Affinity Labelling of Proteinases with Tryptic Specificity by Peptides with C-Terminal Lysine Chloromethyl Ketone

By JOHN R. COGGINS,* WILLIAM KRAY and ELLIOTT SHAW
Biology Department, Brookhaven National Laboratory, Upton, N.Y. 11973, U.S.A.

(Received 20 August 1973)

Methods are described for the synthesis of peptides terminating in Lys-CH₂Cl. The products were examined as affinity labels for several enzymes of trypsin-like specificity which are resistant to Tos-Lys-CH₂Cl. In part, the inertness of the latter may be due to the sulphonamide group, since Z-Lys-CH₂Cl was more effective. However, a number of tripeptides with C-terminal Lys-CH₂Cl were superior in their ability to inactivate subtilisin, thrombin and plasma kallikrein. The possibility of developing enzyme-specific reagents selective for members within the trypsin-like group is demonstrated by Ala-Phe-Lys-CH₂Cl, which readily inactivates plasma kallikrein but not thrombin.

The effectiveness of affinity labelling as a means of locating an active-centre residue of trypsin was demonstrated in studies utilizing the substrate-derived alkylating agent Tos-Lys-CH₂Cl† (TLCK) (Shaw *et al.*, 1965). The rationale on which the design of this reagent was based was the known esterase action of trypsin on N²-tosyl-L-lysine esters and the earlier success of the selective inactivation of chymotrypsin by a substrate-derived chloromethyl ketone (Schoellman & Shaw, 1963). Tos-Lys-CH₂Cl inactivates trypsin by alkylation of N-3 of His-46 of the active centre (Shaw & Springhorn, 1967) after initial formation of a reversible complex (Shaw & Glover, 1970). This feature of affinity labelling, that is, complex-formation between reagent and target enzyme, is considered responsible for the rate enhancement often observed. However, other serine proteinases, which are demonstrably like trypsin in possessing esterase action on lysine derivatives, such as subtilisin (Smith *et al.*, 1966), plasma kallikrein (Colman *et al.*, 1969) and activated Factor X (Radcliffe & Barton, 1972), are not inactivated by Tos-Lys-CH₂Cl. This failure may be due to the tosyl substituent on the α -amino group providing an unfavourable interaction at subsite 2 of the active centre of certain trypsin-like enzymes (terminology of Schechter & Berger, 1967) possibly because of the spatial requirements of the extra oxygen of the sulphonamide group compared with a peptide bond.

In any case the presence of additional subsites in trypsin (Stroud *et al.*, 1971) and presumably also in the related enzymes acting in a more specific way on protein substrates, such as thrombin, plasmin, kallikrein and activated Factor X, suggested that a more effective utilization of the subsites might extend affinity labelling to those enzymes that are not inhibited by Tos-Lys-CH₂Cl. For this reason peptides with C-terminal Lys-CH₂Cl and the simple derivative Z-Lys-CH₂Cl were synthesized.

The acylation of diazomethane by the mixed-anhydride intermediates commonly used in peptide chemistry (Birch *et al.*, 1972) provided a useful starting point for the synthetic work. By this method Boc-Lys (Z) was easily converted into the diazomethyl ketone, which on subsequent treatment with ethanolic HCl gave the chloromethyl ketone with simultaneous deprotection of the α -amino group. The product, Lys(Z)-CH₂Cl hydrochloride, supplied the α -amino component for participation in peptide-bond formation with the mixed anhydride of the next amino acid to be added. A number of tripeptides were prepared with C-terminal Lys-CH₂Cl, the δ -amino group being liberated in the final step with trifluoroacetic acid.

Initial enzymic studies with trypsin, thrombin and subtilisin indicated that more effective affinity labelling was obtained when the tosyl group of Tos-Lys-CH₂Cl was replaced by a benzyloxycarbonyl group or a dipeptide moiety.

Materials and Methods

Materials

Blocked amino acid derivatives were purchased from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A., or from Schwarz-Mann, Orangeburg, N.Y., U.S.A. Ethyl chloroformate was redistilled. All other reagents were of the best commercial grade.

* Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

† The chloromethyl ketones described in this article have been given abbreviations in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [see *Biochem. J.* (1972) 126, 773–780]. Lysine chloromethyl ketone is used as the trivial name for 1-chloro-3,7-diaminoheptan-2-one.

