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INACTIVATION OF THE PLASMINOGEN ACTIVATOR FROM HeLa CELLS BY PEPTIDES OF ARGININE CHLOROMETHYL KETONE

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Summary

The binding specificities of human urinary urokinase (EC 3.4.99.26) and HeLa cell plasminogen activator were studied using peptidyl chloromethyl ketone inhibitors. A ^{125}I-labeled fibrin assay has been developed to yield kinetic information. Reagents of the sequence X-Gly-ArgCH_2Cl were the most effective. The susceptibility of the HeLa cell plasminogen activator differed from that of urokinase in several respects indicating the utility of this type of inhibitor in distinguishing between proteases of this specificity.

Introduction

In the preceding paper [1], the binding specificity of human urokinase was studied using a series of peptide chloromethyl ketone inhibitors. Although urokinase (EC 3.4.99.26) has been the most studied purified activator of mammalian origin, a number of plasminogen activators have been described from a variety of sources which may have greater importance than urokinase [2]. We have chosen to study the plasminogen activator from the human cervical carcinoma HeLa cell line and to compare it with human urinary urokinase.

The effects of plasminogen activators were first observed by Fischer in 1925 [3]. Recent reports have further established that fibrinolytic activity and neoplastic growth are related activities (for review, see Ref. 4). Notable in this field have been reports of Reich and coworkers [5—9] describing roles of plasminogen activator in pathological (neoplastic) and non-pathological physiological states (ovulation, embryogenesis).

Abbreviations: DNS, *N,N*-dimethylaminonaphthalene-5-sulfonyl; Boc, *t*-butoxycarbonyl; Z, carbobenzoxy.

An important, unresolved question is whether the plasminogen activator is the same as or different from urokinase in specificity. Definitive conclusions cannot be made until plasminogen activator and urokinase are obtained from the same species and compared in the same series of experiments.

We report here a comparison, using human urokinase and HeLa plasminogen activator, of the relative susceptibilities of the two enzymes to a series of peptidyl chloromethyl ketones. These techniques have previously defined binding specificities for thrombin [10], kallikrein [11]. The assay uses ^{125}I-labeled fibrinolytic activity coupled through plasmin [5] and this technique has been improved to yield kinetic information about plasminogen activator.

Experimental

Preparation of plasminogen. Plasminogen was prepared from frozen calf serum (Pacific Biologicals, Richmond, CA) by the method of Deutsch and Mertz [12].

^{125}I incorporation into fibrinogen. Fibrinogen (Calbiochem-Behring) was radioiodinated according to the general procedure of Gilbert and Wachsman [13] with modifications designed to enhance the specific radioactivity and lower the ratio of incorporated iodine to fibrinogen. To 5—10 mg fibrinogen (in 1 ml Tris-HCl/KCl buffer) was added 20 μg lactoperoxidase (Calbiochem-Behring) and 3—5 mCi carrier-free ^{125}I (as sodium salt, New England Nuclear). The reaction was initiated by addition of 2 μl H_2O_2 solution (30 μl 30% H_2O_2 in 100 ml H_2O). The solution was allowed to react at ambient temperature for 1—2 h with further addition of 2 μl H_2O_2 at 30-min intervals. The reaction was terminated by the addition of 50 μl of 2% NaN_3 and 50 μl 7% Na_2SO_3. No carrier iodide was added to the reaction mixture. This kept the percentage of modified residues to a minimum without loss of incorporation, which was always 90—99%. Separation of ^{125}I-labeled fibrinogen from $Na^{125}I$ was accomplished using a column of Sephadex G-25 in the Tris-HCl/KCl buffer. Protein fractions of high radioactivity were stored at —20° C. Their useful lifetime was 4—6 weeks before apparent radiation damage resulted in poor clotting percentages. Specific radioactivity ranged from 0.5 to $1.0 \cdot 10^{12}$ cpm/g; the molar ratio of iodine to fibrinogen was 0.10—0.20. Thus, use of carrier-free ^{125}I yields a product essentially identical to unmodified fibrinogen in contrast to that of Gilbert and Wachsman [13] in which 1.2% of the ^{125}I was incorporated and the molar ratio was 5—10 iodines/fibrinogen.

