Proc. Natl. Acad. Sci. USA
Vol. 75, No. 2, pp. 750–753, February 1978
Biochemistry

Direct fluorescent assay of urokinase and plasminogen activators of normal and malignant cells: Kinetics and inhibitor profiles

(fluorogenic substrates/protease inhibitors/protease secretion/plasmin/trypsin-like enzymes)

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Communicated by Roy P. Vagelos, November 23, 1977

ABSTRACT **A direct rate assay for plasminogen activator has been developed using a synthetic fluorogenic peptide substrate, 7-(*N*-Cbz-glycylglycylargininamido)-4-methylcoumarin trifluoroacetate. The assay correlates well with the standard ^{125}I-labeled fibrin plate assay using highly purified urokinase, culture fluids from WI-38, Chinese hamster ovary or HeLa cells, or Rous sarcoma virus-transformed chick fibroblasts as the source of plasminogen activator. The assay is sensitive, rapid, and linear throughout a wide range of enzyme concentrations. With this substrate it is possible to determine inhibitor profiles for the various plasminogen activators, independently of the interfering potential of plasmin. All of the enzymes tested are inhibited by leupeptin and antipain but not by the related aldehydes, elastatinal and chymostatin. The macromolecular inhibitors soybean trypsin inhibitor and trasylol have little or no effect on the plasminogen activators tested. This substrate should be useful for the study of the effect of various agents on functional changes in cells secreting this enzyme and also should allow kinetic measurements of potential inhibitors.**

The enhanced production of plasminogen activator (PA) activity has been shown to be a characteristic of many different cell types. The intracellular and extracellular levels of PA have been demonstrated to be substantially elevated in malignant cells in culture (1–6), cells treated with a tumor promoter (7), activated macrophages (8, 9), established cell lines (10, 11), granulosa cells during ovulation (12), embryonic cells during differentiation (13, 14), and hormone-treated uteri (15). The standard system used for measuring PA in these cells is an indirect, two-step assay in which plasminogen is incubated with a source of PA and the plasmin activity generated is quantitated by using fibrin, casein, or protamine as substrates (16–19). There is a need, however, for a simple, sensitive, direct assay that allows both rapid measurement and kinetic analysis of PA, independent of plasmin generation. In addition, the presence of two proteases of similar specificities in the two-step assay precludes the screening of potential PA inhibitors.

A series of synthetic fluorogenic substrates, specific for a number of serine proteases, utilizing the leaving group 7-amino-4-methylcoumarin (AMC) has been described (20, 21). In a continuation of this approach, we have now prepared a synthetic peptide specific for the cleavage site of PA, incorporating the same leaving group. This compound is Cbz-Gly-Gly-Arg-AMC. We report here the use of this substrate in the direct fluorescent assay of PA from normal and malignant cells, some kinetic parameters of these enzymes, and the effect of various low and high molecular weight protease inhibitors on the various enzymes. The various PAs were analyzed by the direct fluorescent technique in parallel with the indirect standard ^{125}I-labeled fibrin plate assay using purified plasminogens from both canine and bovine sources.

MATERIALS AND METHODS

Enzymes. Highly purified urokinase (UK) was obtained from Alan Johnson, Department of Medicine, New York University Medical School. Human plasmin was obtained from M. Mosseson, Department of Medicine, Downstate Medical Center, SUNY. Purified human granulocyte elastase and cathepsin G were prepared as described (22). Hog pancreatic elastase, chromatographically purified, was obtained from Miles Laboratories. Bovine α-chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp.

Inhibitors. Trasylol was obtained from FBA Pharmaceuticals, and soybean trypsin inhibitor (SBTI) was from Worthington Biochemical Corp. Leupeptin, antipain, elastatinal, and chymostatin were provided by the U.S.–Japan Cooperative Cancer Research Program.

