the assay cuvette and the increase at 340 nm is recorded. For clostripain, benzoyl-arginine ethyl ester is recommended.[3]

Proteolytic Assays.[2] Activity is measured by incubating test samples with a 2.5% solution of azocasein in 0.05 *M* phosphate buffer (pH 5.5–6.0) containing 5 m*M* glutathione and 5 m*M* EDTA at 37°C. After selected time intervals, the reaction is stopped by addition of an equal volume of 10% trichloroacetic acid. Following centrifugation, the optical density of the supernatant solution is measured at 366 nm.

[63] Inactivation of Trypsin-Like Enzymes with Peptides of Arginine Chloromethyl Ketone

By CHARLES KETTNER and ELLIOTT SHAW

Introduction

The value of affinity labels in the identification and characterization of proteases in their physiological environments has been clearly demonstrated by the use of TPCK (tos-PheCH_2Cl), TLCK (tos-LysCH_2Cl), and alanyl peptide chloromethyl ketones. These reagents distinguish among chymotryptic, tryptic, and elastolytic activities by conforming to the primary specificity requirements of the proteases.[1-3] This communication summarizes many of the results we have obtained in the development of selective-affinity labels for individual trypsin-like proteases that function in blood coagulation, fibrinolysis, and hormone processing. This group of enzymes functions by the hydrolysis of either one or two bonds of their physiological substrates, usually at arginyl residues. We have used the approach of incorporating part of the sequence of the physiological substrate in the reagent,[4] thus taking advantage of binding selectivity in both primary and secondary sites.

Synthesis and Properties of Arginine Chloromethyl Ketones

A typical synthesis of peptides of arginine chloromethyl ketones is demonstrated by the preparation of Pro-Phe-Arg$CH_2Cl \cdot 2HCl$.[5] This pro-

[1] G. Schoellmann and E. Shaw, *Biochemistry* **2**, 252 (1963).
[2] E. Shaw, M. Mares-Guia, and W. Cohen, *Biochemistry* **4**, 2219 (1964).
[3] J. C. Powers and P. M. Tuhy, *Biochemistry* **12**, 4767 (1973).
[4] J. R. Coggins, W. Kray, and E. Shaw, *Biochem. J.* **137**, 579 (1974).
[5] C. Kettner and E. Shaw, *Biochemistry* **17**, 4778 (1978).

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cedure has been applied to the synthesis of numerous other reagents with only minor modification. Following the preparation of the first intermediate, Boc-Arg(NO_2)CHN_2 and its conversion to H-Arg(NO_2)CH_2Cl · HCl with anhydrous HCl, Z-Pro-Phe-OH was added by the mixed anhydride procedure. The crystalline product, Z-Pro-Phe-Arg(NO_2)CH_2Cl, was then deprotected with anhydrous HF to yield H-Pro-Phe-ArgCH_2Cl · 2HCl. The addition of the dipeptide to H-Arg(NO_2)CH_2Cl rather than the sequential addition of amino acids is advantageous in that it avoids the dipeptide intermediate obtained in sequential addition. The protected tripeptide arginine chloromethyl ketones are easier to isolate and characterize than the dipeptide intermediate, whose elongation, in addition, results in low yields. Racemization of the residue penultimate to the arginyl residue is a potential problem in the coupling of the N-protected dipeptide to H-Arg(NO_2)CH_2Cl, but we have no indication that this has occurred to a significant extent.

Final products are amorphous white powders that are hygroscopic without exception. The reagents are stable in this form when stored at 4°C desiccated with P_2O_5 *in vacuo* for at least several years. In addition, they are stable up to a year in the form of frozen solutions in 10^{-3} *M* HCl. We have found that it is convenient to prepare a 10^{-2} *M* stock solution for routine use.

An extensive study of the stability of arginine chloromethyl ketones at physiological pH has not been made. However, no change in the biological activity of Phe-Ala-ArgCH_2Cl was observed when it was incubated in 50 m*M* PIPES buffer (pH 7.0) for 24 hr at 25°C. This is in contrast to the instability of Phe-Ala-LysCH_2Cl, which lost half of its biological activity under the same conditions. It is expected that other lysine and arginine chloromethyl ketones will behave similarly, and that at higher pH values the chloromethyl ketones will be progressively less stable. For example, 70% of the biological activity of Phe-Ala-ArgCH_2Cl was lost when it was incubated for 30 min at pH 9.0 and 25°C.

Preparation of H-Arg(NO_2)CH_2Cl · HCl

Boc-Arg (NO_2)-OH (5.0 g, 15.6 mmol) was dissolved in 60 ml of tetrahydrofurane and allowed to react with isobutyl chloroformate (2.06 ml, 15.6 mmol) in the presence of *N*-methylmorpholine (1.72 ml, 15.6 mmol) for 4 hr at −20°C. The mixed anhydride preparation was filtered and the filtrate was added to 120 ml of ethereal diazomethane. After stirring the reaction solution for 30 min at 0°C, 80 ml of ether was added. The product crystallized from the reaction solution in the cold and was isolated by decanting the supernatant and washing the residue with ether. The diazomethyl ketone of Boc-Arg(NO_2)-OH was recrystallized from methanol to yield 2.56 g, mp 150–151°C.

Boc-Arg(NO_2) CHN_2 (1.47 g, 4.30 mmol) was dissolved in a minimal volume of tetrahydrofurane and was allowed to react with ethanolic HCl (20 mmol) at room temperature until nitrogen evolution ceased. Solvent was removed by evaporation and the residue was taken up in 40 ml of 1.8 *N* ethanolic HCl. After stirring the solution for 30 min at room temperature, 1.34 g of an amorphous white solid was obtained by evaporating the solvent and triturating the residue with ether. This product was dried over KOH and P_2O_5 *in vacuo* and was used in subsequent reactions without further purification.