β -Trypsin (Schroeder & Shaw, 1968) and thrombin (Glover & Shaw, 1971) were chromatographically purified by literature methods. Human plasma kallikrein was purified about 1000-fold from Cohn fraction IV₁ (C. A. Sampaio, S. C. Wong & E. Shaw, unpublished work), and its activity, measured in a pH-stat by esterase action on 0.02M-Tos-Arg-OMe, 0.06M in KCl at pH 7.85, is expressed in enzyme units; 1 unit corresponds to the hydrolysis of 1 μ mol of ester/min under these conditions. Subtilisin Novo was obtained from Novo Industries, Copenhagen, Denmark, and dialysed before use (Shaw & Ruscica, 1968).

Methods

Inactivation studies. A stock solution containing 0.01 M-inhibitor in 1 mM-HCl was prepared. Reaction mixtures containing portions of inhibitor solution, enzyme and Tris-HCl buffer, pH 7.0, were maintained at 25°C in a water bath. The final buffer concentration was 0.20M for subtilisin, plasma kallikrein and thrombin, and 0.05M for trypsin and, in the latter case, included 0.02M-Ca²⁺. The enzyme concentrations of trypsin and subtilisin were determined by weight, and that of thrombin was measured by a specific titration procedure (Chase & Shaw, 1969). Details are given in Figs. 1-3. The concentration of plasma kallikrein was 5.5-7.0 enzyme units/ml. This cannot be expressed as a molarity at present. In all cases the enzyme concentrations were sufficiently below the inhibitor concentrations to provide apparent first-order loss of enzymic activity.

Loss of esterase activity was followed by examining samples with 0.167 mM-Z-Lys-ONp in 0.2M-sodium acetate buffer, pH 5.0, for trypsin or in 0.2M-sodium maleate buffer, pH 6.0, for thrombin and kallikrein (Keszdy *et al.*, 1965). The change in ϵ_{340} was measured. At pH 6.0 a double-beam recording spectrophotometer (Beckman DB) was used with substrate in the reference cell to provide compensation for spontaneous hydrolysis of substrate. At pH 5.0, the spontaneous rate was so low that a single-beam instrument (Gilford 2000) was used with observed rates corrected for the low spontaneous rate. Loss of subtilisin esterase activity was measured as described by Shaw & Ruscica (1968).

Inactivation of trypsin was also followed by loss of amidase activity (Shaw & Glover, 1970). When amidase or esterase activity was measured, the reaction was quenched by a decrease in pH until the assay was carried out, as described previously (Shaw & Glover, 1970). This was unnecessary with the other enzymes, since their rates of inactivation were lower.

Synthesis of Z-Lys-CH₂Cl, HCl. A solution of Z-Lys(Boc) (3.0g) in tetrahydrofuran (10ml) was cooled to -10°C and triethylamine (1.25ml) and ethyl chloroformate (0.88ml) were added. The

mixture was left for 9 min at -10°C and then added to an excess of cold ethereal diazomethane (40ml of 0.5M-diazomethane). After 30 min at 0°C the reaction mixture was allowed to warm to 20°C and then extracted in turn with 0.1M-acetic acid (20ml) and saturated aq. NaHCO₃ (20ml). The ethereal layer was dried (MgSO₄), treated with ethanolic 5M-HCl (5ml) at 0°C for 2 min and then immediately extracted in turn with 1M-HCl and saturated aq. NaHCO₃. After drying (MgSO₄) and removal of solvent the protected chloromethyl ketone, Z-Lys(Boc)-CH₂Cl, was obtained as a crystalline residue, m.p. 75-78°C (2.8g; 86% yield). A sample was washed with light petroleum (b.p. 20-40°C) and dried for analysis (Found: C, 58.67; H, 7.04; N, 6.60. Calc. for C₂₀H₂₉ClN₂O₃: C, 58.16; H, 7.08; N, 6.78%). The t-butoxycarbonyl group was removed by treating a solution of Z-Lys(Boc)-CH₂Cl (0.5g) in ethanol (2ml) with ethanolic 5M-HCl (2ml) for 10 min at 20°C. Solvent and excess of HCl were removed by evaporation under reduced pressure (1300Pa) at 20°C and the product was dried under vacuum (130Pa) over NaOH for 2h. The residue was dissolved in water (100ml), filtered to remove some cloudiness and then freeze-dried to yield Z-Lys-CH₂Cl, HCl (0.36g; 85% yield).

