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THE SUSCEPTIBILITY OF UROKINASE TO AFFINITY LABELING BY PEPTIDES OF ARGININE CHLOROMETHYL KETONE

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Summary

Pro-Gly-ArgCH_2Cl, a reagent corresponding to the C-terminal sequence generated in plasminogen on activation by urokinase (EC 3.4.99.26) and probably by other plasminogen activators, was prepared. Pro-Gly-ArgCH_2Cl was effective in the inactivation of urokinase at the 10^{-6} M level (K_i 68 μM and k_2 0.47 min^{-1}). In contrast, only a slow inactivation was obtained by 10^{-2} M *N*-tosyllysine chloromethyl ketone. Glu-Gly-ArgCH_2Cl, *N,N*-dimethylaminonaphthalene-5-sulfonyl-Glu-Gly-ArgCH_2Cl, and Ac-Gly-Gly-ArgCH_2Cl were more reactive than Pro-Gly-ArgCH_2Cl against urokinase by factors of 25, 6, and 3, respectively. The effectiveness of arginine chloromethyl ketones as affinity labels is highly dependent on binding in the S_2 and S_3 sites, thus sequence variations in the reagents exhibited differences in reactivity of up to four orders of magnitude. The most effective reagents had Gly in P_2.

Ac-Gly-Gly-ArgCH_2Cl inactivates urokinase 50 times more rapidly than it does plasmin, thus providing a means of distinguishing the activity of plasmin from its activating protease whereas urokinase is almost inert to Ala-Phe-LysCH_2Cl, a reagent which inactivates plasmin at the 10^{-7} M level.

Introduction

Tissue and cellular plasminogen activators are serine proteases associated with a number of pathological processes which include malignant cell transfor-

Abbreviations: Z, carbobenzoxy; Boc, *t*-butoxycarbonyl; DNS, *N,N*-dimethylaminonaphthalene-5-sulfonyl; OBzl, benzyl ester; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); TLC, thin-layer chromatography.

mation [1,2], inflammation [3], and the promotion of carcinogenesis [4]. The association of plasminogen activators with these processes is marked by notable increases in plasminogen-dependent fibrinolytic activity although the exact contribution of the proteases to the pathological process is not certain. On the other hand, plasma plasminogen activators have an essential role in haemostasis. The formation of plasminogen activator activity is associated with the initial stages of the Hageman factor-mediated blood coagulation pathway, but the identity of the protease remains obscure [5—7]. Clearly, specific inhibitors for plasminogen activators would be helpful in clarifying the physiological roles of these proteases and in establishing their identity.

An approach which has yielding high specific irreversible inhibitors for other physiologically important trypsin-like proteases such as thrombin [8], plasma kallikrein [9] and factor Xa (unpublished data) has been the synthesis of chloromethyl ketones which contain the amino acid sequence at the cleavage site of the physiological substrate for the target protease. Although these proteases have a common primary specificity favoring arginine, secondary sites are sufficiently different to permit such reagents to act selectively.

The urinary plasminogen activator, urokinase (EC 3.4.99.26), was chosen for initial studies and an affinity label was prepared which contains the Pro-Gly-Arg- sequence of plasminogen hydrolyzed by urokinase in the activation of the zymogen [10]. The reactivity of the reagent corresponding to this amino acid sequence, Pro-Gly-ArgCH_2Cl, was compared with that of other arginine chloromethyl ketones in the inactivation of urokinase in order to obtain an insight to the specificity of urokinase. In addition, the selectivity of affinity labels for urokinase was evaluated by comparing their reactivity with the plasminogen activator with that for certain other physiologically important proteases.

Materials and Methods

Analytical procedures. Elemental microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Single column amino acid analyses were performed on a 0.9 × 22 cm column packed with Durrum DC-6A resin on samples hydrolyzed in 2 ml of 6.0 N HCl for 24 h at 110° C in sealed evacuated tubes. TLC was performed on E. Merck precoated silica gel plates (No. 5534) using butanol/acetic acid/water (4 : 1 : 1) as a developing solvent.