Preparation of fibrin plates. The stock solution of ^{125}I-labeled fibrinogen was diluted with distilled H_2O to provide $1—2 \cdot 10^7$ cpm/ml. 100-μl portions were pipetted into each well of a 96 well, flat bottom Linbro tissue culture plate (6-mm diameter wells with a capacity of 200 μl, Linbro Co New Haven, CT). The plates were evaporated to dryness at 45—50° C. Clotting was initiated by addition of 10% calf serum (plasminogen free) and allowed to continue 8—16 h at 37° C. The wells were then given 2—3 60-min washes at 37° C with phosphate-buffered saline. After this treatment, each well contained $3—6 \cdot 10^5$ cpm immobilized and the last wash contained fewer than 3000 cpm. The plates could either be used immediately or stored dry, for a week at —20° C.

Assay of plasminogen activator. Assay mixtures contained 50 nM plasmino-

gen, varying amounts of plasminogen activator or urokinase, plus any other variable constituents, such as inhibitor, remaining from the preincubation. The assay buffer was phosphate-buffered saline (pH 7.4) and the final assay volume was 200 μl. The plate was slowly shaken in a 37° C water bath. 10-μl aliquots were removed at 15—30-min intervals. After 6—8 time points, the residual solution was also removed. Total evaporation was less than 5% at the termination of the assay. Radioactivity was determined with a Beckman 8000 Gamma counter. Graphical displays of the reaction profiles for each well were obtained on a CDC 7600 computer, which also computed linear and quadratic fits of the data. Plasminogen-free controls invariably exhibited no enzymic activity.

Inhibition studies. Time-dependent reactions of inhibitors with either plasminogen activator or urokinase were initiated by the ten-fold dilution of stock solutions of the inhibitor (prepared in 1 mM HCl) into solutions of the proteases in phosphate-buffered saline (pH 7.4). After an arbitrary reaction time of 30 min at 37° C, the reactions were terminated by 20—50-fold dilution into the assay mixture in the fibrin plate wells. Control experiments were performed on any compounds which showed inhibitory properties to determine whether the effects were the result of inhibition of the subsequently formed plasmin. No such examples were found for inhibitors with standard concentrations of less than 0.1 mM. The interpretation of the above-described procedures presumes that there is no reversal of inactivation during the time of the assay.

The rate of inhibition by 0.1 μM DNS-Glu-Gly-ArgCH_2Cl was studied by removing aliquots of the inhibition solution and dilution of the sample into the solution in the fibrin well at 0° C. Upon completion of inhibitory preincubation all plasminogen activation assays were started simultaneously by addition of plasminogen and transfer of the plate to a 37° C bath.

Growth of HeLa cells. Cells were cultured at $0.5 \cdot 10^6$ cells/ml in serum-free Dulbecco modified Eagle's medium containing $1 \cdot 10^{-8}$ M phorbol myristate acetate [14]. Cells were harvested by centrifugation after 36—48 h culture, the time of maximum plasminogen activator activity determined in a time course experiment. The conditioned medium was used without further purification or concentration. The plasminogen activator in as little as 1 μl medium was easily detectable in the standard assay.

Phorbol myristate acetate was obtained from Dr. Peter Borcheart (University of Minnesota). Urokinase was the product of Leo Pharmaceutical Products (Denmark, 9092207 1712E).

Results

Analysis of inhibitors of proteases in this laboratory [1,10,11] has generally involved the direct measurement of the loss of enzymatic activity via a spectrophotometric esterase assay. However, none of the available assays could be adapted to the spectrophotometric analysis of the plasminogen activator of phorbol myristate acetate-stimulated HeLa cells owing to very low levels of the enzyme. We therefore optimized the coupled fibrinolytic assay to provide a relatively rapid kinetic determination of enzyme activity rather than just an end point assay.