Synthesis of Lys(Z)-CH₂Cl, HCl. The preparation of this intermediate was similar to that of Z-Lys-CH₂Cl, HCl described above except that the isomer of the starting material, i.e. Boc-Lys(Z), was used. Crystalline Boc-Lys(Z)-CH₂Cl, m.p. 84-85°C, was obtained in 95% yield (Found: C, 57.98; H, 6.94; N, 6.73%). Removal of the t-butoxycarbonyl group gave a crystalline residue in 92% yield. A sample washed with light petroleum (b.p. 20-40°C) and dried for analysis had m.p. 113-115°C. (Found: C, 51.58; H, 6.35; N, 8.02. Calc. for C₁₅H₂₂Cl₂N₂O₃: C, 51.52; H, 6.41; N, 8.26%).

Synthesis of peptide derivatives of Lys(Z)-CH₂Cl. A standard procedure was adopted, as illustrated by the synthesis of Boc-Ala-Lys(Z)-CH₂Cl. Boc-Ala (0.195g) in tetrahydrofuran (7ml) was converted into the mixed anhydride at -15°C by the addition of triethylamine (0.15ml) and ethyl chloroformate (0.09ml). After 9 min a cold solution of Lys(Z)-CH₂Cl, liberated from its hydrochloride (0.349g; 1mmol) in tetrahydrofuran (10ml) by the addition of triethylamine (0.15ml) just before use, was added. The mixture was left for 20 min at 0°C and then for at least 30 min at 20°C. Protection from moisture was maintained up to this point. Ether (50ml) was added and the organic layer washed with 0.1M-HCl (25ml) followed by saturated aq. NaHCO₃ (25ml) and then dried (MgSO₄). The solvent was removed to give a crystalline residue in 86% yield. A number of protected dipeptide derivatives were obtained by this method (Table 1). The products gave a single spot with t.l.c. on silica gel G plates (Brinkmann;

Westbury, N.Y., U.S.A.) developed with chloroform-acetone (7:3, v/v); spots were detected with I₂ vapour. The antioxidant present in tetrahydrofuran accounted for a second spot and was removed by washing the product with light petroleum (b.p. 20–40°C) or by recrystallization. This was only necessary to obtain analytically pure samples, otherwise the residue was used directly for further syntheses.

For elongation to a tripeptide derivative, of the general type X-Y-Lys-CH₂Cl, a t-butoxycarbonyl derivative such as Boc-Ala-Lys(Z)-CH₂Cl was partially deblocked by treating a solution of 1.1 mmol in ethanol (4ml) with ethanolic 5M-HCl (4ml). After 0.5 h at 20°C the solvent was removed under decreased pressure at 30°C and the residue dried under vacuum (130 Pa) for 2 h to ensure complete removal of HCl. The residue was dissolved in tetrahydrofuran and the dipeptide derivative liberated from its hydrochloride at 0°C as described above for Lys(Z)-CH₂Cl. The neutral fraction obtained after the usual isolation procedure generally crystallized in yields ranging from 75 to 100%. The structures of the products were confirmed by elementary analysis or by quantitative amino acid analysis after acid hydrolysis. In the latter case the fate of Lys-CH₂Cl is not known.

Synthesis of peptide derivatives of Lys-CH₂Cl. In the above procedures the ε-amino group of Lys-CH₂Cl remains blocked by a benzyloxycarbonyl group. To obtain the functional peptide derivatives this and other blocking groups were removed by treating the blocked peptides (0.05 g) with trifluoroacetic acid (0.5 ml) at 50°C for 1 h in a centrifuge tube. The hydrochloride of the product was precipitated by the addition of ethereal 4M-HCl (3 ml) and the product isolated by centrifugation. The product was washed with anhydrous ether (2 × 5 ml); residual solvent was removed with a stream of N₂ and the product dried under vacuum. The yield was quantitative. The products appear to be stable in the solid state, but no systematic study has been made.

