitor plasminogen activation directly, a second assay was used: this was based on the conversion of the single chain of ^{125}I-labelled plasminogen to the two chains of plasmin, which were separated and individually measured after SDS-polyacrylamide gel electrophoresis. The details of both those assays have been described previously [4,21].

Assays for fibrinolysis by plasmin were performed in plastic petri dishes coated with ^{125}I-labelled fibrin, as described above. The assays contained 0.2 μg human plasmin in a final volume of 1 ml 0.1 M Tris-HCl (pH 8.1); the amount of ^{125}I-labelled fibrin solubilized was measured in a gamma-counter.

All the standard assays were performed at 37°C for 1 h. In all assays where

* The plasma used for routine preparations of plasminogen had been stored for unspecified periods, and it appeared important to determine whether the same electrophoretic pattern would be obtained if the protein was rapidly purified from fresh plasma. For this purpose, plasminogen was immediately isolated from several individual normal donors. The respective bloods were collected directly into a mixture of protease inhibitors, yielding final concentrations as above. The plasminogen prepared in this way was electrophoretically indistinguishable from protein isolated from outdated plasma.

inhibitors were tested, the pH was adjusted to the final value of pH 8.1.

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in a stacking system using slab gels (1.2 × 90 × 150 mm) of 11% acrylamide with overlaid stacking gels of 4% acrylamide, as described by Laemmli [25]. Protein was stained with 0.1% Coomassie blue in 50% (w/v) trichloroacetic acid and stained gels were destained in 10% (v/v) acetic acid. A mixture of protein markers was electrophoresed in each gel; these were chymotrypsinogen (M_r 25 000), ovalbumin (M_r 45 000) and bovine serum albumin (M_r 68 000).

Results

Activation of human plasminogen by murine activator

To establish conditions permitting satisfactory quantitation of the activation reaction, the conversion of the single polypeptide chain of ^{125}I-labelled plasminogen to ^{125}I-labelled plasmin was followed by measuring the radioactivity in the two chains of plasmin after they were separated by SDS-polyacrylamide gel electrophoresis. The validity of this approach depends on the assumption that the iodination of the plasminogen is generally random throughout the molecule, yielding a constant specific radioactivity per unit length of polypeptide chain (i.e., no 'hot spots' confined to a single chain of plasmin); such a labeling pattern predicts the formation of fragments with total radioactivity proportional to molecular weight. An experimental test of this assumption gave the results presented in Fig. 1: the relative radioactivity of the heavy and light chains of plasmin exactly reflected the ratio of their molecular weights, thereby validating the assay in this respect.

In this experiment, bovine pancreatic trypsin inhibitor was added to the reaction mixture. When this inhibitor was omitted, the overall recovery of the method was low, as the sum of radioactivity in the individual bands of the gel (plasminogen plus light and heavy chains of plasmin) accounted for only a minor fraction of the total originally present in the reaction as ^{125}I-labelled plasminogen [21]. This loss of protein radioactivity seemed best explained by assuming indiscriminate proteolysis of plasminogen and plasmin accompanying the formation of plasmin. The addition of bovine pancreatic trypsin inhibitor permitted virtually complete (>90%) recovery of the initial radioactivity, all of it in the form of plasminogen and the two chains of plasmin.

We have previously concluded that bovine pancreatic trypsin inhibitor is inert with respect to plasminogen activator, but strongly inhibits the action of plasmin and therefore is a useful tool for analysis of plasminogen activation [3,21]. This conclusion has since been supported by reports from other laboratories [26,27]. There are two further indications that bovine pancreatic trypsin inhibitor does not interact with plasminogen activators: (1) the inhibitor does not inhibit either the catalytic inactivation by diisopropyl phosphorofluoridate or the incorporation of [^{3}H]diisopropyl phosphorofluoridate into urokinase or into plasminogen activators obtained from chicken, mouse or human cells (Danø, K. and Reich, E., unpublished data and ref. 4), (2) preparations of plasminogen activators were not delayed by passage through columns of bovine pancreatic trypsin inhibitor bound to Sepharose which effectively bound plasmin and trypsin under the same conditions (Danø, K. and Reich, E., unpublished data).