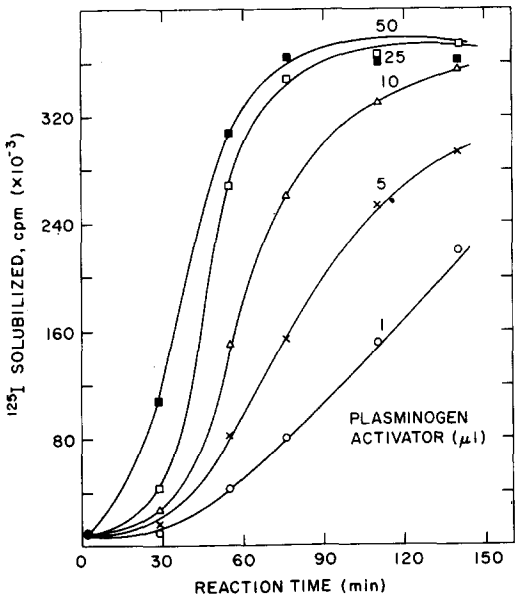

Fig. 1. The solubilization of ^{125}I-labeled fibrin at varying levels of HeLa plasminogen activator. See Experimental for details.

Upon combination of plasminogen and plasminogen activator in a reaction well with ^{125}I-labeled fibrin, there is an initial lag in secondary product (radioactivity) release (Fig. 1) while plasmin is formed followed by an exponential increase. In this phase, the effect of increases in plasminogen activator activity is to greatly enhance the rate of peptide solubilization and to decrease the length of the lag phase. The exponential growth of the reaction rate terminates when all the available fibrin is consumed.

Since the intermediate in this system is another enzyme, a simple, linear relationship between plasminogen activator and the rate of ^{125}I-labeled fibrin solubilization does not exist. To simplify numerical comparisons we have chosen to use the time to reach the inflection point in the reaction profile (the time needed to solubilize half the fibrin clot) in comparisons of plasminogen activator activities.

Wigler and Weinstein [14] originally observed that phorbol myristate acetate stimulated the production of plasminogen activator in HeLa cells in tissue culture. We have corroborated these observations. Under their conditions, we observed that plasminogen activator activity in the medium was 25-fold greater than the phorbol myristate acetate-free control and that there is considerably greater activity in the conditioned medium than in the cell lysate. Loskutoff and Edgington [15] observed similar behavior in cultured endothelial cells which they showed was caused by a cytosol inhibitor of the activator.

Presented in Fig. 2 are reaction profiles typical of a series of survey experiments. All the inhibitors were incubated at 100 μM with the enzyme for 30 min prior to dilution and initiation of the assay. In this experiment there was total inhibition by Ile-Glu-Gly-ArgCH_2Cl and Phe-Ala-ArgCH_2Cl yielded almost total inhibition. Pro-Phe-D-ArgCH_2Cl was the equivalent of no inhibitor and

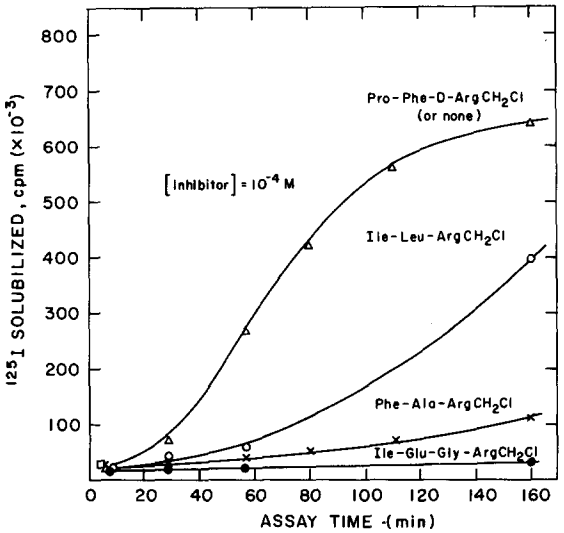

Fig. 2. Levels of fibrinolysis after incubation of HeLa plasminogen activator with 100 μM arginine chloromethyl ketones for 30 min. The time to solubilize half the clot in the absence of inhibitor or with Pro-Phe-D-ArgCH$_2$Cl is 70 min and with Ile-Leu-ArgCH$_2$Cl is 150 min.

