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Synthesis of Peptides of Arginine Chloromethyl Ketone. Selective Inactivation of Human Plasma Kallikrein†

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ABSTRACT: Synthetic procedures have been developed for the preparation of peptides of arginine chloromethyl ketone and applied in the preparation of affinity labels which correspond to the -Pro-Phe-Arg- C terminus of bradykinin, a physiological cleavage site of kallikrein in kininogen. Two such reagents, Ala-Phe-ArgCH_2Cl and Pro-Phe-ArgCH_2Cl, proved to be highly effective as well as selective affinity labels for human plasma kallikrein. For example, Pro-Phe-ArgCH_2Cl inactivates plasma kallikrein 50% in 24 min at a concentration of 2×10^{-8} M, while other trypsin-like proteases are less susceptible in inactivation than kallikrein, differing by a factor of 48 for plasmin and factors of 10^2–10^5 for factor X_a, thrombin, and urokinase. The affinity of human plasma kallikrein for Ala-Phe-ArgCH_2Cl (K_i = 0.078 μM) is about 60 times that for Ala-Phe-LysCH_2Cl (K_i = 4.9 μM), whereas human plasmin exhibits about the same affinity for the former affinity label (K_i = 1.3 μM) as for the latter (K_i = 0.83 μM). The rate constants for the irreversible step of the affinity labeling reaction, k_2, are similar for all affinity labels tested with the individual proteases: 0.35 min^{-1} for plasma kallikrein and 0.18 min^{-1} for plasmin.

An increasing number of serine proteases have been characterized which have very specialized physiological roles that are often limited to the cleavage of one or two peptide bonds (Neurath and Walsh, 1976). Other serine proteases have recently been identified by their biological activities, while their structure and function remain uncertain. Inhibitors are helpful in deducing or confirming the physiological role of enzymes. For example, diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride are useful in the initial characterization of an enzyme as a serine protease, while more specialized inhibitors acting as affinity labels are capable of greater discrimination. Affinity labeling by substrate-derived chloromethyl ketones has permitted further characterization of the activities of serine proteases into chymotryptic, tryptic, and elastolytic (Shaw, 1975). Of these, the serine proteases of trypsin-like specificity comprise a large group of enzymes, including proteases of blood coagulation, fibrinolysis, and fertilization. We have selected this group of proteases, having a common primary specificity, to determine the extent to which selectivity in inactivation can be achieved by affinity labeling.

In earlier work (Coggins et al., 1974; and Shaw, 1975), it was shown that peptides of lysine chloromethyl ketone such as Ala-Phe-LysCH_2Cl inactivate both plasma kallikrein and plasmin at micromolar concentrations of reagent, distinguishing these proteases from thrombin. This selectivity was attributed to differences in the subsites for normal substrate binding. The amino acid sequences at the cleavage sites of the physiological substrates have been determined for a number of regulatory, trypsin-like proteases, revealing that in most

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cases hydrolysis occurs at one or two arginine bonds rather than at lysine peptide bonds. It was important, therefore, to develop a synthetic procedure for the preparation of peptides of arginine chloromethyl ketone and to examine the extent to which the amino acid sequence at the cleavage site of the physiological substrate confers specificity for the target protease. Plasma kallikrein, a highly specific protease whose physiological role is not clear but whose proteolytic formation of kinins by cleavage of the Arg–Ser bond at a -Pro-Phe-Arg-Ser sequence is well documented (Pisano, 1975), was chosen for initial studies. Reagents corresponding to the amino acid sequence of kallikrein's kininogen substrate were prepared to determine if a specific affinity label could be obtained by designing reagents which correspond to the amino acid sequence of the physiological substrate of the target protease. In addition to providing a test for the validity of this approach to selective affinity labeling, these reagents may prove to be useful in deducing the physiological functions of plasma kallikrein.

In earlier synthetic work with arginine chloromethyl ketones, it became evident that cyclization involving the guanidino side chain of arginine represented a difficulty in synthesis (Shaw and Glover, 1970); however, synthesis in which the basic side chain of arginine was in the form of the nitroguanidino group permitted increased yields in the preparation of *N*-α-tosylarginine chloromethyl ketone (Inouye et al., 1974). Consequently, we have utilized the nitro group for protection of the arginine side chain in devising a synthetic scheme leading to peptides of arginine chloromethyl ketone.

Materials and Methods

Z-Ala-Phe-Arg(NO_2)-OH and Z-Pro-Phe-Arg(NO_2)-OH were obtained by saponification of the corresponding methyl esters. The blocked tripeptides were prepared by the addition of blocked amino acids to H-Arg(NO_2)-OMe using the mixed anhydride procedure and deblocking the intermediate, Boc-Phe-Arg(NO_2)-OMe, with ethanolic HCl. H-Lys(Z)-CH_2Cl·HCl was prepared by the method of Coggins et al. (1974).

Z-Ala-Phe-OH and Z-Pro-Phe-OH were purchased from Sigma Chemical Co. Boc-D-Arg(NO_2)-OH was obtained from Vega-Fox Biochemicals.