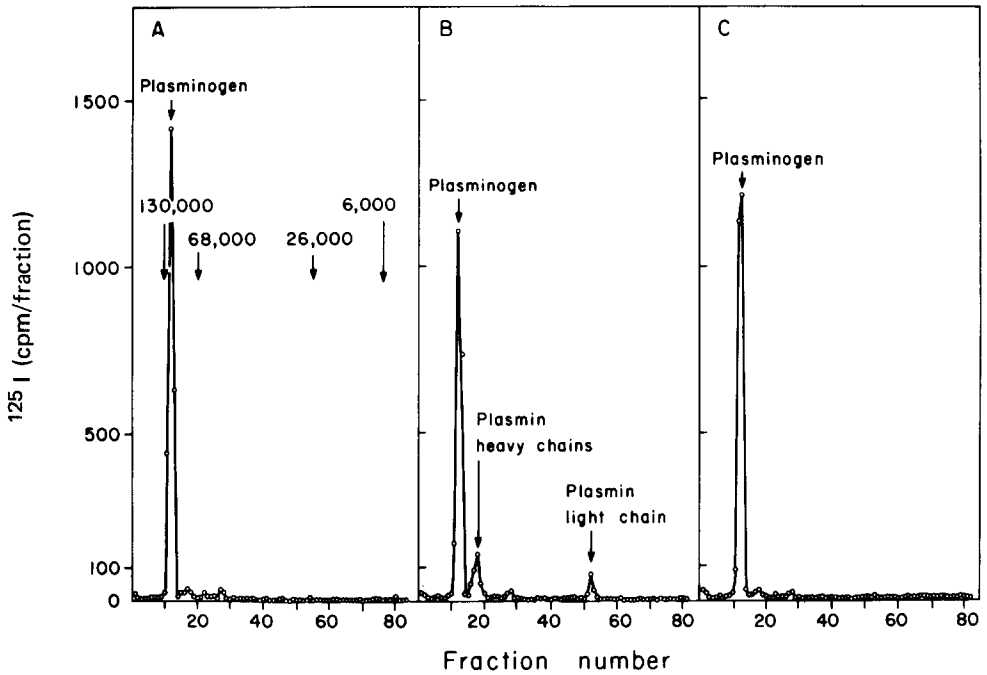

Fig. 1. Radioactivity profile of ^{125}I-labelled plasminogen before and after incubation with activator from transformed mouse cells. 2 μg/ml human ^{125}I-labelled plasminogen; 50 units/ml plasminogen activator from mouse cells and 300 Kallikrein inhibitor units/ml bovine pancreatic trypsin inhibitor were incubated for 1 h at 37°C in 0.1 M Tris-HCl (pH 8.1). The reaction was stopped by the addition of an equal volume of buffer (20% glycerol, 6% SDS, 10% β-mercaptoethanol, 0.125 M Tris-HCl, pH 6.8). After boiling for 2 min, 50-μl samples were applied to a slab gel of 11% polyacrylamide, and electrophoresis was performed. The gel was sectioned longitudinally and each strip was cut into 1.1-mm slices, which were assayed for radioactivity. (A) Plasminogen activator and incubation omitted; (B) incubation with plasminogen activator; (C) incubation without plasminogen activator. In (A) is indicated the localization of marker proteins. When urokinase (2.5 Plough units/ml)was used instead of the murine plasminogen activator, similar results were obtained.

A study of plasminogen activation as a function of enzyme concentration and time illustrates the protective effect of bovine pancreatic trypsin inhibitor on the activator as shown by the results presented in Fig. 2: in the presence of bovine pancreatic trypsin inhibitor, activation was linear for at least 20 min before beginning to level off; this was in contrast to the much shorter duration of the reaction (<5 min) in absence of the inhibitor (Danø, K. and Reich, E., unpublished). The initial rates of activation were determined at different concentrations of plasminogen; in a Lineweaver-Burk plot of the data (Fig. 2), all points fell on a straight line, indicating that the activation reaction was apparently a simple one that obeyed Michaelis-Menten kinetics. The value obtained for the K_m was 180 nM ($\approx$17 μg/ml), indicating that the enzyme is saturated and functioning at maximal rates at the circulating concentration of plasminogen (>2 μM) [16], provided that the affinity for murine plasminogen is the same as that for human plasminogen. *

* An apparent K_m of 30—40 μM was reported recently [40,41] for the activation of human plasminogens with urokinase at 25°C. It is difficult to compare these results with ours since, apart from the differences in plasminogen activators used in the two experiments, the differences in assay conditions could easily account for the discrepancies in apparent K_m values.