Preparation of PA-Containing Culture Fluids. Primary cultures of chicken embryo fibroblasts were prepared, maintained, and infected with Rous sarcoma virus as described (23); these infected cells are designated RSVCEF. Fully transformed cultures of RSVCEF were grown in Eagle's minimal medium supplemented with plasminogen-free fetal bovine serum (24). When the cultures had attained high cell density (1×10^7 cells per 100-mm culture dish), the plates were washed three times with minimal medium and further incubated in serum-free minimal medium. The medium was removed from the cultures every 12 hr and was a source of extracellular PA; it is referred to as harvest fluid (HF). After it was harvested, the HF was immediately centrifuged to remove cells and cellular debris and acidified to pH 3.5 by the addition of 1 M HCl. Ammonium sulfate was added to 70% saturation, and the resulting precipitate was recovered by centrifugation and resuspended in 1/100 the volume of the original HF in 0.05 M glycine·HCl buffer, pH 3.0.

WI-38, HeLa, and Chinese hamster ovary cells were grown in serum-containing medium, washed, and incubated in serum-free Higuchi medium (25). This medium was removed after 24 or 48 hr and was the source of extracellular PA. These HFs were centrifuged, acidified, and concentrated as described for the Rous HF.

All of the HF concentrates were dialyzed for 16 hr against 0.05 M sodium acetate, pH 5.25, to remove the residual ammonium sulfate (at concentrations of 0.1 M and greater, ammonium sulfate inhibits the fluorometric assay). They were

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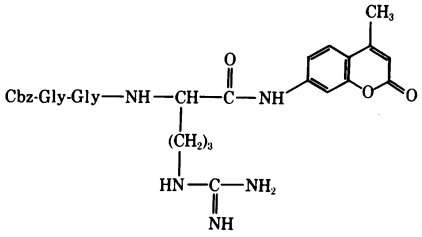
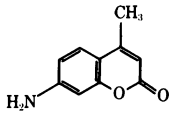
Abbreviations: PA, plasminogen activator; AMC, 7-amino-4-methylcoumarin; UK, urokinase; SBTI, soybean trypsin inhibitor; RSVCEF, Rous sarcoma virus-infected chicken embryo fibroblasts; HF, harvest fluid.

Table 1. Fluorescence properties of substituted 4-methylcoumarins

	Maxima, nm	
	Excitation	Emission
Substrate	325	395
		
Product	345	445
		
Optimum difference for assay purposes	383	455

stored frozen and used as sources of PA in both the fluorescent and fibrin plate assays.

Synthesis of Fluorogenic Substrate. *N*-Cbz-Arg-AMC was prepared essentially as described (21). The compound was purified by chromatography on silica gel (Baker) with $CHCl_3$/MeOH, 70:30 (vol/vol), as the developing solvent. After deblocking with 32% HBr in acetic acid, the Arg-AMC·HBr was coupled to Cbz-Gly-Gly by using isobutylchloroformate (26). Cbz-Gly-Gly was prepared from Cbz-chloride and glycylglycine under Schotten–Baumann conditions. The substrate was purified again on silica gel with $CHCl_3$/MeOH, 90:10 (vol/vol), as the developing solvent. Purity was determined by thin-layer chromatography, amino acid analysis, and nuclear magnetic resonance spectroscopy.

Fluorometric Assay for PA. Enzyme assays with the fluorogenic substrate were conducted at 24° with 0.1–0.5 mM substrate in 0.05 M 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate buffer, pH 7.5/5% (vol/vol) dimethyl sulfoxide; the final volume was 0.5 ml in each assay. Fluorescence of the AMC was monitored continuously by using a Perkin–Elmer 204A spectrofluorometer equipped with a chart recorder. Although both the substrate and its hydrolysis product are highly fluorescent, their excitation and emission wavelengths are distinctly different (20, 21). Activation and emission wavelengths (383 and 455 nm, respectively) were chosen such that AMC retained 20% of its maximal fluorescence but possessed a relative fluorescence 500-fold greater than that of an equimolar amount of Cbz-Gly-Gly-Arg-AMC (Table 1). The instrument was standardized daily so that 10 nM AMC gave 1.0 chart division (0–100 scale).

Fibrin Plate Assay for PA. PA was assayed on ^{125}I-labeled fibrin-coated petri dishes with purified plasminogen-free fibrinogen and purified fetal bovine or dog plasminogen as described (23).