Results and Discussion

Synthesis of inhibitors

In the synthesis of Tos-Lys-CH₂Cl the chloromethyl ketone group was built up by the reaction of the acid chloride of Tos-Lys(Z) with diazomethane followed by conversion of the diazomethyl ketone into the chloromethyl ketone and removal of the benzyloxycarbonyl protecting group (Shaw *et al.*, 1965; Shaw & Glover, 1970). This approach is not promising for acyl derivatives of Lys-CH₂Cl, because acid chloride formation would lead to oxazolone formation and prevent the use of acid-labile blocking groups (such as t-butoxycarbonyl), which are useful in peptide synthesis. The conversion of a carboxyl group into a

diazomethyl ketone by activation as the mixed anhydride (Birch *et al.*, 1972) indicated a promising alternative route for lysine chloromethyl ketones.

Z-Lys(Boc) as the mixed anhydride reacted with ethereal diazomethane to provide the diazomethyl ketone readily, since the overall yield to the chloromethyl ketone was 86%. A limited treatment with ethanolic HCl at room temperature selectively deblocked the ε-amino group and provided Z-Lys-CH₂Cl, whose inhibitory properties are discussed below.

Since it had been shown that peptides terminating in Phe-CH₂Cl could be synthesized by acylation of the amino group of phenylalanine with mixed anhydrides (Moriwaka & Oka, 1970; Powers & Wilcox, 1970) attention was turned to the synthesis of Lys(Z)-CH₂Cl as the basis of an analogous series of peptide chloromethyl ketones directed towards the inhibition of trypsin-like enzymes. This was readily achieved from Boc-Lys(Z) by using the mixed anhydride to obtain the diazomethyl ketone in steps analogous to those described above for the isomer. The free α-amino group in Lys(Z)-CH₂Cl was acylated in good yield by mixed anhydrides of benzyloxycarbonyl- or t-butoxycarbonyl-protected amino acids. Selective exposure of a new α-amino group was achieved by limited treatment with ethanolic HCl and a number of blocked tripeptides with C-terminal Lys(Z)-CH₂Cl were obtained.

Although there was no evidence of side reaction at the chloromethyl ketone group during blocked-peptide synthesis, complete deblocking at the dipeptide stage gave some difficulty. Trifluoroacetic acid had been chosen because of its value in removing the ε-benzyloxycarbonyl group in the synthesis of Tos-Lys-CH₂Cl (Shaw & Glover, 1970). However, all six blocked dipeptides examined lost the chloromethyl ketone group when treated with trifluoroacetic acid at 90°C for 30 min, judging by the disappearance of the methylene proton resonances at 4.1 p.p.m. in C²HCl₃ (Shaw & Glover, 1970). This was also the case at 35°C, when deblocking proceeded more slowly and could be followed in the n.m.r. spectrometer (Varian model T60) by the down-field shift of the benzyl methylene protons (5.2 p.p.m.) accompanying the formation of benzyl trifluoroacetate (5.5 p.p.m.). The chemistry of the side reaction is not known. The chloromethyl ketone group is stable to hot trifluoroacetic acid, as shown by its successful use in the synthesis of Tos-Lys-CH₂Cl and in deblocking tripeptides terminating in Lys(Z)-CH₂Cl (cf. below); nor is the difficulty due to the liberation of the α-amino group at the dipeptide stage, because in derivatives of the general type Boc-X-Lys(Z)-CH₂Cl removal of Boc- caused no difficulty. For the synthesis of unblocked dipeptide derivatives hydrolysis appeared to be satisfactory, since a solution of Z-Ala-Lys(Z)-CH₂Cl in aqueous-ethanolic (1:1,

v/v) 1 M-HCl, when stirred for 0.5 h at 20°C with 10% Pd on carbon in a stream of H₂, gave the desired product.

The generality of this as a deblocking procedure was not explored, since synthesis was directed towards tripeptides. For this purpose, a partially blocked dipeptide such as Ala-Lys(Z)-CH₂Cl was extended by the mixed-anhydride procedure. Blocked tripeptides were readily obtained (Table 1). At this stage the use of trifluoroacetic acid at 50°C provided complete deblocking without loss of chloromethyl ketone function (based on n.m.r. evidence) or alteration of amino acid composition.

Inhibition studies

The biological activities of the newly synthesized derivatives of Lys-CH₂Cl confirmed that replacement of the sulphonamide group of Tos-Lys-CH₂Cl by an amide or peptide derivative leads to more effective active-site-directed inhibitors for trypsin and some related enzymes. Similar results have been obtained with Phe-CH₂Cl derivatives in their action on chymotrypsin (Shaw & Ruscica, 1971; Kurachi *et al.*, 1973) and on subtilisin (Shaw & Ruscica, 1968; Morihara & Oka, 1970).