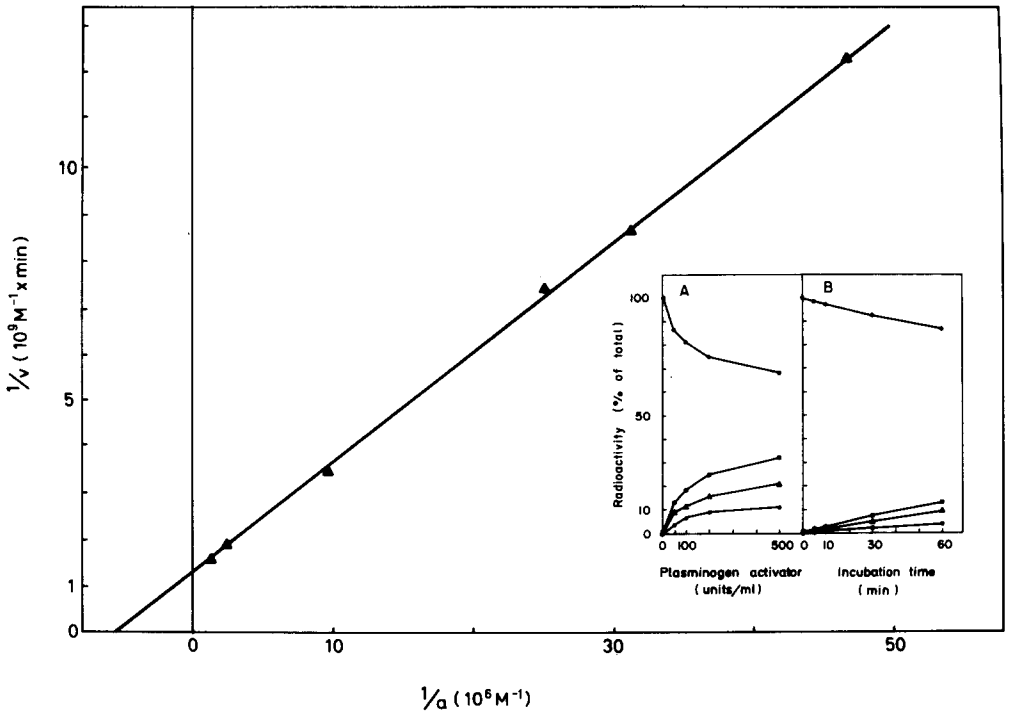

Fig. 2. Double reciprocal plot of initial rate of plasminogen activation as a function of plasminogen concentration. ^{125}I-labelled plasminogen in concentrations ranging from 2—66 μg/ml; bovine pancreatic trypsin inhibitor, 1000 Kallikrein inhibitor units/ml; and plasminogen activator from mouse cells, 50 units/ml, were incubated for 20 min at 37°C in 0.1 M Tris-HCl (pH 8.1). The reaction was stopped by addition of an equal volume of buffer (as described in legend to Fig. 1) and unlabeled, partially activated, plasminogen (100 μg/ml) which provided marker regions for subsequent excision from the gel. 25 μl of each sample were analyzed by gel electrophoresis as described in Fig. 1. The gel was stained, the bands containing plasminogen and the two chains of plasmin were excised, and the radioactivity was counted in a gamma counter. The amount of plasmin formed was obtained by subtraction of the radioactivity which was found in the regions corresponding to plasmin in a control channel in which ^{125}I-labelled plasminogen alone had been subjected to electrophoresis. In separate experiments, the activation of plasminogen was found to be linear with time for the initial 20 min within the concentration range of plasminogen which was used in this experiment. Inset: Plasminogen activation as a function of activator concentration and time. (A): 2 μg/ml ^{125}I-labelled plasminogen and plasminogen activator as indicated were incubated for 1 h at 37°C in 0.1 M Tris-HCl (pH 8.1). (B): Incubation as in (A), except that the reaction was stopped at the indicated time points. The plasminogen activator concentration during the incubation was 50 units/ml. ●———●, plasminogen; ○———○, plasmin light chain; △———△, plasmin heavy chain; □———□, plasmin total.