Preparation of Z-Pro-Phe-Arg(NO_2)CH_2Cl

Z-Pro-Phe-OH (0.43 g, 1.1 mmol) was allowed to react with *N*-methylmorpholine (0.12 ml, 1.1 mmol) in 5 ml of tetrahydrofurane and isobutyl chloroformate (0.15 ml, 1.1 mmol) for 10 min at −20°C. Cold tetrahydrofurane containing triethylamine (0.15 ml, 1.1 mmol) was added to the mixed anhydride preparation and the mixture was immediately added to H-Arg(NO_2)CH_2Cl · HCl (0.31 g, 1.1 mmol) dissolved in 5 ml of cold dimethylformamide. After stirring for 1 hr at −20°C and 2 hr at room temperature, the reaction mixture was filtered, the filtrate was evaporated to dryness, and the residue was dissolved in 3 ml of methanol. The solution was diluted to 100 ml with ethyl acetate and washed with 0.1 *N* HCl, 5% $NaHCO_3$, and saturated aqueous NaCl. The organic phase was briefly dried over anhydrous Na_2SO_4 and was concentrated to yield 0.33 g of crystals, mp 102–105°C.

Preparation of H-Pro-Phe-ArgCH_2Cl · 2HCl

Z-Pro-Phe-Arg(NO_2)CH_2Cl (0.50 g, 0.79 mmol) was treated with approximately 10 ml of anhydrous hydrogen fluoride in the presence of 1 ml of anisole using a KelF hydrogen fluoride line. After allowing the reaction to proceed for 30 min at 0°C, the hydrogen fluoride was removed by distillation and the residue was dissolved in 20 ml of water. The solution was extracted with three 20-ml portions of ether and the aqueous phase was applied to a column containing 20 ml of SP–Sephadex (C-25, H^+ form). The column was washed with 120 ml of water, and the product eluted with 120 ml of 0.4 *N* HCl. After lyophilization of the HCl solution, the residue was transferred to a small container by solution in methanol and concentration under a stream of nitrogen. The product was obtained as 0.36 g of an amorphous white solid by trituration with ether and drying over P_2O_5 and KOH. TLC indicated a single spot, $R_f = 0.21$ by Sakaguchi, and ninhydrin stains after development on silica gel in butanol, acetic acid, and water (4 : 1 : 1).

Kinetics of Affinity Labeling

The reactivity of the chloromethyl ketones with proteases has been determined by incubating the protease with the affinity label, removing timed aliquots, and measuring residual enzymatic activity. The initial concentration of protease was selected so that the concentration of affinity label was at least 10 times greater than that of the enzyme. Values of k_{app}, the apparent, pseudo-first-order rate constant for inactivation of the protease under observation, were determined from slopes of semilogarithmic plots of protease reactivity versus time.[6] Kinetic constants for the affinity labeling mechanism, reaction (1), are shown in Eq. (2). K_i is the dissociation constant for the reversible, substrate-like complex EI, and k_2 is the first-order rate constant for the irreversible alkylation of the protease.

$$E + I \rightleftharpoons EI \xrightarrow{k_2} \text{alkylated protease} \tag{1}$$

$$\frac{1}{k_{app}} = \frac{K_i}{k_2}\frac{1}{I} + \frac{1}{k_2} \tag{2}$$

$$\frac{k_2}{K_i} = \frac{k_{app}}{I} \quad \text{if } I \ll K_i \tag{3}$$

Bimolecular constants, k_2/K_i, were determined from Eq. (2) or estimated by the relationship in Eq. (3). The relationship in Eq. (3) is valid for most of the enzymes we have studied at levels of affinity label that give half-times of inactivation of greater than 15 min.

Determination of individual kinetic constants, K_i and k_2, are less reliable, since k_2 is determined from the intercept on the Y axis of double reciprocal plots of k_{app} versus I. Small variations in the intercept, particularly when it is close to the origin, result in large differences in K_i and k_2. Lineweaver–Burk plots[7] are a more reliable method of measuring K_i.[8] In this approach, the affinity label is incubated with the protease in the presence of substrate and initial velocities of substrate hydrolysis are measured. The substrate sufficiently protects the enzyme from inactivation so that an initial velocity can be measured before inactivation of the protease in the cuvette. (A detailed description can be found in Ref. 8.)

In typical assays, the proteases (10–15 nM) were incubated with the affinity label in 2.00 ml of 50 mM PIPES buffer (pH 7.0) at 25°C. Timed aliquots (200 μl) were removed and diluted in 0.20 M Tris buffer (pH 8.0) containing 0.20 M NaCl, Z-Lys-SBzl as a substrate,[9] and 5,5′-dithiobis-

[6] R. Kitz and I. B. Wilson, *J. Biol. Chem.* **237**, 3245 (1962).

[7] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).

[8] C. Kettner, C. Mirabelli, J. V. Pierce, and E. Shaw, *Arch. Biochem. Biophys.* **202**, 420 (1980).

[9] G. D. J. Green and E. Shaw, *Anal. Biochem.* **93**, 223 (1979).

(2-nitrobenzoic acid) as a chromogen. The final volume of the assay solution was 2.00 ml and the final concentrations of substrate and chromogen were 0.10 m*M* and 0.33 m*M*, respectively. Enzymatic activity was followed for 2–3 min at 412 nm on a Beckman 5230 recording spectrophotometer using a 0.05 chart scale. For the evaluation of the reactivity of highly effective affinity labels, it was occasionally advisable to use lower concentrations of enzymes in order to maintain the concentration of affinity label in excess of that of the protease. It should be noted that under these conditions the rate of inactivation is independent of the initial concentration of protease. Protease concentrations were decreased by increasing the volume of the incubation mixture and proportionally increasing the size of the aliquot (up to 1.0 ml) removed for assay, or by increasing the sensitivity of the spectrophotometer as much as 5-fold.

In almost all cases, Z-Lys-SBzl has been an adequate substrate for the assay of purified enzymes, and in most cases it is comparable in sensitivity to peptide chromogenic substrates.[9] Among the advantages of Z-Lys-SBzl is its ease of synthesis and low cost as well as its adaptability for use with a variety of enzymes with minor modification. An exception is factor X_a, which hydrolyzes this substrate poorly. The chromogenic substrate of Kabi, S-2222, was more suitable for this purpose.