Z-Lys-CH₂Cl rapidly inactivated trypsin. A 50 μM solution produced loss of tryptic activity with a half-time of 0.87 min compared with 23 min for the same concentration of Tos-Lys-CH₂Cl (Shaw & Glover, 1970). Although this rapidity of action made it difficult to examine the effect of inhibitor concen-

tration on the rate of inactivation by sampling methods, it was possible, with great care, to demonstrate saturation kinetics by the double-reciprocal plot method (Fig. 1) (Kitz & Wilson, 1962). The graphically determined *K_i* of 1.3 × 10⁻⁴ M indicates a somewhat tighter binding than with Tos-Lys-CH₂Cl, for which a *K_i* of 2.1 × 10⁻⁴ M was measured (Shaw & Glover, 1970); the respective values for *k₊₂* were 3.1 and 0.16 min⁻¹. These constants refer to the system:

Fig. 1. Saturation kinetics for the inactivation of trypsin by Z-Lys-CH₂Cl

The plot is for data obtained from the amidase assay at 25°C in 0.05 M-Tris-HCl buffer, pH 7.0, with [E] = 30 μM.

Table 1. Synthetic derivatives of Lys-CH₂Cl

Compound	Yield (%)	m.p. (°C)	Formula (mol.wt.)	Analysis (%)		
				C	H	N
Boc-Ala-Lys(Z)-CH ₂ Cl	86		C ₂₃ H ₃₄ ClN ₃ O ₆ (484.0)	57.20	7.04	8.67
			Found	56.73	7.08	8.67
Z-Val-Lys(Z)-CH ₂ Cl	88	122–123	C ₂₈ H ₃₆ ClN ₃ O ₆ (546.0)	61.59	6.65	7.70
			Found	61.77	6.83	7.64
Boc-Phe-Lys(Z)-CH ₂ Cl	83	142–143	C ₂₉ H ₃₈ ClN ₃ O ₆ (560.0)	62.19	6.84	7.50
			Found	62.13	6.78	7.45
Boc-Trp-Lys(Z)-CH ₂ Cl	82	156–157	C ₃₁ H ₃₉ ClN ₄ O ₆ (590.0)	63.11	6.66	9.50
			Found	62.89	6.57	9.23
Boc-Glu(Z)-Lys(Z)-CH ₂ Cl	85	114–115	C ₃₂ H ₄₂ ClN ₃ O ₈ (632.1)	60.80	6.70	6.65
			Found	60.71	6.77	6.57
Boc-Phe-Ala-Lys(Z)-CH ₂ Cl			C ₃₂ H ₄₃ ClN ₄ O ₇ (631.2)	60.40	6.60	8.90
			Found	60.36	6.95	8.61
Boc-Ala-Phe-Lys(Z)-CH ₂ Cl			C ₃₂ H ₄₃ ClN ₄ O ₇ (631.2)	60.40	6.60	8.90
			Found	60.67	6.97	8.87
Boc-β-Ala-Ala-Lys(Z)-CH ₂ Cl			Ala 1.0	β-Ala 0.92		
Boc-Glu(Z)-Ala-Lys(Z)-CH ₂ Cl			Ala 1.0	Glu 0.89		
Boc-Lys(Z)-Ala-Lys(Z)-CH ₂ Cl			Ala 1.0	Lys 1.0		
Boc-Pro-Ala-Lys(Z)-CH ₂ Cl			Ala 1.0	Pro 0.92		
Boc-Leu-Ala-Lys(Z)-CH ₂ Cl			Ala 1.0	Leu 1.1		

The peptide derivatives of Lys-CH₂Cl were also better irreversible inhibitors of trypsin than Tos-Lys-CH₂Cl. For example, 5 μ M-Lys-Ala-Lys-CH₂Cl inactivated trypsin by 50% in 2.1 min compared with the 6 min required for 1 mM-Tos-Lys-CH₂Cl (Shaw & Glover, 1970). This was due mainly to the greater affinity of the enzyme for the inhibitor; the K_i for Lys-Ala-Lys-CH₂Cl was 6.5×10^{-6} M. This substance, in an alternative mode of binding, could be a substrate for trypsin with liberation of the *N*-terminal lysine. However, a charged α -amino group considerably diminishes the binding of lysine derivatives to trypsin (Seely & Benoiston, 1970) and at the high dilutions of inhibitor studied such binding would probably be negligible.