Preparation of Pro-Gly-Arg$CH_2Cl \cdot 2HCl$. H-Arg(NO_2)$CH_2Cl \cdot HCl$ (0.49 g, 1.7 mmol) was prepared and was coupled, using the mixed anhydride method, to Z-Pro-Gly-OH (0.59 g, 1.9 mmol) by the procedures described previously [9]. After removing the reaction solvent, the residue was dissolved in ethylacetate and washed with 0.2 M HCl, 5% $NaHCO_3$, and saturated aqueous NaCl. The crude product was purified by chromatography on a silica gel column packed with 5 μm LiChrosorb SI 60 (E. Merck, 5945143) using ethylacetate containing 3% methanol as a developing solvent. After evaporating the solvent and triturating with ether, 0.34 g of Z-Pro-Gly-Arg(NO_2)CH_2Cl were obtained.

Analysis: for $C_{22}H_{30}N_7O_7Cl$: Calcd.: C = 48.93%, H = 5.61%, N = 18.16%.

Found: C = 48.62%, H = 5.89%, N = 17.88%.

Z-Pro-Gly-Arg(NO_2)CH_2Cl (0.20 g, 0.37 mmol) was treated with approximately 10 ml of anhydrous hydrogen fluoride in the presence of 1 ml of anisole for 30 min at 0° C. HF was removed by evaporation, the residue was taken up in 20 ml of water and extracted with two 20-ml portions of ether. The aqueous phase was applied to a column containing 10 ml of SE-Sephadex (C-25, H^+) which was washed with 100 ml of water. The product was eluted with 120 ml of 0.4 N HCl. After lyophilization and trituration with ether, 110 mg of Pro-Gly-ArgCH_2Cl · 2HCl were obtained.

TLC indicated a single spot at R_F 0.074 by both ninhydrin and Sakaguchi stains. Amino acid analysis of Pro-Gly-ArgCH_2Cl · 2HCl: Pro, 1.00 and Gly, 0.94.

Preparation of Phe-Ala-ArgCH_2Cl · 2HCl. Boc-Phe-Ala-Arg(NO_2)CH_2Cl was prepared by coupling Boc-Phe-Ala-OH (0.58 g, 1.7 mmol) to H-Arg-(NO_2)CH_2Cl · HCl (0.05 g, 1.7 mmol) by the mixed anhydride procedure described previously [9]. After crystallization from ethylacetate, 0.57 g of product (m.p. 110—114° C) were obtained.

Analysis: for $C_{24}H_{36}N_7O_7Cl$: Calcd.: C = 50.56%, H = 6.38%, N = 17.20%.

Found: C = 50.75%, H = 6.45%, N = 16.94%.

Boc-Phe-Ala-Arg(NO_2)CH_2Cl (0.40 g, 0.70 mmol) was deblocked with HF and the product was isolated by the procedure described for the preparation of Pro-Gly-Arg-CH_2Cl · 2HCl to yield 0.28 g of Phe-Ala-ArgCH_2Cl · 2HCl. TLC indicated a single spot, R_F 0.47, by both ninhydrin and Sakaguchi stains. Amino acid analysis of Phe-Ala-ArgCH_2Cl · 2HCl: Phe, 0.97 and Ala, 1.00.

Preparation of Glu-Gly-ArgCH_2Cl · 2 HCl Boc-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl was prepared by coupling Boc-Glu(OBzl)-Gly-OH (0.68 g, 1.7 mmol) to H-Arg-(NO_2)CH_2Cl · HCl (0.50 g, 1.7 mmol) by the mixed anhydride procedure described previously [9]. Boc-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl was crystallized from ethylacetate/hexane to yield 0.56 g.

Analysis: for $C_{26}H_{38}N_7O_9Cl$: Calcd.: C = 49.71%, H = 6.11%, N = 15.61%.

Found: C = 49.69%, H = 6.26%, N = 15.68%.

Boc-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl (0.30 g, 0.48 mmol) was reacted with HF for 70 min at 0° C followed by isolation as described in the preparation of Pro-Gly-ArgCH_2Cl · 2HCl to yield 0.18 g of Glu-Gly-ArgCH_2Cl · 2HCl. TLC indicated a major spot at R_F 0.14 and a trace at R_F 0.31 by both ninhydrin and Sakaguchi stains. Amino acid analysis of Glu-Gly-ArgCH_2Cl · 2HCl: Glu, 1.00 and Gly, 1.00.