Affinity Labeling of Kallikreins

Plasma kallikrein and glandular kallikrein are two distinctive enzymes, differing in their physical properties, specificity, and physiological function. Both enzymes, however, hydrolyze the same Arg-Ser bond of kininogen, liberating the C-terminal of bradykinin (-Phe-Ser-Pro-Phe-Arg-OH).[10] Plasma kallikrein also hydrolyzes factor XII in the initiation of blood coagulation[10] and activates prorenin,[11,12] but the sequence of these substrates is unknown. Less is known about the physiological substrates of glandular kallikreins, although they are implicated in renal function[13] and hormone processing.[14] Therefore, the sequence corresponding to the C-terminal of bradykinin was used in the design of affinity labels for kallikreins.

Plasma Kallikrein. The tripeptide analog corresponding to the C-terminal of bradykinin, Pro-Phe-ArgCH_2Cl, readily inactivates plasma

[10] J. J. Pisano, *in* "Proteases and Biological Control" (E. Reich, D. B. Rifkin, and E. Shaw, eds.), p. 199. Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1975.

[11] N. Yokosawa, N. Takahashi, T. Inagami, and D. L. Page, *Biochim. Biophys. Acta* **569**, 211 (1979).

[12] F. H. M. Derkx, B. N. Bouma, M. P. A. Schalekamp, and M. A. D. H. Schalekamp, *Nature (London)* **280**, 315 (1979).

[13] H. S. Margolius, R. Geller, J. J. Pisano, and A. Sjoerdsma, *Lancet* **2**, 1063 (1971).

[14] M. A. Bothwell, W. H. Wilson, and E. M. Shooter, *J. Biol. Chem.* **254**, 7287 (1979).

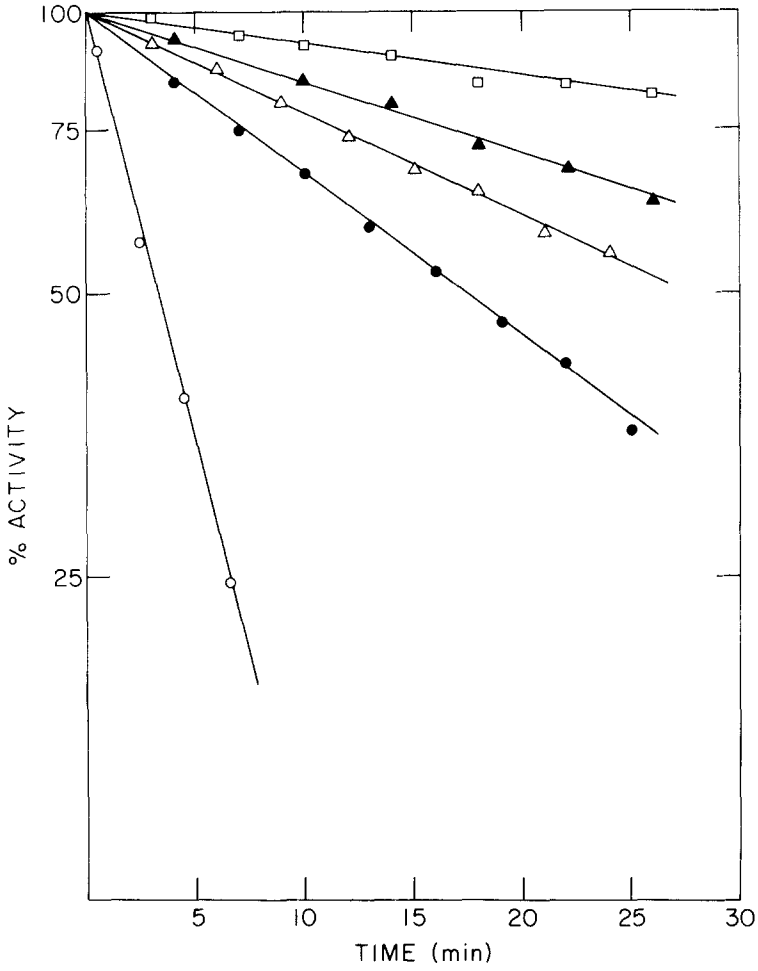

FIG. 1. Selectivity of Pro-Phe-ArgCH_2Cl in the inactivation of plasma kallikrein. The proteases were incubated at 25°C in 50 mM PIPES buffer (pH 7.0) containing Pro-Phe-ArgCH_2Cl at the indicated concentration. Inactivation of each protease was monitored by removing timed aliquots and assaying for esterase activity. ○——○, Kallikrein inactivation, I = 0.20 μM; ●——●, plasmin, I = 2.0 μM; △——△, factor X_a, I = 2.0 μM; ▲——▲, thrombin, I = 15 μM; □——□, urokinase, I = 500 μM.

kallikrein at 2.0×10^{-7} M, and reacts with other trypsin-like proteases more slowly in the presence of higher concentrations of the reagent[5] (Fig. 1). Comparison of values of k_{app}/I shows that plasma kallikrein differs in its reactivity toward Pro-Phe-ArgCH_2Cl from other proteases by a factor of 48 in the case of plasmin, and by factors of 10^2–10^5 for factor X_a, thrombin, and urokinase.[5]

TABLE I
AFFINITY LABELING OF KALLIKREINS[a]

Affinity Label	$10^{-4} \times k_{app}/I$ (M^{-1} min^{-1})[b]		
	Human plasma kallikrein	Human urinary kallikrein	Submaxillary gland protease (mouse)
Ac-Phe-ArgCH$_2$Cl	88	0.81	30
Pro-Phe-ArgCH$_2$Cl	120	0.79	12
Ac-Pro-Phe-ArgCH$_2$Cl	65	0.34	42
Ser-Pro-Phe-ArgCH$_2$Cl	150	0.65	78
Phe-Ser-Pro-Phe-ArgCH$_2$Cl	140	4.20	150
D-Phe-Phe-ArgCH$_2$Cl	2300	18	1500
Glu-Phe-ArgCH$_2$Cl	570	0.32	
DNS-Ala-Phe-ArgCH$_2$Cl	570		
Ala-Phe-ArgCH$_2$Cl	350	0.34	8.4
Phe-Phe-ArgCH$_2$Cl	170	0.63	55
DNS-Pro-Phe-ArgCH$_2$Cl	33	7.1	
Ala-Phe-LysCH$_2$Cl	7.4	0.020	0.19
Pro-Gly-ArgCH$_2$Cl	3.3	0.0029	0.0052
Pro-Phe-HarCH$_2$Cl	0.15	0.031	0.012

[a] Inactivation reactions were conducted at 25°C in 50 mM PIPES buffer (pH 7.0), which was 0.20 M in NaCl, except for plasma kallikrein, for which the NaCl was omitted.
[b] k_{app}/I, the ratio of the apparent, first-order rate constant for the inactivation to the concentration of the affinity label, is an estimate of k_2/K_i, the bimolecular constant for the reaction. Values shown for the first five reagents are actual values of k_2/K_i in the case of plasma and urinary kallikreins.