A comparison of the effectiveness of active-centre-directed inhibitors can be based on their ability to inactivate the enzyme at an inhibitor concentration in the range where first-order loss of enzyme activity occurs. However, the relative effectiveness of inhibitors judged in this way may be concentration-dependent (Shaw & Glover, 1970). The action of inhibitors of relatively low affinity may be described

in terms of a second-order rate constant $k_{obs.}/[I]$; this was useful for chymotrypsin (Shaw & Ruscica, 1971; Kurachi *et al.*, 1973), but for the inhibitors of trypsin derived from Lys-CH₂Cl $k_{obs.}/[I]$ was not constant in the range of inhibitor concentrations suitable for study. This led us to compare the relative effectiveness of these inhibitors by means of the parameter k_{+2}/K_i as determined from plots of $1/k_{obs.}$ versus $1/[I]$. Thompson & Blout (1973) used this method in studies on elastase. It has the significant advantage of taking account of both the efficiency of binding and the rate of irreversible modification. The values of k_{+2}/K_i for Tos-Lys-CH₂Cl, Z-Lys-CH₂Cl and Lys-Ala-Lys-CH₂Cl are 12.8, 410 and $2000 \text{ s}^{-1} \cdot \text{M}^{-1}$ respectively. On this basis their relative effectiveness is in the ratio 1:32:156.

A study of the three-dimensional structure of subtilisin has led to the suggestion that the ineffectiveness of Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl as inhibitors is due to the spatial requirements of the sulphonamide group (Robertus *et al.*, 1972). This cannot be the only reason, since Z-Lys-CH₂Cl is also ineffective (Fig. 2). Incorporation of Lys-CH₂Cl into tripeptides leads to much better inhibitors (Fig. 2). It is known that Bz-Arg binds to subtilisin in a different way from the phenylalanine derivatives. The peptides used in this study should be of value for further crystallographic studies on the binding of basic peptides to subtilisin.

One of the chief goals of the present work was to extend affinity labelling to some of the trypsin-like mammalian serine proteinases. Several of these are homologous with trypsin, for example, plasmin (Robbins *et al.*, 1973), thrombin (Magnusson, 1971) and Factor X (Titani *et al.*, 1972), whereas others are demonstrably trypsin-like in specificity, but structural studies have not yet proceeded to a point that justifies the conclusion of genetic relatedness. This latter group includes the active C' component of complement (Bing, 1970), the kallikrein group (Schachter, 1969) and the sperm proteinase, acrosin (Zaneveld *et al.*, 1972). Because of the crucial physiological role played by these trypsin-like enzymes the development of selective inhibitors is of great interest. To obtain enzyme-specific inhibitors it seemed reasonable to exploit as many of the known structural elements of the physiological substrate as possible. A completely rational approach would also require knowledge of the three-dimensional structure of each enzyme, but such information is not yet available.

It is known that proteolysis of fibrinogen (mol.wt. 370000) by thrombin is largely limited to the *N*-terminal regions of the fibrinogen chains with cleavage of an Arg-Gly bond in each chain, although more than 100 other trypsin-susceptible bonds are available. Since the same specificity is observed after sulphit-olysis or after reduction and carboxymethylation of fibrinogen (Magnusson, 1971), the intact tertiary

Fig. 2. Inhibition of subtilisin by peptide derivatives of Lys-CH₂Cl

The plot shows percentage residual esterase activity at 25°C (measured in 0.2M-Tris-HCl buffer, pH7.0, with $[E]=11 \mu\text{M}$) versus time. ●, Z-Lys-CH₂Cl, 5mM; ○, Ala-Phe-Lys-CH₂Cl, 5mM; ▲, Phe-Ala-Lys-CH₂Cl, 0.25mM; △, Phe-Ala-Lys-CH₂Cl, 0.5mM; ■, Phe-Ala-Lys-CH₂Cl, 2mM.