Fibrinolysis catalyzed by plasmin

As a basis for comparing the inhibitor spectrum of plasmin with that of plasminogen activator, the catalytic activity of plasmin was monitored using a standard fibrinolysis assay, in which proteolysis is measured by the rate of solubilization of ^{125}I-labelled fibrin. To obtain reproducible concentrations of pure plasmin, we used a modification of the method of Alkjaersig et al. [24] in which plasminogen is activated in 50% glycerol. According to Alkjaersig et al. [24], plasminogen was autoactivated in 50% glycerol. We were unable to confirm this claim: our preparations were stable when incubated for up to 96 h at 30°C under these conditions and gave no evidence of activation in the absence

of exogenous activator. We presume that their plasminogen fractions were contaminated with plasminogen activator. In contrast, small amounts of urokinase quantitatively converted plasminogen to plasmin during a 24-h incubation. The plasmin formed in this way was stable for months when stored at 4°C; the two polypeptide chains were electrophoretically indistinguishable from those generated by plasminogen activators in the absence of glycerol and in the presence of bovine pancreatic trypsin inhibitor.

When assayed under the standard conditions of the fibrinolysis assay, the rate of plasmin action was constant for 1 and 2 h at enzyme concentrations up to 0.4 and 0.2 μg/ml, respectively. The rate of proteolysis was linear with the enzyme concentrations up to 0.4 μg/ml for 1-h incubation times. At 0.2 μg/ml, the fibrinolytic activity of plasmin during 1 h was equal to that obtained with the combination of 2 μg/ml plasminogen and 50 units/ml of plasminogen activator.

Plasminogen activated in 50% glycerol is completely converted to plasmin without detectable change in molecular weight. The completion of the reaction and the stability of the product indicate that the proteolytic action of plasmin is largely, and probably completely, suppressed in this solvent. In accordance with this conclusion, only a single stained band of protein, which migrated at the same rate as plasminogen, was observed in SDS-polyacrylamide gels under non-reducing conditions. This effect of glycerol was very sensitive to concentration, since a decrease to 40% glycerol was accompanied by the appearance of several new bands of protein whose molecular weights were lower than 60 000; presumably they were plasmin-catalyzed degradation products of plasmin and/or plasminogen, these being the only two proteins present at significant concentrations in the reaction mixture.

Inhibition of fibrinolysis, of plasminogen activation and of plasmin

All compounds tested were first assayed in the overall fibrinolysis reaction; those possessing inhibitory activity were then examined further in the direct assays for plasminogen activation and plasmin-catalyzed fibrinolysis, respectively. All the assays were performed with 10-fold increments in inhibitor concentrations. A semilogarithmic dose-response curve was plotted and the inhibitor concentration giving 50% inhibition was determined, as illustrated in Fig. 3.

(a) Macromolecular trypsin inhibitors. Both plasminogen activator and plasmin are known to resemble trypsin in their specificity for basic amino acid residues, and several trypsin inhibitors have been reported to inhibit both fibrinolytic and other proteolytic activities of plasma and serum as well as a number of individual enzymes isolated from them (for a review, see ref. 28). A unique, general pattern resembling that already described for bovine pancreatic trypsin inhibitor was found for a spectrum of trypsin inhibitors isolated from a variety of plant and animal sources, including soybean, lima bean, sea anemone, cow colostrum, leeches (bdellin A and B and hirudin), boar seminal plasma and dog submandibularis gland. All of these proteins were active in blocking the overall fibrinolysis assay, the dose response curves in Fig. 3 being qualitatively typical for the entire group. In each case, inhibition was exerted exclusively at the level of plasmin, none of the agents having any significant effect on the activation of plasminogen.