Preparation of DNS-Glu-Gly-ArgCH_2Cl · HCl. H-Glu(OBzl)-Gly-Arg(NO_2)-CH_2Cl · HCl was prepared by treating Boc-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl with 2 ml of anhydrous trifluoroacetic acid for 5 min at room temperature followed by 0.5 ml of ethanolic HCl (5 N) and precipitation with cold ether.

H-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl · HCl (0.50 g, 0.87 mmol) was dissolved in 4 ml of water and 2 ml of dioxane and cooled to 0° C. After addition of $NaHCO_3$ (0.19 g, 2.2 mmol) and dansyl chloride (0.31 g, 1.2 mmol) dissolved in 4 ml of dioxane, the solution was stirred for 2.5 h at room temperature. The

reaction solution was added to 100 ml of ethylacetate and was washed with 5% $NaHCO_3$ and saturated aqueous NaCl. The product was purified by chromatography on a column packed with silica gel 60, 40—63 μm particle size (E. Merck, 9385) using chloroform/methanol (95 : 5) as a developing solvent. After evaporating solvent and triturating with ether, 0.31 g of DNS-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl were obtained.

Analysis: for $C_{33}H_{41}N_8O_9SCl$: Calcd.: C = 52.06%, H = 5.44%, N = 14.72%.

Found: C = 52.03%, H = 5.47%, N = 14.92%.

DNS-Glu(OBzl)-Gly-Arg(NO_2)CH_2Cl (0.29, 0.38 mmol) was reacted with HF for 60 min at 0° C and the product was isolated by the procedure described in the preparation of Pro-Gly-ArgCH_2Cl · 2HCl to yield 0.23 g of DNS-Glu-Gly-ArgCH_2Cl · HCl. TLC indicated a single spot, R_F 0.47, by Sakaguchi stain. The plate was ninhydrin negative.

Preparation of Ac-Gly-Gly-ArgCH_2Cl · HCl. Ac-Gly-Gly-OH (0.25 g, 0.88 mmol) was coupled to H-Arg(NO_2)CH_2Cl · HCl (0.25 g, 0.88 mmol) by the procedure described previously [9] except dimethylformamide was used as a solvent for the formation of the mixed anhydride. After removing the reaction solvent, the residue was crystallized from methanol to yield 0.19 g of Ac-Gly-Gly-Arg(NO_2)CH_2Cl.

Analysis: for $C_{13}H_{22}N_7O_6Cl$: Calcd.: C = 38.28%, H = 5.45%, N = 24.04%.

Found: C = 37.90%, H = 5.07%, N = 23.88%.

Ac-Gly-Gly-ArgCH_2Cl · HCl was prepared by deblocking Ac-Gly-Gly-Arg-(NO_2)CH_2Cl (0.29 g, 0.71 mmol) with HF by the procedure described for the preparation of Pro-Gly-ArgCH_2Cl · 2HCl to yield 0.18 g of product. TLC indicated a single spot, R_F 0.29, by Sakaguchi stain. The plate was ninhydrin negative.

Enzymatic studies. Human urokinase was obtained from Leo Pharmaceutical Products, Denmark; No. 9092207 1712 E. Stock solutions consisting of 1990 Ploug units or 1.1 nmol of the protease established as described in [11] in 2.0 ml of 50 mM Pipes buffer, pH 6.0, which was 0.20 M in NaCl and of the affinity labeling reagent in 1.0 mM HCl were prepared. Aliquots of the affinity labeling reagent, not exceeding 0.200 ml, were diluted to the desired concentration by the addition of 50 mM Pipes buffer, pH 7.0, which was 0.20 M in NaCl to yield a final volume of 2.00 ml. Reactions were initiated by the addition of 50 μl of the stock urokinase to the inhibitor solution at 25° C. At least eight, timed aliquots (200 μl) were removed and residual esterase activity was determined using the thiobenzyl Z-lysinate assay [12]. The apparent, pseudo first-order rate constants for the inactivation of urokinase were determined from the slope of semilogarithmic plots of esterase activity vs. time.