Analytical Procedures. NMR[1] spectra were measured on a Varian T-60 spectrometer; spectral data reported are limited to pertinent bands. Melting points were measured on a Fisher-Johns apparatus and are reported uncorrected. Elemental microanalyses were performed by the Department of Applied Science, Brookhaven National Laboratory, and Galbraith Laboratories, Inc., Knoxville, Tenn. Single-column amino acid analyses were performed on a 0.9 × 22 cm column packed with Durram DC-6A resin following hydrolysis in 2 mL of 6 N HCl for 24 h at 110 °C in sealed evacuated tubes. Thin-layer chromatography was performed on E. Merck precoated silica gel plates (catalog no. 5534/0001).

Enzymatic Studies. Human plasma kallikrein was purified from Cohn fraction IV-1 by a previously published procedure (Sampaio et al., 1974). Human plasminogen was purified from Cohn fraction III (Deutsch and Mertz, 1970; Liu and Mertz, 1971) and activated with streptokinase (Robbins and Summaria, 1970). Human α-thrombin, a gift from Dr. John Fenton, was purified by the procedure of Fenton et al. (1977), and bovine factor X_a, a gift from Dr. Yale Nemerson, was purified by the procedure of Jesty and Esnouf (1973). Human urokinase was purchased from Leo Pharmaceutical Products, Denmark, no. 9092207 1712E. Protease concentrations were determined by active-site titrations using methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973) by the procedure described by Coleman et al. (1976) or with *p*-nitrophenyl *p*-guanidinobenzoate (Chase and Shaw, 1970).

Inactivations of plasma kallikrein by the affinity labels were performed in 50 mM Pipes buffer (pH 7.0) at 25 °C. A 1.00-mL dilution of a stock solution of the chloromethyl ketone in 1.0 mM HCl was diluted to 10.0 mL with 55.5 mM Pipes buffer (pH 7.0). The reaction was initiated by adding 50 μL of plasma kallikrein to yield a final concentration of 5.0 nM for the protease. At least seven 1.00-mL aliquots were removed at time intervals and assayed for esterase activity. The apparent, pseudo-first-order rate constants for the inactivation reactions were determined from the slopes of semilogarithmic plots of esterase activity vs. time. The rate constants for the inactivation of the other proteases studied were determined by the general procedure described above with some modifications.

Inactivations of plasmin, thrombin, and factor X_a were conducted in 2.00 mL of buffer and 0.200-mL aliquots were removed for assays of activities of the proteases, while the inactivations of urokinase were conducted in 1.00 mL of buffer and 0.100-mL aliquots were removed for assay. Buffer solutions for thrombin and urokinase were 0.20 M in NaCl, and the buffer solution for factor X_a was 0.20 M in NaCl and 1.0 mM in $CaCl_2$. The initial concentrations of plasmin, thrombin, urokinase, and factor X_a in the reaction solutions were 15, 26, 27, and 69 nM, respectively.

Plasma kallikrein, plasmin, thrombin, and urokinase were assayed for esterase activity in a 2.00-mL final volume of 0.10 M Tris buffer (pH 8.0) containing 0.10 M NaCl, 0.10 mM thiobenzyl benzoxycarbonyllysinate as a substrate in the presence of 0.33 mM 5,5′-dithiobis(2-nitrobenzoic acid) as a chromogen. A higher concentration of substrate (1.33 mM) was used in the assay for factor X_a. Hydrolysis of the thioester was monitored at 412 nm using a Beckman DB spectrophotometer (Green and Shaw, 1978).

Kinetic constants were determined for the inactivation reactions of plasma kallikrein and plasmin by the equation (eq 1) described by Kitz and Wilson (1962) in which the reversible dissociation constant, K_i, and the first-order rate constant, k_2, are described in terms of the apparent, pseudo-first-order rate constant, k_{app}, and the concentration of the affinity label, I. At least five values of k_{app} were determined at different concentrations of the affinity labels, and the kinetic constants, K_i and k_2, were determined from double-reciprocal plots of k_{app} vs. I after determining the best straight line by the least-squares method.

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

Values of k_{app} were determined for factor X_a, thrombin, and urokinase at several concentrations of the affinity labels to first establish the concentration range which k_{app} is proportional to I. K_{app}/I was then used as an estimate of k_2/K_i according to the relationship in eq 2 (Kitz and Wilson, 1962)

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

if $K_i \gg I$.

Biological Assays. The ability of thrombin to clot fibrinogen

[1] Abbreviations used are: TLCK, *N*-α-tosyllysine chloromethyl ketone; NMR, nuclear magnetic resonance; Boc, *tert*-butoxycarbonyl; Z, benzoxycarbonyl; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; LC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

was determined by the method of Kline (1955). For the bioassay of human plasma kallikrein, the ability to lower blood pressure in the intact rat was measured as described by Ohanian et al. (1977).

A sample of plasma kallikrein (0.64 nmol) was incubated with Pro-Phe-ArgCH_2Cl (0.32 nmol) in 0.50 mL of 50 mM Pipes buffer (pH 7.0) in order to obtain a sample of partially inactivated protease to be used in establishing the correlation between protease esterase activity and biological activity. After 20 min, the esterase activity had decreased by 52% and no further change was observed. The biological activity of the partially inactivated protease (4 μg) was compared with that of the completely inactivated sample and with that of a control by monitoring the depression of rat arterial blood pressure.