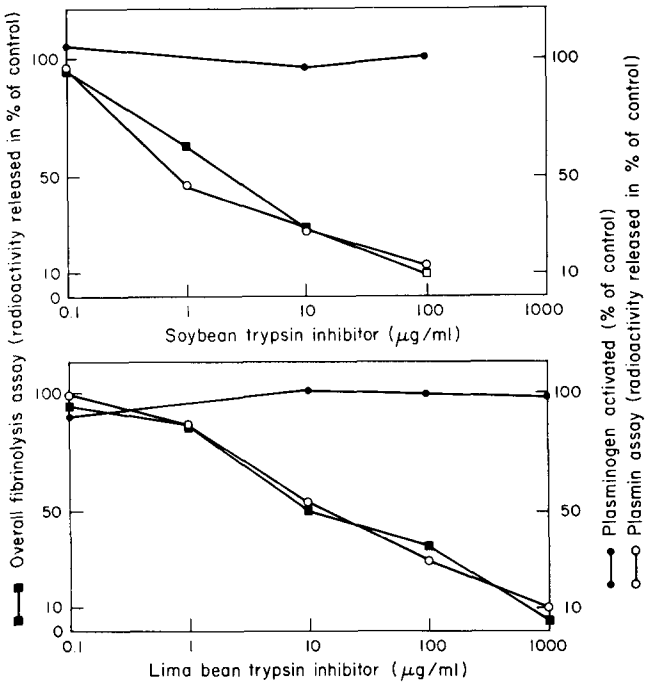

Fig. 3. Effect of soybean and lima bean trypsin inhibitors on plasminogen activation, plasmin assay, and overall fibrinolysis assay. The experimental conditions were as described in Materials and Methods and in the legend to Fig. 1. All the assays were incubated for 1 h at 37°C. The assays for plasminogen activation and the overall fibrinolysis assays contained plasminogen activator from transformed mouse cells, 50 units/ml.

(b) Dyes and related compounds. Both cationic and anionic dyes inhibited overall fibrinolysis; the more potent of these blocked both stages of the reaction, although to slightly different extents (Table I). We have not performed

TABLE I

EFFECT OF DYES AND RELATED COMPOUNDS ON THE ACTIVATION OF HUMAN PLASMINOGEN BY ACTIVATOR FROM TRANSFORMED MOUSE CELLS, PLASMIN CATALYZED FIBRINOLYSIS, AND THE OVERALL FIBRINOLYSIS ASSAY

Experimental conditions were as described in the legend to Fig. 3. In the assay for plasminogen activation and the overal fibrinolysis assay the concentration of plasminogen was 2 μg/ml (22 nM) and that of plasminogen activator 50 units/ml. In the plasmin assay, the plasmin concentration was 0.2 μg/ml 2.2 nM). For each inhibitor the concentration inhibiting 50% was obtained from a semilogarithmic dose-response curve with 10-fold increments in concentrations as in Fig. 3.

Inhibitor	Concentration inhibiting 50%		
	Plasminogen activation (mM)	Plasmin assay (mM)	Overall fibrinolysis assay (mM)
Trypan blue	0.0059	0.018	0.0067
Coomassie blue	0.0092	0.0031	0.0033
Evans blue	0.051	0.043	0.032
Germanin	0.070	0.40	0.026
Acridine orange	0.086	0.060	0.024
Orange IV	0.38	0.068	0.059
Phenol red	>1.0	0.17	0.150

kinetic analyses of the inhibition by any of these agents and the precise mechanism of inhibition by each remains to be established.

The action of trypan blue on plasminogen activator is of some interest, as its potency makes this dye one of the few effective inhibitors of the reaction. Plasminogen activator is strongly adsorbed to hydrophobic surfaces and the binding of trypan blue may possibly be mediated by the same part of the protein structure as that responsible for adsorption.