Kinetic studies were based on the affinity labeling mechanism for serine proteases, Eqn. 1 [13]. Values of K_i, the reversible dissociation constant for the protease-inhibitor complex, and of k_2, the first-order rate constant for the alkylation of the active-site histidine, were determined by the graphic solution of Eqn. 2 [14] where k_{app} is the apparent, pseudo first-order rate constant and

I is the concentration of the affinity label. At least seven values

$$E + I \rightleftharpoons EI \xrightarrow{k_2} \text{alkylated protease} \quad (1)$$

$$\frac{1}{k_{app}} = \frac{K_i}{k_2}\frac{1}{I} + \frac{1}{k_2} \quad (2)$$

of k_{app} were determined at different concentrations of the affinity labels and kinetic constants were determined from double-reciprocal plots of k_{app} vs. I after determining the best straight line by the least squares method.

Additional kinetic constants, k_{app}/I, were determined for human urokinase, plasma kallikrein, plasmin, and bovine thrombin by a procedure we have previously described [9]. Values of k_{app}/I for purified human urinary kallikrein (17 tosylarginine methyl ester units/mg), a gift from Dr. Jack V. Pierce, were measured by the procedure described for urokinase except $2.5 \cdot 10^{-3}$ unit [15] were incubated with the affinity labels and the protease was assayed using 0.30 mM substrate. Values of k_{app} were measured at several concentrations of the affinity label to establish that this constant was proportional to I in the range studied. The constant, k_{app}/I, was then used as an estimate of k_2/K_i according to the relationship of Eqn. 3 [14].

$$\frac{k_{app}}{I} = \frac{k_2}{K_i} \quad \text{if } I << K_i \quad (3)$$

Results

Syntheses were developed for Pro-Gly-ArgCH_2Cl and a number of closely related peptides containing arginine chloromethyl ketone which were of interest in exploring the relation of structure to ability to inactivate urokinase. Pro-Gly-Arg is the sequence in plasminogen at which cleavage of the arginyl peptide bond results in activation [10,16]. The effectiveness of the newly synthesized compounds as affinity labels for urokinase was compared with that of a group containing sequences specific for other proteases.

The rates of inactivation of urokinase were measured at concentrations of reagents so that the half-times for inactivation were in the range of 20—30 min. This rate of inactivation is favorable for accurate rate measurement and was generally obtainable with concentrations of affinity labeling reagent for which the rates of inactivation were proportional to the concentration used, therefore below a level producing saturation effects.

Pro-Gly-ArgCH_2Cl was in fact much more effective in inactivating urokinase than many tripeptides containing arginine chloromethyl ketone or lysine chloromethyl ketone. It was superior to Tos-LysCH_2Cl, a commonly used reagent for the inactivation of trypsin-like enzymes, by four orders of magnitude (Table I). However, a number of reagents having in common a glycine residue in the P_2 position were even more effective (Fig. 1). Thus, Glu-Gly-ArgCH_2Cl inactivated urokinase at least 20 times more rapidly than Pro-Gly-ArgCH_2Cl as shown by the values of k_{app}/I in Table I. DNS-Glu-Gly-ArgCH_2Cl and Ac-Gly-Gly-ArgCH_2Cl were less effective, differing in reactivity from Glu-Gly-ArgCH_2Cl by factors of 4 and 8, respectively.

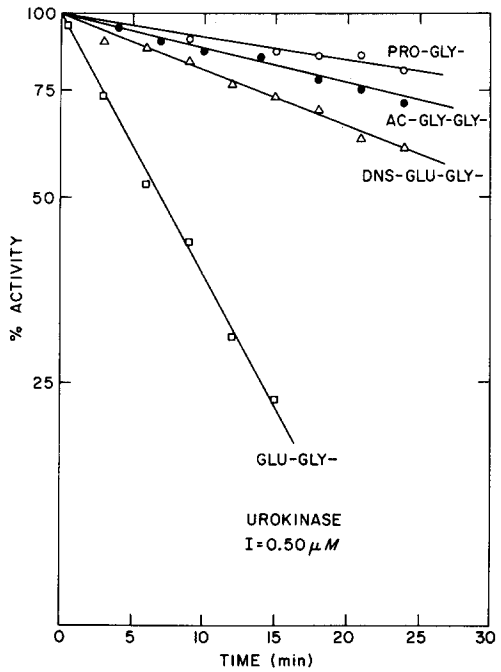

Fig. 1. Comparison of the reactivity of urokinase with different N-substituted arginine chloromethyl ketones. The reagents were incubated at a concentration of 0.50 μM with urokinase at pH 7.0 and 25°C. The inactivation of urokinase was monitored by removing timed aliquots and assaying for esterase activity.