The reactivity of plasma kallikrein has been determined with two series of reagents. The first consists of di- through pentapeptide analogs corresponding to the sequence of kininogen to determine the influence of binding in the P_3–P_5 sites on reagent effectiveness. The affinity of the acetylated dipeptide analog, Ac-Phe-ArgCH$_2$Cl, was comparable to that of Phe-Ser-Pro-Phe-ArgCH$_2$Cl (Table I), indicating that the P_3–P_5 binding sites contribute little to the specificity of kallikrein for kininogen.

In the second series of reagents, the reactivity of plasma kallikrein was determined with chloromethyl ketones in which amino acid substituents in the Pro-Phe-ArgCH$_2$Cl sequence were replaced by other residues. The importance of the P_1 Arg on reagent effectiveness is clearly shown by the 50- and 800-fold greater reactivity of the arginine chloromethyl ketone than the corresponding lysine and homoarginine analogs. Similarly, the importance of the P_2 Phe is shown by the 36-fold greater reactivity of Pro-Phe-ArgCH$_2$Cl than Pro-Gly-ArgCH$_2$Cl. However, in P_3, replacement of Pro by Ala, Phe, or Glu resulted in reagents of comparable or superior reactivity, indicating that the P_3 residue is of minor importance in determining the specificity of plasma kallikrein.

A reagent with a P_3 D-Phe was prepared for kallikrein due to the favorable effect obtained with this substituent in a related case. D-Phe-Phe-ArgCH_2Cl is the most effective reagent for plasma kallikrein obtained, inactivating it with a $t_{1/2}$ of 15 min at a reagent concentration of $2.0 \times 10^{-9} M$; however, the selectivity of this reagent for the inactivation of plasma kallikrein is no greater than that of Pro-Phe-ArgCH_2Cl.

As shown in Fig. 2, D-Phe-Phe-ArgCH_2Cl reacts stoichiometrically

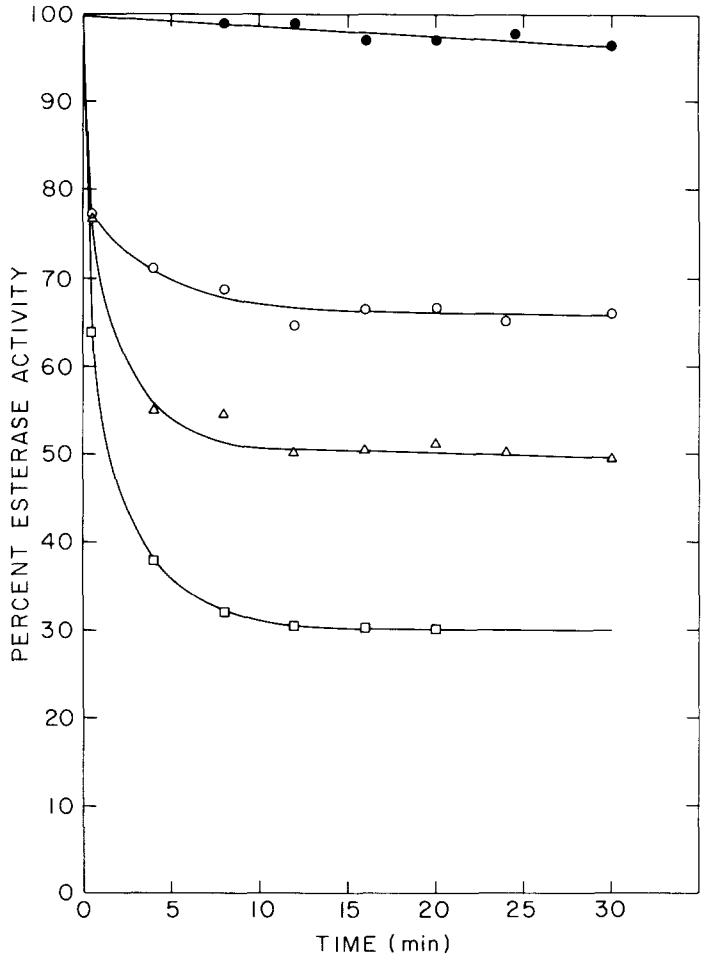

FIG. 2. Inactivation of human plasma kallikrein by D-Phe-Phe-ArgCH_2Cl. Plasma kallikrein ($1 \times 10^{-7} M$) was incubated with the reagent at the indicated concentrations in 50 mM PIPES buffer, pH 7.0 (final volume 1.54 ml). Reagent concentrations were as follows: ●——●, 0; ○——○, $2.5 \times 10^{-8} M$; △——△, $5 \times 10^{-8} M$; □——□, $7.5 \times 10^{-8} M$. Timed aliquots (20 μl) were removed and diluted into 2.00 ml of the Z-Lys-SBzl assay solution[9] and the change in absorbance monitored on a 0.10 recorder scale.

with plasma kallikrein, inactivating it in proportion to the level of affinity label. Titration of plasma kallikrein by D-Phe-Phe-ArgCH_2Cl has the advantage that when coupled with a sensitive colorimetric assay such as provided by Z-Lys-SBzl,[9] considerably lower concentrations of kallikrein can be determined than is possible with the usual *p*-nitrophenyl guanidinobenzoate (NPGB) titration.

Human Urinary Kallikrein. The differences between the specificity of glandular and plasma kallikrein are clearly illustrated by the differences in the kinetic constants in Table I for the two enzymes.[8] In all cases, the arginine chloromethyl ketones were more reactive with plasma kallikrein than urinary kallikrein and the pattern of reactivities of the affinity labels was distinct for each protease. As expected for enzymes hydrolyzing the same bond of kininogen, reagents with a P_1 Arg and P_2 Phe were the most effective of the inhibitors tested for both proteases.