(c) Inorganic ions. A previous indication that the overall fibrinolysis reaction was strongly inhibited by elevated concentrations of sodium chloride [1] prompted us to survey the effects of a variety of inorganic substances. As seen in Table II, a number of these inhibited plasmin, plasminogen activator, or both. Zn^{2+} was particularly potent in blocking plasminogen activation, and rather less so in its effect on plasmin. Also prominent were the inhibitory activities of the rare earth metals, although these did not conform to a single pattern; the effect of each of these appeared to be distinctive, showing a preference either for plasminogen activation (Pr^{3+}, Yb^{3+}), or plasmin (Eu^{3+}, Cd^{2+}) or, in the case of Y^{3+}, equal activity against both enzymes.

We did not identify any anion that was capable of inhibiting either reaction significantly at concentrations below millimolar.

(d) Amino acids and related compounds. It is known that plasmin has a preference for lysine residues in protein substrates, consistent with the inhibition of enzymatic activity by lysine and by its derivative, ε-aminocaproic acid. By analogy, it might be expected that plasminogen activator, which hydrolyses an arginyl-valine bond in its substrate plasminogen [19,20], should be preferentially inhibited by arginine or its derivatives, and this was partly the case. As

TABLE II

EFFECT OF INORGANIC SALTS ON THE ACTIVATION OF HUMAN PLASMINOGEN BY ACTIVATOR FROM TRANSFORMED MOUSE CELLS, PLASMIN CATALYZED FIBRINOLYSIS, AND THE OVERALL FIBRINOLYSIS ASSAY

Experimental conditions were as described in the legend to Table I.

Inhibitor	Concentration inhibiting 50%		
	Plasminogen activation (mM)	Plasmin assay (mM)	Overall fibrinolysis assay (mM)
$ZnCl_2$	0.010	0.14	0.010
$PrCl_3$	0.023	0.22	0.010
YCl_3	0.031	0.037	0.014
$YbCl_3$	0.031	0.11	0.023
$LaCl_3$	0.070	0.068	0.045
$EuCl_3$	0.14	0.014	0.0084
$CdCl_2$	0.14	0.039	0.07
$FeCl_2$	0.16	0.12	0.13
$FeCl_3$	0.23	0.036	0.036
$HgCl_2$	0.45	0.20	0.30
$NiCl_2$	0.49	0.62	0.22
$CoCl_2$	1.1	1.1	0.74
$NaSO_3$	1.3	41	0.82
$BaCl_2$	1.5	320	2.9
NaCl	34	>1000	30

seen in Table III, the arginine compounds were in general more potent than the corresponding lysine derivatives as inhibitors of plasminogen activation, and potency within the arginine series was increased by eliminating a negative charge on the carboxyl group. However, the lysine derivative ϵ-aminocaproic acid, previously reported to inhibit urokinase-catalyzed hydrolysis of low molecular weight substrates [16], was equipotent with the arginine compounds as an inhibitor of plasminogen. The presence of a free carboxyl in ϵ-aminocaproic acid suggests that it may not be homologous with the arginine derivatives, and may bind differently to plasminogen activator. This possibility should be tested by more detailed enzymatic analysis or direct measurements of binding equilibria.

A comparison of the relative susceptibilities of plasminogen activation and plasmin to ϵ-aminocaproic acid indicates that the inhibition of the overall fibrinolysis reaction at lower concentrations of this compound is due principally to an effect on plasminogen activation.

(e) Various inhibitors. This group (Table III) includes a variety of compounds and it is heterogeneous with respect to the binding mode and inhibitory mechanisms. *p*-Nitrophenyl-*p'*-guanidino benzoate [29], diisopropyl phosphorofluoridate [30], leupeptin [31] and antipain [32] all bind covalently to

TABLE III

EFFECT OF AMINO ACIDS AND VARIOUS INHIBITORS ON ACTIVATION OF HUMAN PLASMINOGEN BY ACTIVATOR FROM TRANSFORMED MOUSE CELLS, PLASMIN CATALYZED FIBRINOLYSIS, AND THE OVERALL FIBRINOLYSIS ASSAY

Experimental conditions were as described in the legend to Table I.