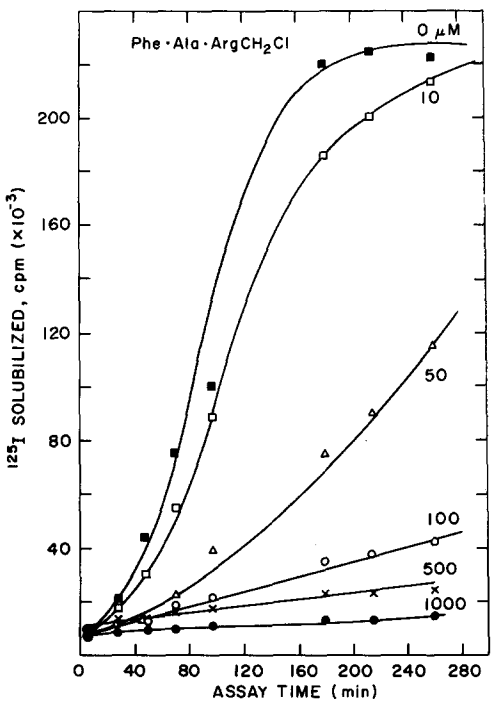

Fig. 3. Inactivation of HeLa plasminogen activator by Phe-Ala-ArgCH$_2$Cl. The time to solubilize half the fibrin clot in the absence of inhibitor is 80 min, 100 min for 10 μM, and 240 min for 50 μM. The times for the higher inhibitor concentrations are too long for accurate estimates.

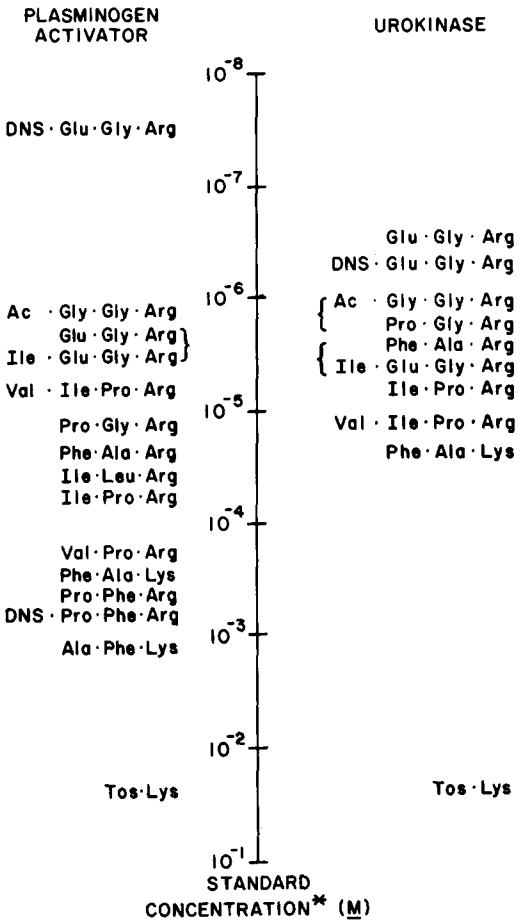

Fig. 4. The relative effectiveness of chloromethyl ketone inhibitors with plasminogen activator and urokinase. The position of the chloromethyl ketone on the log concentration scale indicates the approximate value of its 'standard concentration'. Inhibitors with close to identical values are signified by brackets. * The concentration of inhibitor which increases the time of the mid-point in the reaction profile to twice that of the uninhibited control.