structure of fibrinogen itself is not required, although that of the *N*-terminal region may be (Blomback *et al.*, 1968). Even in the absence of demonstrable peptidase action at lysine residues, thrombin was slowly and specifically alkylated at an active-centre histidine by Tos-Lys-CH₂Cl (Glover & Shaw, 1971), an observation in accord with its known esterase action (Magnusson, 1971). Attempts to make inhibitors with structures more closely related to the proteolytic specificity of thrombin, for example by using Arg-CH₂Cl, have encountered difficulties in synthesis (Shaw & Glover, 1970), and so alterations of the Tos-Lys-CH₂Cl structure offer the best means at present for exploring the effect of subsite utilization on the affinity labelling of thrombin. The action of 1 mM-Tos-Lys-CH₂Cl is slow (Fig. 3); at 0.1 mM, inactivation was not detectable during a 2 h incubation. Replacement of the sulphonamide group by an amide as in Z-Lys-CH₂Cl resulted in a more effective irreversible inhibitor (Fig. 3). At least two peptide derivatives, Glu-Ala-Lys-CH₂Cl and Phe-Ala-Lys-CH₂Cl, were even more active (Fig. 3). For the purposes of comparison, k_{+2}/K_i for the inactivation of

thrombin was determined for these and other derivatives with the results: inhibitor (k_{+2}/K_i , s⁻¹·M⁻¹), β-Ala-Ala-Lys-CH₂Cl (1.2), Pro-Ala-Lys-CH₂Cl (2.0), Lys-Ala-Lys-CH₂Cl (4.7), Z-Lys-CH₂Cl (7.4), Glu-Ala-Lys-CH₂Cl (12) and Phe-Ala-Lys-CH₂Cl (15.8). These values represent an estimated increase in effectiveness of two orders of magnitude over the action of Tos-Lys-CH₂Cl. In the peptide derivatives, alanine in the P₂ position (terminology of Schechter & Berger, 1967) corresponds to the sequence of bovine fibrinopeptide B (Blomback *et al.*, 1965) whereas fibrinopeptide A has valine in this position. Further work will be required to determine the effect of valine at P₂ and whether there is any advantage in elongating the peptide chain.

The results observed with human plasma kallikrein were similar. The inertness of Tos-Lys-CH₂Cl had been noted before (Colman *et al.*, 1969), but Z-Lys-CH₂Cl was an inhibitor (C. A. Sampaio, S. C. Wong, & E. Shaw, unpublished results). When a number of peptide derivatives of Lys-CH₂Cl were examined for inhibition of kallikrein the following results were obtained: inhibitor (k_{+2}/K_i , s⁻¹·M⁻¹), β-Ala-Ala-Lys-CH₂Cl (2.2), Pro-Ala-Lys-CH₂Cl (3.1), Lys-Ala-Lys-CH₂Cl (7.8) and Glu-Ala-Lys-CH₂Cl (14). Thus their effectiveness in inhibiting kallikrein and thrombin was similar. Since this group of peptide derivatives did not give any indication of selectivity between thrombin and kallikrein an attempt was made to exploit the fact that the physiological action of kallikrein in kinin liberation involves cleavage at a Phe-Arg sequence (Schachter, 1969). Ala-Phe-Lys-CH₂Cl was synthesized and the results obtained were very encouraging, since the susceptibility of kallikrein was greatly increased. The k_{+2}/K_i was 445 s⁻¹·M⁻¹. On the other hand the sensitivity of thrombin was diminished, 1 mM-Ala-Phe-Lys-CH₂Cl requiring 50.3 min for 50% inhibition. So Ala-Phe-Lys-CH₂Cl showed a high degree of selectivity as an inhibitor of kallikrein. This selectivity may be due to the phenylalanine side chain providing increased affinity for subsite 2 in kallikrein but being too bulky for the corresponding region of the active centre of thrombin. Further studies with peptide derivatives of Lys-CH₂Cl and in particular measurements of k_{+2} and K_i values for thrombin and kallikrein will establish whether or not the route to selectivity lies solely in exploiting differences in K_i as the present work suggests. An additional and more interesting possibility is that certain substrate side chains have a mechanistic importance that will be reflected in enhancement of k_{+2} , the rate of alkylation of the enzyme.

We are grateful for the assistance of Mr. George Latham Jr. and Mr. John Ruscica. This work was supported by the U.S. Atomic Energy Commission and Public Health Service grant 17849 from the National Institute of General Medical Sciences.