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 THF and was 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 h 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 with ether. The diazomethyl ketone of Boc-Arg(NO_2)-OH was recrystallized from methanol to yield 2.56 g of product (mp 150–151 °C; $[\alpha]^{24}_D$ −57.2 in DMF).

Anal. Calcd for $C_{12}H_{21}N_7O_5$: C, 41.97; H, 6.18; N, 28.56. Found: C, 41.84; H, 6.27; N, 28.45.

Boc-Arg(NO_2)CHN_2 (1.47 g, 4.30 mmol) was dissolved in a minimum volume of THF 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 titurating 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 H-D-Arg(NO_2)CH_2Cl·HCl. H-D-Arg(NO_2)CH_2Cl·HCl was prepared from Boc-D-Arg(NO_2)-OH (1.00 g, 3.10 mmol) by the exact procedure described for the L enantiomer, except the reaction was run on 1/5 scale. Boc-D-Arg(NO_2)CHN_2 (mp 150–151 °C; $[\alpha]^{24}_D$ = 50.7 in DMF) was obtained in a yield of 0.17 g and was treated with HCl to yield 0.13 g of H-D-Arg(NO_2)CH_2Cl·HCl.

Preparation of Z-Pro-Phe-Arg(NO_2)CH_2Cl. Z-Pro-Phe-OH (0.43 g, 1.10 mmol) was allowed to react with *N*-methylmorpholine (0.12 mL, 1.10 mmol) in 5 mL of THF for 10 min at −20 °C. Cold THF (20 mL) containing triethylamine (0.15 mL, 1.10 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, 1.10 mmol) dissolved in 5 mL of cold DMF. After stirring for 1 h at −20 °C and 2 h 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 was 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).

NMR spectrum in acetone-d_6: δ 4.40 (s, 2 H). Anal. Calcd for $C_{29}H_{37}N_7O_7Cl$: C, 55.18; H, 5.92. Found: C, 54.94; H, 5.73.

Preparation of Z-Pro-Phe-D-Arg(NO_2)CH_2Cl. Z-Pro-Phe-D-Arg(NO_2)CH_2Cl was prepared from H-D-Arg-(NO_2)CH_2Cl·HCl (0.34 g, 1.20 mmol) and Z-Pro-Phe-OH (0.47 g, 1.20 mmol) by the above procedure described for Z-Pro-Phe-Arg(NO_2)CH_2Cl. Crystallization from ethyl acetate yielded 0.44 g of product (mp 123–126 °C).

NMR spectrum in acetone-d_6: δ 4.50 (s, 2 H). Anal. Calcd for $C_{29}H_{37}N_7O_7Cl$: C, 55.18; H, 5.92. Found: C, 55.05; H, 5.96.

Preparation of Z-Ala-Phe-Arg(NO_2)CH_2Cl. Z-Ala-Phe-OH (0.79 g, 2.10 mmol) was allowed to react with *N*-methylmorpholine (0.23 mL, 2.10 mmol) and isobutyl chloroformate (0.28 mL, 2.10 mmol) in 5 mL of THF for 10 min at −20 °C. Cold THF (30 mL) containing triethylamine (0.29 mL, 2.10 mmol) was added to the mixed anhydride preparation, and the mixture was immediately added to H-Arg(NO_2)CH_2Cl·HCl (0.54 g, 2.10 mmol) dissolved in 5 mL of DMF. After stirring for 1 h at −20 °C and 2 h at room temperature, 30 mL of THF was added and the mixture was filtered. The filtrate was evaporated to yield a residue which crystallized after adding methanol. The chloromethyl ketone was recrystallized from ethyl acetate to yield 0.64 g of product.

NMR spectrum in acetone-d_6: δ 4.33 (s, 2 H). Anal. Calcd. for $C_{27}H_{34}N_7O_7Cl$: C, 53.68; H, 5.68; N, 16.23. Found: C, 53.58; H, 5.60; N, 16.06.

Preparation of Z-Ala-Phe-Lys(Z)CH_2Cl. Z-Ala-Phe-OH (0.46 g, 1.20 mmol) was allowed to react with *N*-methylmorpholine (0.14 mL, 1.20 mmol) and isobutyl chloroformate (0.16 mL, 1.20 mmol) for 10 min in 5 mL of THF at −20 °C. Cold THF (20 mL) containing triethylamine (0.17 mL, 1.20 mmol) was added, and the mixture was immediately added to H-Lys(Z)CH_2Cl·HCl (0.43 g, 1.20 mmol) dissolved in 5 mL of cold DMF. The mixture was stirred for 1 h at −20 °C and 2 h at room temperature. Additional THF was added, the reaction mixture was filtered, and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate and washed with 0.2 N HCl, 5% $NaHCO_3$, and saturated aqueous NaCl. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to yield a partially crystalline residue. Recrystallization from methanol yielded 0.35 g of product (mp 188–190 °C).

Anal. Calcd. for $C_{35}H_{41}N_4O_7Cl$: C, 63.19; H, 6.22; N, 8.42. Found: C, 63.33; H, 6.14; N, 8.37.