Pro-Gly-ArgCH_2Cl, however, was more effective than arginine chloromethyl ketones which contained residues other than Gly in the P_2 position. The role of binding in secondary sites in determining the selectivity of urokinase is apparent in the large differences observed among arginine chloromethyl ketones with different substituents in the P_2 and P_3 positions. For example, urokinase differed in its reactivity with Pro-Phe-ArgCH_2Cl and Glu-Gly-ArgCH_2Cl by four orders of magnitude. On the other hand, the contribution of arginine in the P_1 position is illustrated by the ten-fold greater reactivity of Phe-Ala-ArgCH_2Cl compared to its lysine analog.

The difference in the effectiveness of Glu-Gly-ArgCH_2Cl and the other reagents in Table I in the inactivation of urokinase is probably due to differences in affinity since analysis of the reactivity of peptides of arginine chloromethyl ketone with other proteases indicated that the rate constants for the alkylation step of the affinity labeling mechanism, k_2, often were relatively constant for individual proteases [8,9]. This has been found to be the case for the 26-fold difference in the reactivities of Glu-Gly-ArgCH_2Cl and Pro-Gly-ArgCH_2Cl with urokinase after evaluating values of K_i and k_2 for these reagents by the method of Kitz and Wilson [14]. The values of k_2 for the inactivation of urokinase by Glu-Gly-ArgCH_2Cl and Pro-Gly-ArgCH_2Cl were almost identical, 0.46 min^{-1} and 0.47 min^{-1}, respectively, whereas differences in the dissociation constant, K_i, for reversible binding of the two reagents were comparable to differences in their reactivity. The K_i for Glu-Gly-ArgCH_2Cl was $1.8 \cdot 10^{-6}$ M

TABLE I

COMPARISON OF THE REACTIVITY OF UROKINASE WITH PEPTIDES OF ARGININE AND LYSINE CHLOROMETHYL KETONES

Inactivations were conducted at 25°C in 50 mM Pipes buffer, pH 7.0, containing 0.20 M NaCl. The initial concentration of urokinase was 13 nM. $t_{1/2}$ is the half-time for the first-order inactivation of urokinase at the indicated concentration of the affinity labeling reagent. k_{app}/I is the ratio of the apparent, first-order rate constant for inactivation to the concentration of the affinity label. These values were calculated from the values of $t_{1/2}$ using the relationship, $k_{app} = \ln 2/t_{1/2}$, and the indicated concentration of the affinity label.

Affinity label	Concn. (μM)	$t_{1/2}$ (min)	k_{app}/I ($M^{-1} \cdot min^{-1}$) ($X10^{-4}$)
Glu-Gly-ArgCH$_2$Cl	0.20	17.0	20.4
DNS-Glu-Gly-ArgCH$_2$Cl	0.50	28.9	4.80
Ac-Gly-Gly-ArgCH$_2$Cl	0.50	54.0	2.57
Pro-Gly-ArgCH$_2$Cl	2.0	44.0	0.79
Val-Pro-ArgCH$_2$Cl	10.0	12.8	0.54
Ile -Pro-ArgCH$_2$Cl	10.0	17.8	0.39
Phe-Ala-ArgCH$_2$Cl	10.0	23.9	0.29
Val-Ile -Pro-ArgCH$_2$Cl	20.0	19.6	0.18
Phe-Ala-LysCH$_2$Cl	100.0	28.7	0.024
Gly-Val-ArgCH$_2$Cl	100.0	41.3	0.017
Ile -Leu-ArgCH$_2$Cl	200.0	23.9	0.014
Ala-Phe-ArgCH$_2$Cl	500.0	23.6	0.0059
Pro-Phe-ArgCH$_2$Cl	500.0	91	0.0015
Ala-Phe-LysCH$_2$Cl	2 500	83	0.0003
Tos-LysCH$_2$Cl	50 000	85	0.00002

and for Pro-Gly-ArgCH$_2$Cl was $6.9 \cdot 10^{-5}$ M.