Inhibitor	Concentration inhibiting 50%		
	Plasminogen activation (mM)	Plasmin assay (mM)	Overall fibrinolysis assay (mM)
p-Nitrophenyl-*p'*-guanidino benzoate	0.000006	0.000022	0.000005
Diisopropyl phosphorofluoridate	0.16	0.14	0.14
N-Acetyl-L-arginine methyl ester	0.17	8.6	0.14
L-Arginine methyl ester	0.29	4.9	0.26
Benzamidine	0.33	0.58	0.30
Aminomethyl cyclohexane carboxylic acid	0.35	0.038	0.028
ϵ-Amino caproic acid	0.35	1.0	0.28
Leupeptin	0.43	0.022	0.028
L-Lysine methyl ester	1.3	3.7	2.7
L-Cysteine methyl ester	1.9	9.0	2.9
Antipain	8.9	0.080	0.11
L-Lysine	9.0	33	2.4
N-α-Tosyl-L-lysylchloromethyl ketone	13	13	3.9
L-Arginine	14	340	11
Agmantine sulphate	25	36	4.8
	μg/ml	μg/ml	μg/ml
Human placental urokinase inhibitor (Kawano)	72	>100	49
Protamine sulphate	270	280	130
Concanavalin A	>1000	72	57

active sites of serine enzymes, but they differ with respect to mechanisms and reversibility. Of these, *p*-nitrophenyl-*p'*-guanidino benzoate was by far the most potent inhibitor, especially during short periods of incubation, but its effects on the two stages of the fibrinolysis reaction were not very different. The peptide aldehydes leupeptin and antipain also inhibited both plasmin and plasminogen activator, although plasmin was the more sensitive of the two under our assay conditions. Since diisopropyl phosphorofluoridate phosphorylates the active sites of most serine enzymes, its ability to inhibit both plasmin and plasminogen activator was not surprising. The low potency of diisopropyl phosphorofluoridate with these enzymes reflects, to a large extent, the kinetics of its reaction, since longer periods of incubation greatly increased its potency in molar terms; for example, 50% inhibition of overall fibrinolysis rates was produced when 2 μM diisopropyl phosphorofluoridate was incubated with plasminogen activator for 6 h at 37°C.

Benzaminidine and aminomethyl cyclohexane carboxylic acid are both reversible competitive inhibitors of some serine proteases [13,33]; these compounds are presumed to interact with trypsin-like proteases at the substrate binding site, by virtue of their resemblance to positively charged amino acid residues. Both compounds were active at the two stages of the fibrinolysis reaction, but whereas benzamidine was equally potent against both, aminomethyl cyclohexane carboxylic acid inhibited plasmin more effectively than plasminogen activator. This preference is of some interest, since aminomethyl cyclohexane carboxylic acid is a rigid analogue of ϵ-aminocaproic acid, and, in contrast, the latter preferentially inhibited the activation reaction under the same conditions.

Two proteins were observed to inhibit the overall fibrinolysis reaction. One

TABLE IV

EFFECT OF INHIBITORS ON THE ACTIVATION OF PLASMINOGEN CATALYZED BY UROKINASE

Experimental conditions were as described in the legend to Table I, except that urokinase (Leo, 2.5 Plough units/ml) was used instead of the murine plasminogen activator.

Inhibitor	Concentration inhibiting 50% (mM)
p-Nitrophenyl-*p'*-guanidino benzoate	0.000015
$PrCl_3$	0.0016
Coomasie blue	0.0040
YCl_3	0.0075
Trypan blue	0.015
$ZnCl_2$	0.033
Diisopropyl phosphorofluoridate	0.086
Benzamidine	0.9
L-Arginine methyl ester	1.1
Aminomethyl cyclohexane carboxylic acid	1.6
ϵ-Aminocaproic acid	1.9
L-Arginine	86
	μg/ml
Human placental urokinase inhibitor (Kawano)	82
Soybean trypsin inhibitor	>1000
Lima bean trypsin inhibitor	>1000

of these was the placental urokinase inhibitor isolated by Kawano et al. [14]; it selectively blocked the plasminogen activation reaction, although rather high concentrations were required. In contrast, the other protein, Concanavalin A, selectively inhibited plasmin, again at rather high concentrations; no attempt was made to identify the basis of this effect.