Submaxillary Gland Protease. Our interest in the specificity differences among trypsin-like proteases led us to examine the reactivity of a series of arginine chloromethyl ketones toward the arginine-specific protease[15] of the mouse submaxillary gland, which functions as the epidermal growth factor-binding protein. Reagents prepared specifically for kallikrein proved to be most effective in the inactivation of this enzyme (Table I). The pattern of reactivity of the arginine-specific protease with the affinity labels is distinct from both plasma and urinary kallikrein, but the overall results suggest that this protease is kallikrein-like in specificity. In fact, the differences observed between the arginine-specific protease and human urinary kallikrein may be due at least in part to species differences, since previous results[8] have shown considerable differences between species for glandular kallikreins. Subsequent to these observations, Bothwell *et al.*[14] reported the independent conclusion that this protease exhibited considerable kininogenase activity and was immunologically similar to other mouse glandular kallikreins. Thus this result represents an example in which the specificity of a protease for a physiological substrate was deduced from its reactivity toward peptides of arginine chloromethyl ketone.

Affinity Labeling of Factor X_a

Factor X_a, a protease common to both the intrinsic and extrinsic blood coagulation pathways, hydrolyzes two bonds in the conversion of prothrombin to thrombin. The hydrolysis sites in bovine prothrombin are[16]

[15] M. Boesman, M. Levy, and I. Schenkein, *Arch. Biochem. Biophys.* 175, 463 (1976).

[16] S. Magnusson, T. E. Petersen, L. Sottrup-Jensen, and H. Claeys, *in* "Proteases and Biological Control" (E. Reich, D. B. Rifkin, and E. Shaw, eds.), p. 123. Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1975.

TABLE II
REACTIVITY OF FACTOR X_a WITH AFFINITY LABELS[a]

Affinity labels	Concentrations (M)	$t_{1/2}$ (min)	$10^{-4} \times k_{app}/I$ (M^{-1} min^{-1})
DNS-Glu-Gly-ArgCH$_2$Cl	2.5×10^{-9}	12.8	2200
Ile-Glu-Gly-ArgCH$_2$Cl	2.5×10^{-8}	14.4	190
Ac-Glu-Gly-ArgCH$_2$Cl	2.0×10^{-8}	24.7	140
Glu-Gly-ArgCH$_2$Cl	2.5×10^{-7}	17.5	16
Glu-Phe-ArgCH$_2$Cl	2.0×10^{-7}	25.1	14

[a] Inactivation reactions of factor X_a (44 ng/ml) were conducted at 25°C in 50 mM PIPES buffer (pH 7.0), which was 0.20 M in NaCl and 1.0 mM in $CaCl_2$. Rates of inactivation were determined by removing at least four timed aliquots (0.50 ml) and diluting with 0.60 ml of 1.67×10^{-4} M Bz-Ile-Glu-Gly-Arg-*p*NA in 0.20 M Tris buffer (pH 8.0), 0.20 M in NaCl, followed by observation at 405 nm.

-Ile-Glu-Gly-Arg↓-Ile and -Ile-Glu-Gly-Arg↓-Ser-

Reagents corresponding to this sequence were prepared and shown to be the most effective affinity labels for bovine factor X_a (Table II).[17]

Several aspects of the specificity of factor X_a are unique. Addition of the dansyl group (DNS) to Glu-Gly-ArgCH$_2$Cl increased the reactivity of the reagent 140-fold, yielding an affinity label effective at 2.5×10^{-9} M. For other plasma proteases this change yielded only a 10- to 20-fold increase in reactivity.[18] Acetylation or the addition of Ile to Glu-Gly-ArgCH$_2$Cl increased the reactivity of the reagent about 10-fold, whereas for other proteases there was little effect. Reagents containing Val, Pro, and Lys in the P_2 position were at least 2 orders of magnitude less effective than Glu-Gly-ArgCH$_2$Cl; however, factor X_a can readily accommodate a P_2 Phe.

Of the reagents tested, DNS-Glu-Gly-ArgCH$_2$Cl and Ac-Glu-Gly-ArgCH$_2$Cl were the most selective affinity labels for factor X_a, inactivating it about 20 times more readily than human plasma kallikrein and at least 50 times more effectively than thrombin and plasmin.

Affinity Labeling of Thrombin

Thrombin, the last protease in the blood-coagulation cascade, catalyzes the conversion of fibrinogen to fibrin by the hydrolysis of two peptide bonds, liberating fibrinopeptides A and B. It also hydrolyzes the "pro" portion of thrombin and activates factor XIII. The amino acid

[17] C. Kettner and E. Shaw, *Thromb. Res.* **22**, 645 (1981).
[18] C. Kettner and E. Shaw, *Biochim. Biophys. Acta* **569**, 31 (1979).

sequences preceding the hydrolyzed bonds are -Gly-Val-Arg-, -Ile-Pro-Arg-, and -Val-Pro-Arg- for the A chain of fibrinogen,[19] prothrombin,[16] and factor XIII,[20] respectively. Initially, chloromethyl ketones corresponding to each of these sequences were prepared.[21] Both reagents with a penultimate Pro inactivated thrombin with a $t_{1/2}$ of 17–22 min at 7.5×10^{-8} *M*, whereas Gly-Val-ArgCH_2Cl was about 20 times less effective. The degree of selectivity in inactivation of thrombin by the reagents containing a P_2 Pro was demonstrated by the 20-, 120-, and 150-fold greater reactivity of Ile-Pro-ArgCH_2Cl with thrombin than with plasma kallikrein, factor X_a, and plasmin, respectively.

An earlier observation in this laboratory indicated that thrombin was more susceptible to an inhibitor with a D-amino acid in the P_3 position, and Bajusz *et al.*[22] showed that a substrate with a D-Phe in the P_3 position was particularly well cleaved by thrombin. Subsequently, D-Phe-Pro-ArgCH_2Cl was prepared and its reactivity with thrombin was determined.[23] As shown in Fig. 3, 5×10^{-10} *M* D-Phe-Pro-ArgCH_2Cl inactivates 2.3×10^{-10} *M* thrombin within a few minutes, whereas it was at least 3 orders of magnitude less reactive with other trypsin-like proteases. When D-Phe-Pro-ArgCH_2Cl is allowed to react with a molar excess of thrombin, it acts as a titrant, rapidly inactivating thrombin stoichiometrically and providing a means of determining the concentrations of solutions of thrombin as low as 2.5×10^{-9} *M*. (For an example of titration data, cf. Ref. 23 or compare the titration of plasma kallikrein by D-Phe-Phe-ArgCH$_2$Cl, Fig 1.)