To determine to what extent the reagents effective in inactivating urokinase are selective, the reactivities of urokinase with Glu-Gly-ArgCH$_2$Cl, DNS-Glu-Gly-ArgCH$_2$Cl, Ac-Gly-Gly-ArgCH$_2$Cl, and Pro-Gly-ArgCH$_2$Cl were compared with the reactivities of the plasma proteases, plasma kallikrein, thrombin, and plasmin. All reagents in Table II except for DNS-Glu-Gly-ArgCH$_2$Cl inactivated urokinase more effectively than plasmin as shown by the differences in the second-order constant for their inactivation reactions. For example, Ac-Gly-Gly-ArgCH$_2$Cl inactivated plasmin by 50% in 70 min at a concentration

TABLE II

SELECTIVITY OF ARGININE CHLOROMETHYL KETONES IN THE INACTIVATION OF TRYPSIN-LIKE PROTEASES

k_{app}/I is the ratio of the apparent, first-order rate constant for inactivation to the concentration of the affinity label. Values of k_{app} were measured at 25°C, pH 7.0.

Affinity label	k_{app}/I ($M^{-1} \cdot min^{-1}$) ($X10^{-4}$)				
	Uro-kinase	Plasma kallikrein	Thrombin	Plasmin	Urinary kallikrein
Glu-Gly-ArgCH$_2$Cl	20	16	1.9	1.3	0.00095
DNS-Glu-Gly-ArgCH$_2$Cl	4.2	140	26	28	0.18
Ac-Gly-Gly-ArgCH$_2$Cl	2.6	1.4	0.74	0.053	0.00054
Pro-Gly-ArgCH$_2$Cl	0.79	3.3	1.2	0.091	0.0017

of $2.0 \cdot 10^{-5}$ M while urokinase was inactivated by 50% in 54 min at a concentration of $5.0 \cdot 10^{-7}$ M. Plasma kallikrein, thrombin, and urokinase were similar in their reactivities with most of the reagents in Table II while in contrast human urinary kallikrein was almost inert to inactivation by Glu-Gly-Arg-CH_2Cl, Ac-Gly-Gly-ArgCH_2Cl and Pro-Gly-ArgCH_2Cl, requiring concentrations of reagents 3—5 orders of magnitude greater than effective with urokinase. A unique difference in the reactivity of urokinase and the other proteases is the 9—20-fold greater reactivities of the plasma proteases and the 180-fold greater reactivity of urinary kallikrein with DNS-Glu-Gly-ArgCH_2Cl than with Glu-Gly-ArgCH_2Cl while the bulky DNS residue in the P_4 position was restrictive for urokinase, decreasing the effectiveness of the reagent four-fold.

Discussion

Urokinase has been the object of a number of studies involving synthetic inhibitors acting reversibly [17,18], or irreversibly by acylation [19], sulfonylation [20], or alkylation [21]. The present work extends to this enzyme alkylation by peptides of arginine chloromethyl ketone which have proven to be very effective with other trypsin-like enzymes [8,9,22].

The preparation of Pro-Gly-ArgCH_2Cl, an affinity label corresponding to the amino acid sequence of plasminogen cleaved by urokinase in zymogen activation, yielded a reagent effective in the inactivation of urokinase, but lacking the high degree of reactivity and selectivity of reagents for thrombin [8] and plasma kallikrein [9] corresponding to their respective physiological substrates. In fact, Pro-Gly-ArgCH_2Cl was more effective in the inactivation of plasma kallikrein and thrombin than in the inactivation of urokinase.