Inhibition of urokinase-catalyzed plasminogen activation

In view of the catalytic similarities between plasminogen activators produced in cell culture and urinary urokinase [4], it was of interest also to compare the responses of these enzymes to inhibitors; hence a selection of compounds was tested using amounts of urinary urokinase that were catalytically similar to the levels previously studied with transformed murine fibroblast activator. In general (Table IV), no qualitative differences were observed between the two enzymes: all substances inhibiting the murine activator also inhibited urinary urokinase, although there were some quantitative differences in both absolute and relative potencies. Of particular significance is the finding that two macromolecular trypsin inhibitors were inert with urokinase, just as they were with murine cell activator.

Discussion

Several of the results summarized in this paper seem worthy of comment. Reports from a number of laboratories have established that plasminogen exists in many forms differing in polypeptide chain length and *N*-terminal amino acid residues [34—36], isoelectric pH [18,31] and carbohydrate content [38]. In addition, plasma, urine and cell culture medium contain a variety of enzymes capable of activating plasminogen to plasmin. Although it is known that plasminogen activators resemble trypsin in their requirement for arginine and lysine residues, their proteolytic specificity for macromolecular substrates appears to be remarkably limited, since the ability of these enzymes to attack proteins other than plasminogen has never been demonstrated convincingly. It follows that the recognition sites of plasminogen activators for macromolecular substrates must be exacting in their specificity, and an assessment of the physiological significance, if any, of multiple activators, plasminogens and natural and synthetic inhibitors, must be based on a direct study of the plasminogen activation reaction itself, rather than on the use of small synthetic substrates.

With the preceding considerations in mind, the assay that we have used in the present work appears to be satisfactory. Although the conversion of ^{125}I-labelled plasminogen to ^{125}I-labelled plasmin as monitored by SDS-polyacrylamide gel electrophoresis is somewhat laborious and time-consuming, these disadvantages are outweighed by the fact that the results bear unambiguously on the activation reaction, and they are not subject to uncertainties arising from the indiscriminate proteolytic action of plasmin, which is autolytic and inactivates both plasminogen and the activators. Furthermore, the kinetic properties of the system conform to those expected of single enzymatic reactions; this permitted, a reliable determination of the K_m of plasminogen activators with plasminogen as substrate and further applications for quantitative characterization of different plasminogens, activators and inhibitors appear reasonable.

A number of general conclusions can be drawn from the spectrum of inhibitory compounds identified in this study:

(1) the inhibitor profiles for plasminogen activators of human and murine origin appear qualitatively identical; taken together with the fact that they are serine proteases [3,4,17,39], these results also establish the activators secreted by transformed and neoplastic cultures as enzymes with catalytic properties homologous to those of human urokinase.

(2) The plasminogen activators, and urokinase, are remarkable for their resistance to all but one of the macromolecular protease inhibitors tested. The spectrum of substances tested included agents known to inhibit most known classes of trypsin-like enzymes, even those with narrow substrate specificity (e.g. thrombin). The failure of plasminogen activators to interact with such inhibitors re-emphasizes the exacting macromolecular binding specificity of these enzymes, already reflected in their restricted range of substrates.

(3) The inhibitor spectrum of plasminogen activators differs clearly from that of plasmin; however, there are also many similarities, and these demonstrate the pitfalls in the use of overall fibrinolysis reactions for assaying potential inhibitors of the activation reaction.

(4) We have identified several new (but not specific) inhibitor of plasminogen activation, including, among others, a group of large aromatic dyes (trypan blue, Coomassie blue), their derivatives (germanin), and a number of polyvalent cations (Zn^{2+}, La^{3+} and other rare earths). While the properties of these substances make them less useful as general probes for the biological roles of plasminogen activation, they are likely to be convenient adjuncts in isolation and purification of plasminogen activators and other proteases.

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