Studies by Collen have demonstrated that D-Phe-Pro-ArgCH_2Cl is an effective anticoagulant *in vivo*.[24] No significant drop in plasma fibrinogen levels was observed when rabbits received an intravenous injection of 1.0 mg of affinity label before an intravenous infusion of 0.5 mg of thrombin over a 5-min period. Rabbits receiving only thrombin died due to massive thrombosis in the right heart before the infusion of the thrombin was complete. This effective level of D-Phe-Pro-ArgCH_2Cl is considerably less than its LD_{50} in mice, which is greater than 50 mg/kg.

[19] S. Iwanaga, P. Wallen, N. J. Grondahl, A. Nenschen, and B. Blombach, *Eur. J. Biochem.* **8**, 189 (1969).

[20] T. Takagi and R. F. Doolittle, *Biochemistry* **13**, 750 (1974).

[21] C. Kettner and E. Shaw, *in* "Chemistry and Biology of Thrombin" (R. L. Lundblad, J. W. Fenton, II, and K. G. Mann, eds.), p. 129. Ann Arbor Sci. Publ., Ann Arbor, Michigan, 1977.

[22] S. Bajusz, E. Barabas, E. Szell, and D. Bagdy, *in* "Peptides: Chemistry, Structure and Biology" (R. Walter and J. Meienhofer, eds.), p. 603. Ann Arbor Sci. Publ., Ann Arbor, Michigan, 1975.

[23] C. Kettner and E. Shaw, *Thromb. Res.* **14**, 969 (1979).

[24] D. Collen, O. Matsuo, J. M. Stassen, C. Kettner, and E. Shaw, *J. Lab. Clin. Med.* (submitted for publication).

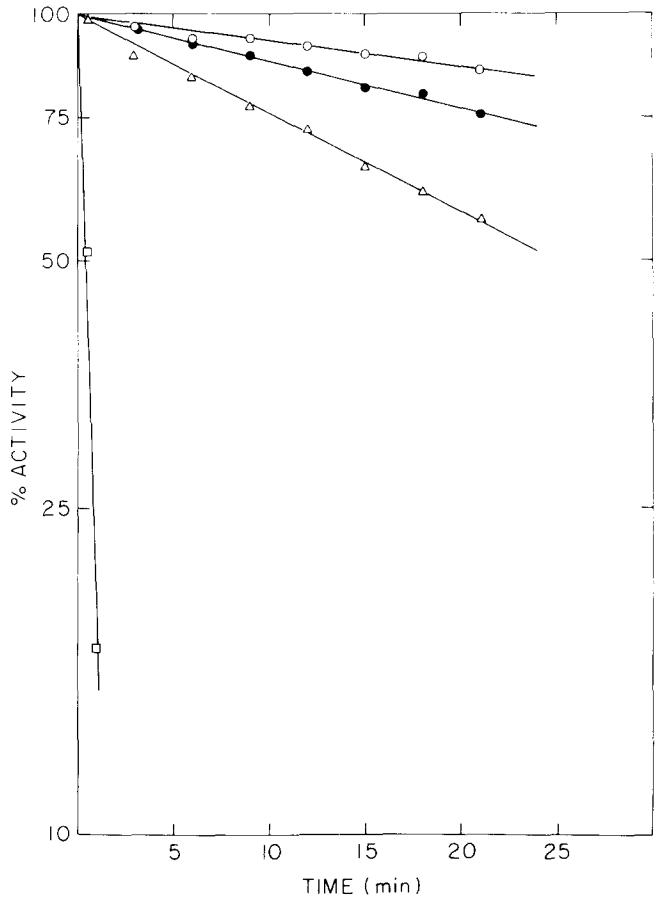

FIG. 3. Selectivity of D-Phe-Pro-ArgCH_2Cl in the inactivation of thrombin. The proteases were incubated with D-Phe-Pro-ArgCH_2Cl at the indicated concentrations at 25°C in 50 mM PIPES buffer, pH 7.0; timed aliquots were removed for measurement of residual esterase activity.[9] The initial concentrations of thrombin, plasma kallikrein, factor X_a, and plasmin were 0.23, 2.5, 14, and 1.8 nm, respectively. ○——○, Plasmin inactivation, $I = 6 \times 10^{-8} M$; ●——●, factor X_a, $I = 6 \times 10^{-8} M$; △——△, kallikrein, $I = 6 \times 10^{-8} M$; □——□, thrombin, $I = 5 \times 10^{-10} M$.

Affinity Labeling of Plasmin

Plasmin hydrolyzes multiple bonds of its physiological substrate, fibrin, during the lysis of blood clots. The specificity of plasmin for fibrin *in vivo* does not appear to be highly dependent on the sequence of its substrate, but rather on the binding of plasmin and plasminogen on the sur-

TABLE III
SELECTIVITY OF AFFINITY LABELS IN INACTIVATION OF PLASMA PROTEASES[a]