Fig. 3. Inhibition of thrombin by peptide derivatives of Lys-CH₂Cl

The plot shows percentage residual esterase activity at 25°C (measured in 0.2M-Tris-HCl buffer, pH 7.0, with [E] = 4 μM) versus time. ●, Tos-Lys-CH₂Cl, 1 mM; ○, Z-Lys-CH₂Cl, 50 μM; ■, Glu-Ala-Lys-CH₂Cl, 50 μM; □, Phe-Ala-Lys-CH₂Cl, 50 μM; ▲, Phe-Ala-Lys-CH₂Cl, 0.1 mM.

References

- Bing, D. H. (1970) *J. Immunol.* **105**, 1289-1291
- Birch, P. L., El-Obeid, H. A. & Akhtar, M. (1972) *Arch. Biochem. Biophys.* **148**, 447-451
- Blomback, B., Blomback, M. & Grondahl, N. J. (1965) *Acta Chem. Scand.* **19**, 1789-1791
- Blomback, B., Blomback, M., Henschen, A., Hessel, B., Iwanaga, S. & Woods, K. R. (1968) *Nature (London)* **218**, 130-134
- Chase, T. & Shaw, E. (1969) *Biochemistry* **8**, 2212-2224
- Colman, R. W., Mattler, L. & Sherry, S. (1969) *J. Clin. Invest.* **48**, 11-22
- Glover, G. & Shaw, E. (1971) *J. Biol. Chem.* **246**, 4594-4601
- Kezdy, F. J., Lorand, L. & Miller, K. D. (1965) *Biochemistry* **4**, 2302-2308
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245-3249
- Kurachi, K., Powers, J. C. & Wilcox, P. E. (1973) *Biochemistry* **12**, 771-777
- Magnusson, S. (1971) *Enzymes*, 3rd edn., **3**, 278-321
- Morihara, K. & Oka, T. (1970) *Arch. Biochem. Biophys.* **138**, 526-531
- Powers, J. C. & Wilcox, P. E. (1970) *J. Amer. Chem. Soc.* **92**, 1782-1783
- Radcliffe, R. D. & Barton, P. G. (1972) *J. Biol. Chem.* **247**, 7735-7742
- Robbins, K. C., Bernabe, P., Arzadon, L. & Summari, L. (1973) *J. Biol. Chem.* **248**, 1631-1633
- Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C. & Wilcox, P. E. (1972) *Biochemistry* **11**, 2439-2449
- Schachter, M. (1969) *Physiol. Rev.* **49**, 509-547
- Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157-162
- Schoellman, G. & Shaw, E. (1963) *Biochemistry* **2**, 252-255
- Schroeder, D. D. & Shaw, E. (1968) *J. Biol. Chem.* **243**, 2943-2949
- Seely, J. H. & Benoiton, N. L. (1970) *Can. J. Biochem.* **48**, 1122-1131
- Shaw, E. & Glover, G. (1970) *Arch. Biochem. Biophys.* **139**, 298-305
- Shaw, E. & Ruscica, J. (1968) *J. Biol. Chem.* **243**, 6312-6313
- Shaw, E. & Ruscica, J. (1971) *Arch. Biochem. Biophys.* **145**, 484-489
- Shaw, E. & Springhorn, S. (1967) *Biochem. Biophys. Res. Commun.* **27**, 391-397
- Shaw, E., Mares-Guia, M. & Cohen, W. (1965) *Biochemistry* **4**, 2219-2224
- Smith, E. L., Markland, F. S., Kasper, C. B., De Lange, R. J., Landon, M. & Evans, W. H. (1966) *J. Biol. Chem.* **241**, 5974-5976
- Stroud, R. M., Kay, L. M. & Dickerson, R. E. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 125-140
- Thompson, R. C. & Blout, E. R. (1973) *Biochemistry* **12**, 44-47
- Titani, K., Hermanson, M. A., Fujikawa, K., Ericsson, L. H., Walsh, K. A., Neurath, H. & Davie, E. W. (1972) *Biochemistry* **11**, 4899-4903
- Wright, C. S., Alden, R. A. & Kraut, J. (1972) *J. Mol. Biol.* **66**, 283-289
- Zaneveld, L. J. D., Polakoski, K. & Williams, W. (1972) *Biol. Reprod.* **6**, 30-39