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 HF in the presence of 1 mL of anisole using a KelF, HF line similar to that described by Sakakibara et al. (1967). After allowing the reaction to proceed for 30 min at 0 °C, HF 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 was eluted with 120 mL of 0.4 N HCl. After lyophilization of the HCl solution, the residue was transfrerred to a small container by dissolving in methanol and concentrating under a stream of nitrogen. The product was obtained as 0.36 g of an amorphous, white solid by triturating with ether and drying over P_2O_5 and KOH.

TLC indicated a single spot, R_f 0.21, by both ninhydrin and Sakaguchi stains after developing with butanol–acetic acid–water (4:1:1). Amino acid analysis of Z-Pro-Phe-Arg(NO_2)-CH_2Cl: Pro, 1.00; Phe, 0.90. Amino acid analysis of Pro-Phe-ArgCH_2Cl·2HCl: Pro, 1.00; Phe, 0.88.

Preparation of H-Ala-Phe-ArgCH_2Cl·2HCl. Z-Ala-Phe-Arg(NO_2)CH_2Cl (0.40 g, 0.66 mmol) was treated with HF, and the product was isolated by the procedure described for

the preparation of Pro-Phe-ArgCH_2Cl·2HCl. After drying in vacuo, with KOH and P_2O_5, 0.29 g of product was obtained as a white solid.

TLC with butanol–acetic acid–water (4:1:1) indicated a single spot, R_f 0.34, by both ninhydrin and Sakaguchi stains. Amino acid analysis of Z-Ala-Phe-Arg(NO_2)CH_2Cl: Ala, 1.00; Phe, 0.87. Amino acid analysis of Ala-Phe-ArgCH_2Cl·2HCl: Ala, 1.00; Phe, 0.89.

Preparation of H-Ala-Phe-LysCH_2Cl·2HCl. Z-Ala-Phe-Lys(Z)CH_2Cl (0.32 g, 0.48 mmol) was treated with HF and the product was isolated by the procedure described for the arginine chloromethyl ketones. After drying over P_2O_5 and KOH in vacuo, 0.19 g of product was obtained in the form of a white powder.

The product exhibited a single spot (R_f 0.29) on TLC by UV and by ninhydrin stain after development with butanol–acetic acid–water (4:1:1). Amino acid analysis of Z-Ala-Phe-Lys(Z)CH_2Cl: Ala, 1.00; Phe, 0.91. Amino acid analysis of Ala-Phe-LysCH_2HCl·2HCl: Ala, 1.00; Phe, 0.85.

Results and Discussion

Synthesis of Arginine Chloromethyl Ketones. Two synthetic procedures have been developed for the preparation of peptides containing a terminal nitroarginine chloromethyl ketone: (1) direct activation of blocked peptides by treatment with isobutyl chloroformate for 3–4 h followed by treatment with diazomethane and HCl (reaction 3) and (2) the addition of blocked peptide to H-Arg(NO_2)CH_2Cl (reaction 4).

Z-Pro-Phe-Arg(NO_2)-OH
$$\rightarrow \text{Z-Pro-Phe-Arg}(NO_2)CH_2Cl \quad (3)$$

Z-Pro-Phe-OH + H-Arg(NO_2)CH_2Cl
$$\rightarrow \text{Z-Pro-Phe-Arg}(NO_2)CH_2Cl \quad (4)$$

Preparation of peptides of nitroarginine chloromethyl ketone through the reactions illustrated in reaction 3 was the initial synthetic approach taken due to the apparent inertness of H-Arg(NO_2)CH_2Cl to coupling reactions; however, further investigation revealed that H-Arg(NO_2)CH_2Cl could be coupled to either Z-Ala-Phe-OH or Z-Pro-Phe-OH by the mixed anhydride method (reaction 4).

Preparation of chloromethyl ketones from either Z-Pro-Phe-Arg(NO_2)-OH or Z-Ala-Phe-Arg(NO_2)-OH resulted in mixtures of products which required LC fractionation in order to obtain analytically pure samples. In each case, two distinctly different products were isolated whose elemental composition corresponded to the desired chloromethyl ketones. For example, preparation of the chloromethyl ketone from Z-Pro-Phe-Arg(NO_2)-OH and fractionation of the products by LC yielded a sample (mp 102–105 °C) in a yield of 18% and a sample (mp 122–125 °C) in a yield of 8.5%. NMR analysis of both products revealed the expected bands for the peptide moiety, but the $-COCH_2Cl$ bands of the chloromethyl ketone portions of the molecules were distinctly different and corresponded to the L-Arg and D-Arg derivatives, respectively, on the basis of the evidence given below. These observations indicated that the nitroarginine residue had been racemized probably as a consequence of the long exposure to isobutyl chloroformate required for complete reaction.