Affinity label	$10^{-4} \times k_{app}/I$ (M^{-1} min^{-1})[b]			
	Human plasma kallikrein	Bovine factor X_a	Bovine thrombin	Human plasmin
D-Phe-Phe-ArgCH$_2$Cl	2320	19.3	45	36.7
DNS-Glu-Phe-ArgCH$_2$Cl	770	42.1	2.62	85
Glu-Phe-ArgCH$_2$Cl	568	13.8	0.154	14.5
DNS-Ala-Phe-ArgCH$_2$Cl	566	49	—	95
Ala-Phe-ArgCH$_2$Cl	440	2.22	0.17	7.83
Phe-Phe-ArgCH$_2$Cl	171	4.7	—	2.64
Pro-Phe-ArgCH$_2$Cl	140	1.33	0.12	1.86
DNS-Glu-Gly-ArgCH$_2$Cl	141	2200	26	28.6
D-Phe-Pro-ArgCH$_2$Cl	47.5	27.2	69,000	4.3
DNS-Pro-Phe-ArgCH$_2$Cl	33.2	2.26	—	2.08
Ile-Glu-Gly-ArgCH$_2$Cl	28.6	190	3.03	0.45
Ala-Lys-ArgCH$_2$Cl	18.9	0.0158	—	0.44
Ile-Leu-ArgCH$_2$Cl	18.8	0.385	5.2	0.317
Glu-Gly-ArgCH$_2$Cl	15.8	16.0	2.2	1.00
Leu-Gly-Leu-Ala-ArgCH$_2$Cl	8.25	27.9	15.3	3.33
Gln-Gly-ArgCH$_2$Cl	4.3	14	1.67	1.00
D-Val-Leu-LysCH$_2$Cl	3.5	—	12.0	5.45
Pro-Gly-ArgCH$_2$Cl	3.3	2.0	1.2	0.11
Val-Pro-ArgCH$_2$Cl	2.96	0.028	54.0	0.31
Phe-Ala-ArgCH$_2$Cl	2.85	1.37	9.0	0.10
Val-Ile-Pro-ArgCH$_2$Cl	2.2	2.18	73.0	0.26
Ile-Pro-ArgCH$_2$Cl	2.0	0.34	42.0	0.27
Gly-Val-ArgCH$_2$Cl	1.6	0.036	1.9	0.051
Val-Val-ArgCH$_2$Cl	1.47	0.037	2.1	0.034
Ac-Gly-Gly-ArgCH$_2$Cl	1.40	5.59	0.74	0.053
Glu-Gly-HarCH$_2$Cl	0.057	0.004	—	0.005

[a] Inactivation reactions were conducted at 25°C in 50 m*M* PIPES buffer, pH 7.0. The incubation solutions also contained 0.20 *M* NaCl for bovine thrombin and 0.20 *M* NaCl and 1.0 m*M* $CaCl_2$ for factor X_a.

[b] k_{app}/I, the bimolecular constants for the inactivation reactions, are the ratios of the apparent, pseudo-first-order rate constants for the inactivation of the proteases to the concentration of the affinity label used in the inactivation.

face of fibrin.[25] In consequence, the usual approach for the development of a selective-affinity label is not applicable to plasmin. In the course of our studies of plasma proteases, effective affinity labels for plasmin have been obtained (Table III), and several aspects of the response of plasmin are worthy of note.

[25] B. Wiman and D. Collen, *Nature (London)* **272**, 549 (1978).

The difference in the affinity of plasmin for Ala-Phe-ArgCH$_2$Cl and the Lys analog indicated that plasmin has a 2-fold greater preference for the lysine chloromethyl ketone.[5] However, this difference in affinity is relatively minor when the influence of residues in the P_2 and P_3 sites are examined. For example, there is a 400-fold difference in the reactivity of Glu-Phe-ArgCH$_2$Cl and Val-Val-ArgCH$_2$Cl.

DNS-Ala-Phe-ArgCH$_2$Cl is the most effective affinity label for plasmin, inactivating it 50% in 18 min at 4.0×10^{-8} M, and other arginine chloromethyl ketones containing a P_2 Phe were among the most effective reagents, indicating a preference of plasmin for this P_2 residue. The reactivity of plasmin is also dependent on the P_3 residue, as shown by the 2- to 8-fold difference in the reactivity of plasmin with Pro-Phe-ArgCH$_2$Cl and its analogs containing a P_3 Glu, Ala, and Phe. Finally, addition of DNS in the P_4 position or substitution of a D-residue in the P_3 position resulted in reagents with enhanced reactivity as observed for other plasma proteases.[17,18]

Affinity labels that will distinguish plasmin from other trypsin-like proteases have not been obtained. However, urokinase reacts very poorly with arginine chloromethyl ketones containing a P_2 Phe; therefore, such reagents readily distinguish plasmin from urokinase and probably from other plasminogen activators.

Affinity Labeling of Plasminogen Activators

Aside from the role of plasminogen activators in hemostasis, tissue and plasma activators are associated with a number of pathological processes, which include malignant cell transformation,[26,27] inflammation,[28] and the promotion of carcinogenesis.[29]

The urinary plasminogen activator, urokinase, hydrolyzes a single Arg-Val bond in the Pro-Gly-Arg-Val- sequence of plasminogen in the activation process.[30] The affinity label corresponding to this sequence, Pro-Gly-ArgCH$_2$Cl, was effective in the inactivation of urokinase at the 10^{-6} M level (K_i = 68 μM, k_2 = 0.47 min^{-1}).[18] Measurement of the inactivation rate, k_{app}/I, for Glu-Gly-ArgCH$_2$Cl, DNS-Glu-Gly-ArgCH$_2$Cl, and Ac-Gly-Gly-ArgCH$_2$Cl revealed that these reagents were even somewhat

[26] L. Ossowski, J. C. Unkeless, A. Tobia, J. P. Quigley, D. B. Rifkin, and E. Reich, *J. Exp. Med.* **137**, 112 (1973).

[27] J. C. Unkeless, A. Tobia, L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich, *J. Exp. Med.* **137**, 85 (1973).

[28] J.-D. Vassalli, J. Hamilton, and E. Reich, *Cell* **11**, 695 (1977).

[29] M. Wigler and I. B. Weinstein, *Nature (London)* **259**, 232 (1976).

[30] L. Sottrup-Jensen, M. Zajdel, H. Claeys, T. E. Petersen, and S. Magnusson, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2577 (1975).

more reactive than Pro-Gly-ArgCH_2Cl 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, resulting in differences in reactivity of up to 4 orders of magnitude. These differences in the susceptibility of urokinase suggested that the series of affinity labels would be useful in evaluating differences among plasminogen activators from various sources.