Ile-Leu-ArgCH_2Cl was of intermediate effectiveness. A large excess of urokinase resulted in the solubilization of approximately the same amount of ^{125}I-labeled fibrin.

Subsequent experiments investigated the concentration dependence of the inhibitors at concentrations in their range of intermediate inhibition. Data for Phe-Ala-ArgCH_2Cl are presented in Fig. 3. The inhibitor at 10 μM displaces the reaction profile only slightly to the right whereas 1000 μM completely inhibits. Phe-Ala-ArgCH_2Cl at 50 μM displaces the profile considerably, the time to solubilize half the fibrin increasing from 80 min (no inhibitor) to 240 min.

For the comparison of inhibitors, it was decided to establish for each the 'standard concentration' for inhibition, defined as that concentration required to increase the time to solubilize half of the fibrin radioactivity to twice that time required by the uninhibited control.

TABLE I

COMPARISON OF FIBRINOLYTIC AND ESTEROLYTIC ASSAYS FOR MONITORING UROKINASE INHIBITION

Fibrinolytic *		Esterolytic **	
Inhibitor	Standard concn. (μM)	Inhibitor	k_{app}/I ($M^{-1} \cdot min^{-1}$) ($\times 10^{-4}$)
Glu-Gly-ArgCH$_2$Cl	0.4	Glu-Gly-ArgCH$_2$Cl	20.4
DNS-Glu-Gly-ArgCH$_2$Cl	0.7	DNS-Glu-Gly-ArgCH$_2$Cl	4.80
Ac-Gly-Gly-ArgCH$_2$Cl	3	Ac-Gly-Gly-ArgCH$_2$Cl	2.57
Pro-Gly-ArgCH$_2$Cl	3	Pro-Gly-ArgCH$_2$Cl	0.79
Ile-Glu-Gly-ArgCH$_2$Cl	5	Ile-Pro-ArgCH$_2$Cl	0.39
Phe-Ala-ArgCH$_2$Cl	5	Ile-Glu-Gly-ArgCH$_2$Cl	0.30
Ile-Pro-ArgCH$_2$Cl	7	Phe-Ala-ArgCH$_2$Cl	0.29
Val-Ile-Pro-ArgCH$_2$Cl	30	Val-Ile-Pro-ArgCH$_2$Cl	0.18
Phe-Ala-LysCH$_2$Cl	50	Phe-Ala-LysCH$_2$Cl	0.024

* See text for details.

** See preceding paper [1] for details.

In each set of experiments, the effects of Phe-Ala-ArgCH$_2$Cl in the 1—100 μM range were determined in order to have some internal monitor of day-to-day consistency. For six determinations of the standard concentration the mean was 35 ± 12 μM, (range 20—50 μM). A similar series of experiments was performed with human urinary urokinase, and the relative effectiveness of the inhibitors for both plasminogen activator and urokinase is presented in Fig. 4. This presentation can be used to compare relative effectiveness of various inhibitors toward one enzyme and effectiveness of one inhibitor for both enzymes.

The utility of the fibrinolytic assay compared with the spectrophotometric assay can best be judged by comparison of the results obtained from urokinase inhibition (Table I). The ranking of inhibitors is from most to least effective.

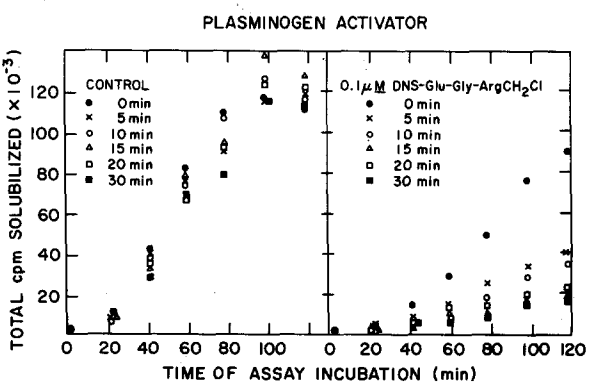

Fig. 5. Dependence of the rate of ^{125}I-labeled fibrin solubilization on the length of preincubation of HeLa plasminogen activator with 0.10 μM DNS-Glu-Gly-ArgCH$_2$Cl for the time indicated on the figure. The time required to solubilize half of the available fibrin was 45 to 55 min for the control while after preincubation with 0.10 μM DNS-Glu-Gly-ArgCH$_2$Cl for 5 min the time was increased to approximately 160 min.