RESULTS

Specificity and Kinetics of the Fluorometric Assay. Cbz-Gly-Gly-Arg-AMC is not only an excellent substrate for UK but also can be used to detect the presence of PA, either directly or after concentration in cultures with lower levels of activity, in the serum-free medium bathing various cells. Using this substrate, we measured PA activity secreted from HeLa, Chinese hamster ovary, and WI-38 cells and RSVCEF.

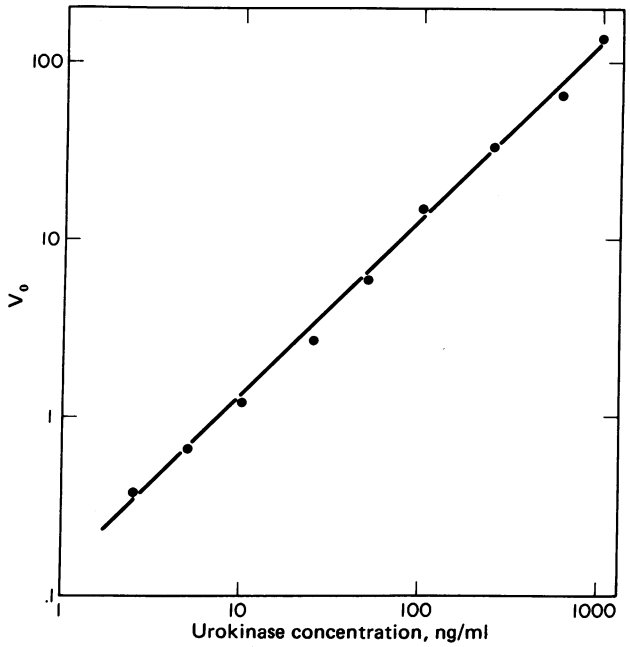


FIG. 1. Initial velocity versus urokinase concentration. Initial velocity (V_0) is expressed as nmol of AMC released per min.

As shown in Fig. 1, the limit of detection of UK with this substrate and a reaction time as short as 5 min is 2.5 ng of protein per ml, and the rate of hydrolysis is proportional to enzyme concentration over at least a 400-fold range. The sensitivity could easily be increased by longer incubation times. The rate of hydrolysis of RSVCEF HF concentrate also is linear over at least a 100-fold range (Fig. 2). The K_m for UK with this fluorogenic substrate is 4×10^{-4} M; with the RSVCEF concentrate it is 6×10^{-4} M.

Neither UK nor the RSVCEF activator hydrolyzed, at a significant rate, the fluorogenic substrates for elastase, trypsin, and chymotrypsin reported previously (11). Conversely, Cbz-Gly-Gly-Arg-AMC is not significantly hydrolyzed by pancreatic elastase, granulocyte elastase, chymotrypsin, or granulocyte cathepsin G. However, like many arginine peptides, the substrate is hydrolyzed by trypsin, thrombin, and plasmin. The possible presence of the latter enzyme is monitored in our assay systems both by the plasminogen dependence

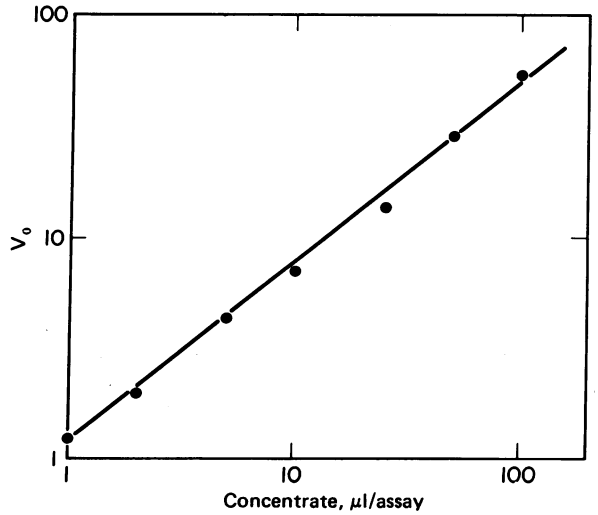


FIG. 2. Initial velocity versus concentration of HF concentrate from RSVCEF. Initial velocity (V_0) is expressed as nmol of AMC released per min.