The plasminogen activator secreted by HeLa cells following exposure to the tumor promoter, phorbol myristic acetate,[28] was also examined. Because of the low concentration of protease, use was made of the coupled assay with plasminogen and ^{125}I-labeled fibrin to measure activity. Since values of k_{app}/I are difficult to obtain in the nonlinear ^{125}I-labeled fibrin assay, values of I_{50}, defined in Fig. 4, were determined for the affinity labels. The validity of this approach was established by the good correlation obtained in the case of urokinase between values of k_{app}/I and I_{50}. As shown in Fig. 4,[31] the HeLa cell plasminogen activator and urokinase are similar in their susceptibilities toward affinity labels in many respects. Reagents containing a P_2 Gly were the most effective for both enzymes, reagents with a P_2 Pro and Ala were intermediate in effectiveness, and those containing a P_2 Phe were the least reactive. However, distinctive differences exist between the responses of the two plasminogen activators. Most notable is the effect of adding a residue to the P_4 position. For example, the addition of DNS to Glu-Gly-ArgCH_2Cl increased its reactivity with the HeLa cell enzyme by an order of magnitude, but decreased its reactivity with urokinase.

Thus far we have not obtained an affinity label that will distinguish either the HeLa cell enzyme or urokinase from all plasma proteases. For example, the most effective inhibitor of the HeLa cell plasminogen activator, DNS-Glu-Gly-ArgCH_2Cl, reacts with factor X_a at the 10^{-9} M level.[17] On the other hand, Ac-Gly-Gly-ArgCH_2Cl is 50 times more reactive with urokinase than plasmin, providing a means of distinguishing these two proteases.

Selectivity in Affinity Labeling

This section summarizes the results obtained in the affinity labeling of plasma trypsin-like enzymes by providing values of k_{app}/I for the reactivity of the affinity labels with plasma kallikrein, plasmin, factor X_a, and thrombin. In addition to making the data on the reactivities of the chloromethyl ketones accessible, Table III illustrates the fact that a

[31] P. Coleman, C. Kettner, and E. Shaw, *Biochim. Biophys. Acta* **569**, 41 (1979).

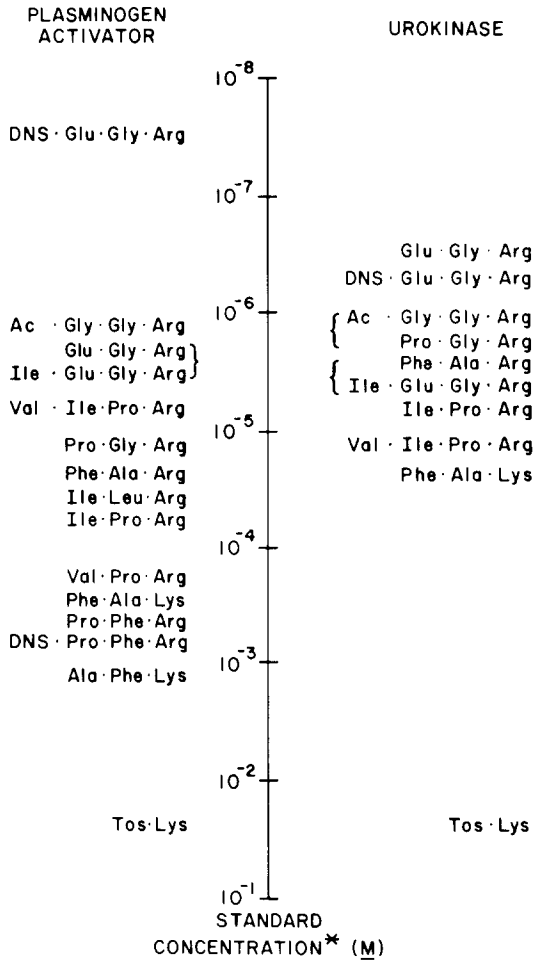

FIG. 4. The relative effectiveness of chloromethyl ketone inhibitors with plasminogen activator (from HeLa cells) and urokinase. The position of the chloromethyl ketone on the log concentration scale indicates the approximate value of its "standard concentration." Inhibitors with close to identical values are designated by brackets. (*) The concentration of inhibitor that increases the time of the midpoint in the reaction profile of ^{125}I-labeled fibrin solubilization to twice that of the uninhibited control. From Coleman *et al.*[31]

unique pattern of reactivity is obtained for each protease. These patterns should be useful in the identification of proteases in their physiological environments. In crude systems the reactivity of the proteases with affinity labels may be less than that obtained with purified enzymes, particularly if their physiological substrate is present; however, the relative reactivities can be expected to be characteristic (Table III).

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[64] Affinity Methods Using Argininal Derivatives

By SHIN-ICHI ISHII and KEN-ICHI KASAI

Leupeptin, which is produced by several strains of Streptomyces, has been known as a potent inhibitor for trypsin-family proteases including trypsin, plasmin, kallikrein, and urokinase.[1,2] It is a mixture of tripeptide derivatives; α-amino group of L-leucyl-L-leucyl-L-argininal is acylated by either acetyl or propionyl group. The strong inhibitory action has been explained by formation of a covalent hemiacetal adduct between an aldehyde group in the inhibitor and a serine hydroxyl group in the enzyme-active site.[3] We shall obtain useful biospecific affinity adsorbents if leupeptin or its essential moiety can be immobilized. Because the guanidino group of the terminal argininal is also indispensable for the activity, there remains no functional group suitable for immobilization reaction. In order to use leupeptin as an immobilized ligand for affinity adsorbents, appropriate modification is required.

We have developed a new method to expose an α-amino group by using thermolysin. Thermolysin was found to hydrolyze exclusively the leucyl–leucyl bond. However, the direct thermolysin digestion of leupeptin gave complex products and yield of the expected leucylargininal was very low. This would be due to the reaction of the exposed α-amino group with the aldehyde group in the same or the other molecule. Thus the aldehyde group was protected prior to thermolysin digestion and the product was handled under the protected form throughout a series of reaction steps. Finally, the aldehyde group was regenerated (see Scheme I).

[1] T. Aoyagi, S. Migita, M. Nanbo, F. Kojima, M. Katsuzaki, M. Ishizuka, T. Takeuchi, and H. Umezawa, *J. Antibiot.* **22**, 558 (1969).
[2] H. Umezawa, this series, Vol. 45, p. 678.
[3] H. Kuramochi, H. Nakata, and S. Ishii, *J. Biochem. (Tokyo)* **86**, 1403 (1979).

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