Other reagents, Glu-Gly-ArgCH_2Cl, DNS-Glu-Gly-ArgCH_2Cl, and Ac-Gly-Gly-ArgCH_2Cl, proved to be more effective than Pro-Gly-ArgCH_2Cl in the inactivation of urokinase. Substitution of Glu for Pro in the P_3 position of Pro-Gly-ArgCH_2Cl enhanced the affinity of urokinase for the reagent 40-fold while a three-fold enhancement was obtained by substitution of Ac-Gly- for Pro in the P_3 position. In contrast to the P_3 position, the importance of the P_2 glycyl residue in determining the selectivity of urokinase for the single -Arg-Val- bond of the -Pro-Gly-Arg-Val-Val- sequence of plasminogen [10] is clearly demonstrated. Bulkier substituents in the P_2 position were progressively less effective inactivators of urokinase. In all cases, tripeptide analogs were more effective than the corresponding tetrapeptide analogs. For example, Glu-Gly-ArgCH_2Cl is four times more effective than DNS-Glu-Gly-ArgCH_2Cl and Ile-Pro-ArgCH_2Cl is twice as effective as Val-Ile-Pro-ArgCH_2Cl. An additional example is the 68-fold greater reactivity of Glu-Gly-ArgCH_2Cl than Ile-Glu-Gly-ArgCH_2Cl with urokinase (unpublished observation). The restrictive nature of the S_4 subsite of urokinase appears to be a feature distinguishing urokinase from the plasma proteases examined. For the latter, tetrapeptide analogs were similar in reactivity to the corresponding tripeptide analogs and in several cases enhanced reactivities, similar to that found with DNS-Glu-Gly-ArgCH_2Cl, were observed.

The lower reactivity of urokinase with the reagent corresponding to the amino acid sequence of its physiological substrate than that expected from the reactivities of the plasma proteases, thrombin [8] and plasma kallikrein [9],

with their appropriate affinity labels was not due to differences in the susceptibility of the active-site histidine to alkylation since the first-order rate constant for the alkylation step of the affinity labeling reaction was comparable to those measured for thrombin [8], plasmin, and plasma kallikrein [9]. The lower reactivity of urokinase most probably reflects its high degree of specificity. A much larger binding region of a unique secondary structure of the polypeptide chain at the urokinase cleavage site of plasminogen, which is not obtained with the tripeptide analogs, may be essential in determining the selectivity of urokinase. The relatively low reactivity of urokinase with Pro-Gly-ArgCH_2Cl is consistent with the observation of Sottrup-Jensen et al. [10] that urokinase does not hydrolyze a 38 residue, carboxymethylated fragment of plasminogen encompassing the region cleaved by urokinase in the activation of plasminogen. Also consistent with the high degree of specificity of urokinase, large differences in the reactivity of urokinase with arginine chloromethyl ketones containing different substituents in the P_2 and P_3 binding sites were observed. The difference in the reactivity of urokinase with the most effective and least effective arginine chloromethyl ketone spans four orders of magnitude. A similar comparison for thrombin [8] revealed differences of three orders in magnitude while for the less specific proteases, trypsin and acrosin, differences of 180 and 100 were obtained [22].

A selective affinity label for urokinase has not emerged from these initial studies in which the reactivity of the urinary plasminogen activator was compared with the reactivities of plasma proteases. However, in contrast to the similar reactivities of plasma kallikrein and urokinase with Glu-Gly-ArgCH_2Cl, Ac-Gly-Gly-ArgCH_2Cl, and Pro-Gly-ArgCH_2Cl, human urinary kallikrein was decisively insensitive to these reagents thus providing a means for distinguishing between the two urinary proteases. Powerful reagents are now available which may be useful in determining the roles of urokinase and other plasminogen activators in their physiological environments. For example, Ac-Gly-Gly-Arg-CH_2Cl is 50 times more reactive with urokinase than with plasmin, thus providing a means of distinguishing the activity of plasmin from its activating protease. Other affinity labels readily inactivate plasmin distinguishing it from urokinase. The most effective, Ala-Phe-LysCH_2Cl [9], inactivates plasmin at the 10^{-7} M level while urokinase only slowly reacts with this reagent even at 10^{-3} M.

In addition, the large differences in reactivity of urokinase with various arginine chloromethyl ketones has made it possible to distinguish urokinase from the plasminogen activator secreted by HeLa cells [23]. The series of arginine chloromethyl ketones described here should be useful in characterizing plasminogen activators from other sources [23] and may lead to reagents of therapeutic value.

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