The orders are not identical, but they are certainly similar enough to indicate that they are measuring the same event.

The comparative effectiveness of 0.1 μM DNS-Glu-Gly-ArgCH$_2$Cl with the two proteases was also determined as a function of time in contrast to previous experiments in which a reaction time of 30 min was used. As shown in Fig. 5, there is a progressive decline in activity for the plasminogen activator with the time of incubation with 0.10 μM DNS-Glu-Gly-ArgCH$_2$Cl. Under similar reaction conditions, 0.10 μM DNS-Glu-Gly-ArgCH$_2$Cl had no effect on urokinase since it resembled that of the control.

Discussion

The results obtained from this method are not easily quantified, but one parameter, the length of time to solubilize half of the radioactive label, is reproducible, and serves as a basis for standardizing the relative effectiveness of the inhibitors. A 'standard concentration' of an inhibitor was defined as that concentration which, under the conditions of the experiment, will double the length of time necessary to solubilize half the radioactivity. As an internal monitor Phe-Ala-ArgCH$_2$Cl was tested in each series of inhibitor experiments to normalize variations between days; however, the values obtained were so consistent that normalization was not necessary. A second check may be made by comparing the relative ranking of the urokinase inhibitors derived from the fibrinolytic assays and the values for k_{app}/I for urokinase obtained from the spectrophotometric assays. The rankings of the fibrinolytic assays are predictive of the spectrophotometrically obtained rankings. Thus the assay is reproducible from day-to-day, and accurately reflects the relative efficiency of the various inhibitors.

The sequence around the site of hydrolysis in the activation of the single chain plasminogen to the two chain plasmin is Cys-Pro-Gly-Arg-Val, where Arg-Val is the hydrolyzed peptide bond [16]; therefore Pro-Gly-ArgCH$_2$Cl was synthesized for study [1] in the expectation that an arginine chloromethyl ketone derivative would satisfy the primary specificity of urokinase and the additional residues confer reactivity and selectivity relative to other proteases cleaving at arginyl residues.

Inhibitors terminating in the lysyl group have 10—20% of the effectiveness of the corresponding arginyl inhibitor against both the activator and urokinase. Similar preferences have also been observed with human thrombin [10], human plasma kallikrein [11], acrosin and trypsin (Ref. 17 and unpublished observations). Plasmin prefers the lysyl moiety in the first position [11].

The glycyl group in the P_2 position (using the nomenclature of Schechter and Berger [18]) would be expected to be the preferred residue and this is consistent with our observation. For each enzyme X-Gly-ArgCH$_2$Cl are the best inhibitors tested.

Inhibitors with proline in the P_3 position are far less inhibitory than those with the glutamyl group. Each enzyme exhibits this preference, and with each enzyme the prolyl group is only slightly preferred to the phenylalanyl residue.

Significant differences between the specificities of plasminogen activator and

urokinase are exhibited in the P_4 site. The addition of DNS moiety to Glu-Gly-ArgCH_2Cl greatly increases the inhibitory reaction with the activator but decreases the ability to interact with urokinase. The same order of effectiveness applies with urokinase when assayed spectrophotometrically.

In addition to differences in the relative effectiveness of the two compounds with one enzyme, the comparison of the levels of inhibition by one inhibitor against the two enzymes also indicates that the enzymes are different. DNS-Glu-Gly-ArgCH_2Cl is an order of magnitude more inhibitory against plasminogen activator than urokinase. The opposite order of effectiveness occurs with Glu-Gly-ArgCH_2Cl; the difference is slightly less than an order of magnitude. Thus, by two major differences, plasminogen activator and urokinase can be distinguished from one another using a pair of similar inhibitors.