The subsequent preparation of H-Arg(NO_2)CH_2Cl·HCl in good yields and the development of a method of extending the peptide chain (reaction 4) provided a means of identifying the configuration of the products obtained by reaction 3. When the mixed anhydride of either Z-Pro-Phe-OH or Z-Ala-Phe-OH was prepared and coupled to H-Arg(NO_2)CH_2Cl (reaction 4), the products obtained were identical to the major components obtained by reaction 3. Furthermore, the coupling of Z-Pro-Phe-OH to H-D-Arg(NO_2)CH_2Cl by reaction 4 yielded a product identical to the secondary product obtained by reaction 3. Finally, Z-Pro-Phe-Arg(NO_2)CH_2Cl and Z-Pro-Phe-D-Arg(NO_2)CH_2Cl prepared by reaction 4 were eluted as single peaks from the LC column initially used to purify the products from reaction 3 and corresponded in position to the major and secondary products obtained from reaction 3.

Final products were obtained by deblocking Z-Pro-Phe-Arg(NO_2)CH_2Cl and Z-Ala-Phe-Arg(NO_2)CH_2Cl prepared by reaction 4 with anhydrous hydrogen fluoride using a modification of the procedure described for the synthesis of tosyl-ArgCH_2Cl (Inouye et al., 1974). Uncorrected amino acid analyses following 24-h hydrolysis revealed that phenylalanine was approximately 10% lower for both compounds; however, the results are consistent with the amino acid analyses of the blocked compounds and are probably due to the inherent resistance of the -Phe-ArgCH_2Cl linkage to acid hydrolysis. Final products are homogeneous in TLC to both ninhydrin and Sakaguchi stains. The success achieved with the HF-deblocking procedure for the arginine chloromethyl ketones prompted the application of this method in deblocking Z-Ala-Phe-Lys(Z)CH_2Cl, since previous methods had yielded heterogeneous products requiring extensive purification. The peptidyl lysine chloromethyl ketone obtained after HF deblocking proved to be homogeneous, eliminating the necessity of further purification.

Both the arginine and lysine chloromethyl ketones were stored either in the dry state desiccated in the cold or as a frozen 10 mM solution in 1.0 mM HCl. No detectable change has been observed in these compounds over an extended period of time under these conditions.

Comparison of Lysine and Arginine Chloromethyl Ketones in the Inactivation of Plasmin and Plasma Kallikrein. The availability of methods for synthesizing peptides of arginine chloromethyl ketone has permitted us to examine initially the effect of changes in the P_1 position (using the notation of Schecter and Berger, 1967) on the effectiveness of reagents corresponding to the sequence of kallikrein physiological substrate, -Pro-Phe-Arg-(-P_3-P_2-P_1-), in the inactivation of human kallikrein and plasmin. Ala-Phe-LysCH_2Cl, a reagent previously shown to be highly effective in the inactivation of plasma kallikrein (Coggins et al., 1974), is similar in its reactivity with both plasmin and plasma kallikrein (Shaw, 1975). Differences in the rates of inactivation of these proteases by 0.50 μM Ala-Phe-LysCH_2Cl are less than twofold.

The affinity labeling reagent Ala-Phe-ArgCH_2Cl, which corresponds to kallikrein's physiological substrate in both the P_2 and P_1 positions, proved to be much more effective in the inactivation of plasma kallikrein than its lysine analogue. Plasma kallikrein is inactivated by 0.10 μM Ala-Phe-ArgCH_2Cl approximately ten times faster than a fivefold higher concentration of Ala-Phe-LysCH_2Cl. In contrast, plasmin was inactivated less effectively by Ala-Phe-ArgCH_2Cl than by its lysine analogue.

The difference in the reactivity of the arginine and lysine chloromethyl ketones was analyzed further by determining the dissociation constant, K_i, of the reversible, substrate-like complex, EI, and the first-order rate constant, k_2, for the irreversible alkylation step of the mechanism for affinity labeling shown in reaction 5.

$$E + I \rightleftarrows EI \xrightarrow{k_2} \text{alkylated protease} \quad (5)$$

TABLE I: Comparison of Kinetic Constants in the Inactivation of Plasma Kallikrein and Plasmin by Arginine and Lysine Chloromethyl Ketones.[a]

affinity label	plasma kallikrein			plasmin		
	K_i (μM)	k_2 (min^{-1})	k_2/K_i (M^{-1} min^{-1} $\times 10^{-3}$)	K_i (μM)	k_2 (min^{-1})	k_2/K_i (M^{-1} min^{-1} $\times 10^{-3}$)
Ala-Phe-ArgCH$_2$Cl[b]	0.078	0.35	4400	1.3	0.18	140
Ala-Phe-LysCH$_2$Cl[c]	4.9	0.35	72	0.83	0.18	210
Pro-Phe-ArgCH$_2$Cl[d]	0.24	0.36	1500	4.2	0.15	37

[a] The reversible dissociation constants of the substrate-like complex, K_i, and the first-order rate constants of the irreversible steps of the reaction, k_2, were determined by the method of Kitz and Wilson (1962) at pH 7.0 and 25 °C. [b] The concentration range of Ala-Phe-ArgCH$_2$Cl over which values of k_{app} were measured in determining the kinetic constants was 0.0083–0.025 μM for plasma kallikrein and 0.17–0.76 μM for plasmin. [c] The concentration range of Ala-Phe-LysCH$_2$Cl was 0.47–1.4 μM for plasma kallikrein and 0.12–0.50 μM for plasmin. [d] The concentration range of Pro-Phe-ArgCH$_2$Cl over which values of k_{app} were measured in determining the kinetic constants was 0.16–0.05 μM for plasma kallikrein and 0.55–2.0 μM for plasmin.