Table 2. Comparison of fibrinolytic and fluorometric assays for plasminogen activator

Plasminogen	PA	Fibrinolytic assay: Incubation time, hr	^{125}I released, cpm	Ratio RSVCEF/UK
Dog, 7 μg	5 μl RSVCEF	1.5	1,314	1.37
	5 μl UK	1.5	960	
	5 μl RSVCEF	4.0	4,812	1.13
	5 μl UK	4.0	4,263	
	5 μl RSVCEF	7.0	13,550	1.01
	5 μl UK	7.0	13,406	
	10 μl RSVCEF	1.5	2,930	1.15
	10 μl UK	1.5	2,539	
	10 μl RSVCEF	4.0	11,900	1.38
	10 μl UK	4.0	8,607	
	10 μl RSVCEF	7.0	27,887	1.20
	10 μl UK	7.0	23,237	
Fetal bovine, 7 μg	5 μl RSVCEF	1.5	14,863	11.94
	5 μl UK	1.5	1,245	
	10 μl RSVCEF	1.5	20,162	10.16
	10 μl UK	1.5	1,985	

PA	Fluorometric assay: AMC released, nmol/min	Ratio, RSVCEF/UK
10 μl RSVCEF	5.3	1.23
10 μl UK	4.3	
20 μl RSVCEF	14.0	1.31
20 μl UK	10.7	

* UK solution contained 5 μg of protein per ml.

of the fibrin plate assays and the response to inhibitors described below.

Correlation of Fibrinolytic and Fluorometric Assays. We correlated the enzyme activity responsible for the hydrolysis of Cbz-Gly-Gly-Arg-AMC with PA activity measured in the standard fibrinolysis assay. As shown in Table 2, we found an excellent correlation of RSVCEF PA/UK ratios between the fluorometric assay and the fibrin plate assay with dog plasminogen. Fetal bovine plasminogen appears to be much more sensitive to the RSVCEF enzyme. With dog plasminogen, the relative activities of the PAs in concentrates from HeLa, Chinese hamster ovary, and WI-38 cells were determined in the two assays. On the basis of assigning the HeLa concentrate an activity of 1.0, the HeLa/hamster ovary/WI-38 ratio in the fibrinolytic assay was 1.0:1.1:6.9; in the fluorometric assay it was 1.0:0.95:8.5, which is again a good correlation. It should be emphasized that, in the absence of plasminogen, there was little or no detectable fibrinolytic activity with 10 μl of either UK or any of the concentrates added to the fibrinolytic system. Therefore, the activity being detected in these fluids is indeed PA and not plasmin. The fluorogenic substrate, added as a stock concentrate in dimethylsulfoxide, has no effect on the fibrin plate assay; therefore, neither the substrate itself nor the vehicle at the concentrations used is inhibitory to either PA or plasmin.

Inhibitor Studies. Using the fluorogenic substrate, we determined the effect of various specific protease inhibitors on UK and the enzymes derived from various cell lines. Table 3 shows that leupeptin and antipain are inhibitors of both UK and the RSVCEF enzyme with K_i values of approximately the same order of magnitude. These two compounds are competitive inhibitors, based on Dixon plots. On the other hand, neither chymostatin, an inhibitor of chymotrypsin-like enzymes, nor elastatinal, a specific inhibitor of elastase (27), inhibited these activators at a concentration of 100 μg/ml. As expected, both UK and the RSVCEF enzyme were inhibited by diisopropylfluorophosphate.