Another such pair of similar inhibitors is Ile-Pro-ArgCH_2Cl and Val-Ile-Pro-ArgCH_2Cl. The tetrapeptide analog is more effective than the tripeptide by an order of magnitude with plasminogen activator, whereas the opposite order is true for urokinase with a smaller separation. Again, the urokinase results are consistent by either method of measurement.

The residue in the P_4 position is much more influential for plasminogen activator than for many other of the proteases studied by this technique [1]. Both the addition of Val to Ile-Pro-ArgCH_2Cl and of DNS to Glu-Gly-ArgCH_2Cl enhance the effectiveness ten-fold or greater. These same two additions result in a loss of effectiveness against urokinase. The residue occupying the P_4 position in the substrate (plasminogen) is cystine, a bulky structural group. Mere bulk alone in the P_4 position will not suffice to promote binding as Ile-depressed inhibition. Rather, some specific interaction must occur which valyl performs to a small extent, but which dansyl satisfies to a much greater extent. But the dansyl group in P_4 is not sufficient in itself to enhance the inhibitory capacity of a compound which cannot meet P_2 and P_3 requirements at least not in this instance. The effectiveness of DNS-Pro-Phe-ArgCH_2Cl is no different from Pro-Phe-ArgCH_2Cl.

Based on the primary structure of the substrate for these enzymes, one would expect the best inhibitor to be Pro-Gly-ArgCH_2Cl. For neither enzyme is this true. It has been found with thrombin that the inhibitor based on fibrinogen is not as effective as inhibitors based on thrombin-cleaved sequences from factor XIII and prothrombin, physiologically significant control points in the coagulation process [10]. Since factor XIII and prothrombin are at much lower concentrations than fibrinogen, it follows that they must bind to thrombin effectively at their normal concentrations if control is to operate efficiently and therefore probably have a greater affinity for thrombin than for fibrinogen. The results with both urokinase and plasminogen activator are possibly analogous to those with thrombin; however, rather than confirming a known control point this information might suggest that there are other important physiological substrates.

Recent results by Nieuwenhuizen et al. [19,20] and Huseby et al. [21] are consistent with the results of this study. Huseby et al. [21] report the synthesis of *N*-Z-Gly-Gly-Arg-4-methoxy-2-naphthylamide, a substrate for both human urokinase and porcine kidney plasminogen activator obtained in tissue culture without comparison of relative binding, but that type of analysis was per-

formed by Nieuwenhuizen et al. [19,20]. They compared Boc-Val-Gly-Arg-β-naphthylamide and Val-Gly-Arg-β-naphthylamide as substrates for human urokinase and human uterine plasminogen activator. Urokinase exhibited preferential binding and catalysis of the smaller substrate. Uterine plasminogen activator was completely inactive toward the smaller substrate, preferring the compound with the bulky Boc group in the P_4 position. Each observation is consistent with our results using chloromethyl ketone inhibitors.

Åstedt and Holmberg [22] reported immunological identity of urokinase and a plasminogen activator from an ovarian carcinoma. This appears to be inconsistent with our observations; however, immunological techniques test primary structural similarity and kinetic techniques test binding similarity. In addition, there are probably several plasminogen activators. Some may be similar to and others different from urokinase.

Finally, besides showing that plasminogen activator is different from urokinase, we have shown that almost any tripeptide chloromethyl ketone is a better inhibitor of plasminogen activator than tosyllysine chloromethyl ketone, the one most often used diagnostically. One of these inhibitors, DNS-Glu-Gly-ArgCH_2Cl, is six orders of magnitude more effective than tosyllysine chloromethyl ketone. However, it is probably not selective for plasminogen activator [11].

Acknowledgements

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