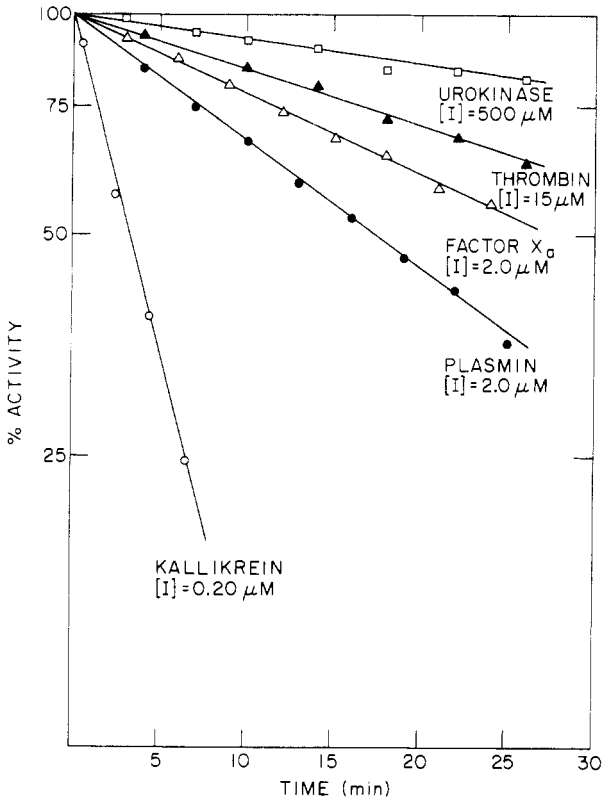

FIGURE 1: Selectivity of Pro-Phe-ArgCH$_2$Cl in the inactivation of plasma kallikrein. The proteases were incubated at 25 °C in 50 mM Pipes buffer (pH 7.0) containing Pro-Phe-ArgCH$_2$Cl at the indicated concentration. Inactivation of the protease was monitored by removing timed aliquots and assaying for esterase activity.

The results in Table I clearly demonstrate the importance of the nature of the basic residue in affinity labels for trypsin-like proteases. Modification of the sequence of Ala-Phe-LysCH$_2$Cl to correspond to the arginine residue of kallikrein's physiological substrate in the P_1 position increased the affinity for plasma kallikrein 60-fold, whereas plasmin exhibited similar binding constants for both the lysine and arginine chloromethyl ketones. Differences in the effectiveness of the chloromethyl ketones, obtained from the second-order constant, k_2/K_i, show that the Ala-Phe-LysCH$_2$Cl is threefold more effective in the inactivation of plasmin than plasma kallikrein, while Ala-Phe-ArgCH$_2$Cl inactivates kallikrein 30 times more effectively than plasmin. Similar differences were obtained between the reactivities of plasma kallikrein and plasmin with Pro-Phe-ArgCH$_2$Cl. The difference in the binding constants of the arginine and lysine chloromethyl ketones is the major factor contributing to the difference observed in the reactivity of these proteases, but plasmin and kallikrein also differ in the rate constant for the alkylation step of their reactions. In fact, the k_2 observed for plasma kallikrein is approximately twice that for plasmin, thus contributing to the difference in the reactivity of kallikrein and plasmin. The consistency of the values of k_2 for each protease with the affinity labels in Table I suggests that the conformations of the reversible complexes of the proteases with both the lysine and arginine chloromethyl ketones are identical and that differences in the values of k_2 for kallikrein and plasmin reflect differences in the conformation of the active sites of these proteases.

The differences in the affinities of plasma kallikrein and plasmin for the lysine and arginine chloromethyl ketones reflect the differences in the specificities of these proteases for their physiological substrate. The enhanced affinity of plasma kallikrein for the arginine chloromethyl ketone is attributable to the homology of this reagent with the C-terminal sequence of bradykinin, a peptide hormone liberated from kininogen by kallikrein. Plasmin, on the other hand, cleaves many peptide bonds during the digestion of fibrin clots, favoring lysyl bonds (Weinstein and Doolittle, 1972). The relatively small difference in the affinity of plasmin for the lysine and arginine chloromethyl ketones reflects its more general specificity.

Selectivity of Pro-Phe-ArgCH$_2$Cl and Ala-Phe-ArgCH$_2$Cl in the Inactivation of Plasma Kallikrein. The foregoing results have demonstrated that the esterase activity of plasma kallikrein can be differentiated from that of plasmin by a reagent corresponding to the amino acid sequence of kallikrein's physiological substrate in the P_1 and P_2 positions. This distinction was attributable to the higher affinity of kallikrein for arginine in the P_1 position. As an additional test of the validity of this approach to designing the affinity labels specifically for their target proteases, the susceptibilities of human thrombin, urokinase, and bovine factor X_a to the reagents specifically designed for kallikrein were determined. These proteases, like kallikrein, are highly specific regulatory enzymes which hydrolyze only one or two bonds of their physiological substrate; since these cleavages are at arginyl bonds, differentiation on the basis of primary specificity is not likely to be a significant factor.