Table 3. K_i values of leupeptin and antipain with two plasminogen activators

Enzyme	K_i, M: Leupeptin	Antipain
Urokinase	2.6×10^{-5}	6×10^{-5}
RSVCEF PA	1.6×10^{-5}	2×10^{-5}

The response of PAs from various sources to several known inhibitors of trypsin-like enzymes is of considerable interest. As shown in Table 4, all of the enzymes tested are inhibited by both leupeptin and antipain, but in all cases leupeptin is the better inhibitor. UK and the enzymes from HeLa, hamster ovary, and WI38 cells have similar leupeptin/antipain inhibitory ratios but are not inhibited by trasylol or SBTI. The RSVCEF enzyme exhibits a leupeptin/antipain ratio similar to that of the other PAs but is inhibited slightly by SBTI. Plasmin is inhibited by much lower concentrations of all these inhibitors, again clearly demonstrating that we are measuring PA, not plasmin, in the extracellular fluids.

DISCUSSION

We have developed a direct rate assay for PA that uses a synthetic fluorogenic peptide. The assay results correlate with those of the standard fibrin plate assay using culture fluids from various cells as the source of PA. The assay is sensitive, rapid, and allows for the determination of kinetic constants and inhibitor profiles.

The sequence of the synthetic peptide was chosen to optimize the activity with PA. Kettner *et al.* (28) prepared a peptide chloromethyl ketone, with the sequence Ac-Gly-Gly-Arg-CH_2Cl, which inhibits UK 23-fold better than it inhibits plasmin (8), identifying this sequence as being desirable. Huseby *et al.* (29) recently reported the detection of UK activity by using a similar peptide linked to the leaving group 4-methoxy-2-naphthylamine; however, the leaving group was detected indirectly by using a fast blue dye. In another approach to the direct assay of PA, Dano and Reich (30) reported a use of the macromolecular substrate, radiolabeled plasminogen. They determined hydrolysis of this substrate by electrophoresis, measuring the appearance of the heavy and light chains of plasmin.

Use of the substrate reported in this study has allowed us to

Table 4. Inhibition of PAs from various sources and plasmin by inhibitors of trypsin-like enzymes

Enzyme	ID_{50},* μM: Leupeptin	Antipain	Trasylol	SBTI
Urokinase	67	165	>12	>4.5
RSVCEF PA	38	100	>12	1.4
HeLa PA	38	165	>12	>4.5
WI-38 PA	110	165	12	>4.5
CHO PA†	89	200	>12	>4.5
Plasmin	1.1	6.6	0.046	0.0031

Substrate: 0.5 nM Cbz-Gly-Gly-Arg-AMC in 0.05 M pH 7.5 buffer, containing 5% dimethyl sulfoxide.

* 50% inhibitory dose, the concentration that inhibits 50% of the enzyme activity under assay conditions described in text.

† CHO, Chinese hamster ovary.

detect directly PAs from cultures of transformed fibroblasts (RSVCEF), a malignant cell line (HeLa), and two nonmalignant cell types (WI-38 and Chinese hamster ovary). In addition, purified UK was detected and compared to the PAs from the cultures. With all of the PAs tested, the fluorometric assay showed an excellent correlation with the fibrin plate assay (Table 2); however, the fluorometric assays were performed more rapidly and in the absence of plasminogen. The striking difference between the activation of canine and bovine plasminogens by UK and the RSVCEF activator should be noted. However, differences between plasminogens from different sources with respect to their activation by various PAs have been reported (24). The fluorometric assay was demonstrated to be linear throughout a wide range of enzyme concentrations and allowed determination of K_m values with different enzymes for the substrate and K_i values for inhibitors. Thus, with this assay it was possible to establish an inhibitor profile for this important enzyme. The macromolecular inhibitors SBTI and Trasylol had little or no effect on the PAs tested, in agreement with the observations by Dano and Reich (30). On the other hand, we found inhibition with leupeptin and antipain but not with the related aldehydes elastatinal and chymostatin. We also found that leupeptin and antipain were competitive inhibitors.

This direct assay should facilitate monitoring of enzyme purification and studying functional changes in cells as measured by changes in enzyme secretion, as well as the large-scale testing of PA inhibitors independently of the interfering potential of plasmin.

Note Added in Proof. Since this manuscript was initially submitted, a similar substrate for urokinase has been reported (31).

A part of this work was performed at the Marine Biological Laboratory, Woods Hole, MA. A part of this work was supported by grants from the National Institutes of Health (CA16740) and the American Cancer Society (BC163) awarded to J.P.Q.

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