The susceptibility of plasmin, factor X_a, thrombin, and urokinase to inactivation by Pro-Phe-ArgCH$_2$Cl is compared with the susceptibility of plasma kallikrein in Figure 1. Plasma kallikrein is rapidly inactivated by 0.20 μM Pro-Phe-ArgCH$_2$Cl, while plasmin is inactivated at a slower rate by a tenfold higher concentration of this reagent. The regulatory proteases (factor X_a, thrombin, and urokinase) are inactivated by Pro-Phe-ArgCH$_2$Cl at even slower rates by elevated con-

TABLE II: Selectivity of Pro-Phe-ArgCH$_2$Cl and Ala-Phe-ArgCH$_2$Cl in the Inactivation of Trypsin-Like Proteases.

protease	concn of affinity labels (μM)	Pro-Phe-ArgCH$_2$Cl		Ala-Phe-ArgCH$_2$Cl	
		$t_{1/2}$[a] (min)	k_{app}[b]/I (M^{-1} min^{-1} $\times$ 10^{-3})	$t_{1/2}$[a] (min)	k_{app}[b]/I (M^{-1} min^{-1} $\times$ 10^{-3})
plasma kallikrein[c]	0.020	24.0	1500	10.0	4400
plasmin[c]	1.00	23.9	37	9.1	140
factor X_a	1.00	52.0	13	20.3	34
thrombin	25.0	23.5	1.2	16.5	1.7
urokinase	500	91.0	0.015	23.6	0.059

[a] $t_{1/2}$ is the half-time for the pseudo-first-order inactivation of the respective proteases at pH 7.0 and 25 °C; $t_{1/2} = \ln 2/k_{app}$. [b] k_{app}/I is the ratio of the apparent pseudo-first-order rate constant for the inactivation reactions:the concentration of affinity label. k_{app}/I is an estimate of k_2/K_i, the second-order constant for the reaction. [c] Values of k_{app}/I shown are actual values of k_2/K_i from Table I.

centrations for thrombin and urokinase of 15 and 500 μM, respectively.

Rates of inactivation for the proteases shown in Figure 1 are reported in Table II as the time for 50% inactivation by single concentrations of the affinity label. To permit comparison, estimated second-order constants are also reported as the ratio of the apparent, first-order rate constant, k_{app}, to the concentration of the affinity label, I. Plasma kallikrein differs from plasmin and factor X_a in its susceptibility to inactivation by Pro-Phe-ArgCH$_2$Cl by factors of 48 and 107, respectively, while thrombin and urokinase differ by factors of approximately 1200 and 100 000. These differences in the reactivity of Pro-Phe-ArgCH$_2$Cl with the proteases were determined by the rates of loss of esterase activity. As expected, identical rates of inactivation of thrombin were obtained with the fibrinogen clotting assay. Similarly, results show a correlation between loss of esterase activity of plasma kallikrein and its ability to lower rat arterial blood pressure. A similar correlation has been shown between the esterase activity and the plasminogen activating activity of urokinase (Coleman, Kettner, and Shaw, in preparation).

Inactivation data are also reported in Table II for Ala-Phe-ArgCH$_2$Cl in order to evaluate the contribution of the P_3 residue in determining the selectivity of these reagents. Although Pro-Phe-ArgCH$_2$Cl corresponds to the amino acid sequence of kallikrein's physiological substrate in the P_1-P_3 positions, Ala-Phe-ArgCH$_2$Cl was somewhat more reactive. This reagent was also more effective than Pro-Phe-ArgCH$_2$Cl in the inactivation of all other proteases tested, so essentially the same pattern of selectivity was obtained for both Ala-Phe-ArgCH$_2$Cl and Pro-Phe-ArgCH$_2$Cl.

The lower affinity of plasma kallikrein for Pro-Phe-ArgCH$_2$Cl than for Ala-Phe-ArgCH$_2$Cl suggests that proline in the P_3 position is not significant in determining the selectivity of plasma kallikrein for its physiological substrate, but other possibilities exist. The charged imino group of proline may be more disruptive to binding than the amino group of alanine, or perhaps the amino acid residue in the P_4 position is required for alignment of the P_3 proline residue to optimize its binding.

The preceding results have demonstrated that effective as well as selective affinity labels can be obtained for plasma kallikrein by designing chloromethyl ketones which correspond to the amino acid sequence of its physiological substrate. The 60-fold higher affinity of kallikrein for arginine provided the basis for distinguishing the activities of plasma kallikrein and plasmin. On the other hand, the 10^2-10^5-fold difference in the effectiveness of Pro-Phe-ArgCH$_2$Cl in the inactivation of plasma kallikrein and the other regulatory proteases is probably due to differences in the secondary specificity of these proteases.

This approach to obtaining selective affinity labels has been successfully applied to thrombin (Kettner and Shaw, 1977) and is presently being extended to other trypsin-like proteases of physiological importance. These reagents should be helpful in the clarification of the role of these proteases in their complex physiological environment.

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Importance of C^{4a}- and N^5-Covalent Adducts in the Flavin Oxidation of Carbanions†

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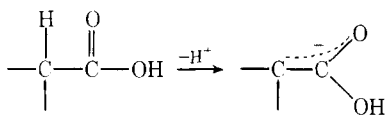
ABSTRACT: The reaction of nitroalkanes, furoin, and phenacyl alcohol with 7- and 8-cyano-3,10-dimethylisoalloxazine (7-$CNFl_{ox}$, 8-$CNFl_{ox}$), 5-ethyl-3-methyllumiflavinium ion ($Fl_{ox}{}^+Et$), and 2,6-dichloroindophenol (DCI) have been studied. Nitromethane anion forms an adduct on reaction with $Fl_{ox}{}^+Et$. The condensation reaction has been shown (nuclear magnetic resonance) to occur through addition of the methylene carbon of the nitromethane anion to the 4a position of $Fl_{ox}{}^+Et$. This adduct undergoes spontaneous dissociation to its components in acid. It does not undergo conversion in base to reduced flavin (1,5-FlEtH), NO_2^-, and CH_2O. For this reason one may conclude that *nitroalkane anion oxidation by flavin does not involve the intermediacy of a 4a-covalent intermediate*. Oxidation of nitroalkane anion through formation of an N^5-flavin adduct is discussed in terms of the peculiar requirements of the substrate when compared with carbanions derived from normal flavoenzyme substrates. It is concluded that the mechanism of nitroalkane oxidation by flavin must bear no relationship to the flavin oxidation of normal substrates. Furoin and phenacyl alcohol (compounds containing the -CH(OH)—CO- functionality) are oxidized by 8-$CNFl_{ox}$, 7-$CNFl_{ox}$, DCI and by the N^5-blocked flavin $Fl_{ox}{}^+Et$. These reactions are found to be zero order in oxidant at the concentrations of oxidants ($\sim 1.0 \times 10^{-5}$ M) and reductant ($\sim 10^{-3}$ to 10^{-4} M) employed. This finding, and that of an $^1H/^2H$ kinetic isotope effect of 3.5, as well as certain equalities of the determined rate constants establish the rate-determining step in all these oxidations to be carbanion formation. That the N^5-blocked flavin ($Fl_{ox}{}^+Et$) serves just as well as an oxidant as do substituted and unsubstituted isoalloxazines provides evidence that the *flavin oxidation of carbanions of general structure (-C^-(OH)—CO-) does not require the formation of an intermediate N^5-flavin adduct*. This conclusion when taken with the fact that the N^5-flavin adduct formed on reaction of dihydroflavin with -C(=O)—CO- is not an intermediate in the reduction of -C(=O)—CO- to -CH(OH)—CO- by dihydroflavin shows that N^5 adducts do not arise as intermediates in these oxidations. Remaining mechanisms (4a addition and radical pair formation) are discussed. 4a addition of carbanions to $Fl_{ox}{}^+Et$ is shown to be subject to considerable steric strain by the instability of the nitroethane adduct and the inability to observe the formation of the 2-nitropropane adduct.

The carbon acid substrates which are oxidized by flavoenzymes are divisible into two groups dependent upon the acidity of the C–H bond which ultimately gives up its bonding electrons to the flavin cofactor. In the first group, the pK_a of the C–H function is so great that oxidation cannot involve the transfer of electrons from a derived carbanion (glucose, NADH, etc.). The second grouping consists of such substrate types as carbonyl compounds, carboxylic acids (α-amino, α-hydroxy, and others) or esters which may undergo α-C–H bond dissociation to provide a resonantly delocalized carbanion as the species which undergoes oxidation. (Presumably the carboxylic acid substrates are bound to the enzyme in the undissociated form allowing resonance stabilization of the α-carbanion (eq 1).)



(1)

The evidence for the formation of intermediate carbanion species in these enzymatic oxidations derives from studies of competitive oxidation and halide elimination and others (succinic acid dehydrogenase, see Tober et al., 1970; D-amino acid oxidase, see Cheung & Walsh, 1976; lactic acid oxidase, see Walsh et al., 1973; Ghisla & Massey, 1977). The nonenzymatic (flavin) oxidation of carbanion forming carbon acids has been a topic of investigation in this laboratory (see Discussion section).

An understanding of the mechanism of such flavoenzymes as the amino acid oxidases and lactic acid oxidase, etc. is obtained only when it is understood just how the electron pair of the appropriate carbanions are transferred to oxidized flavin. One may envision the formation of covalent intermediates [at either the 4a position (I and II) or N^5 position (III and IV)] or caged radical pairs as intermediates in these oxidations.

In the present study we have included the N^5-blocked 5-ethyl-3-methyllumiflavin ($Fl_{ox}{}^+Et$)[1] as oxidant and nitroal-

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[1] Abbreviations used: $Fl_{ox}{}^+Et$, 5-ethyl-3-methyllumiflavinium; DCI, 2,6-dichloroindophenol; 7-$CNFl_{ox}$ and 8-$CNFl_{ox}$, 7- and 8-cyano-3,10-dimethylisoalloxazine, respectively; Mops, morpholinopropanesulfonic acid.

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