strated that Y^{3+}, lanthanides, as well as many other divalent cations, fail to activate the apoenzyme.

References

Birnbaum, E. R., Gomez, J. E., and Darnall, D. W. (1970), *J. Amer. Chem. Soc. 92*, 5287.

Colman, P. M., Weaver, L. H., and Matthews, B. W. (1972), *Biochem. Biophys. Res. Commun. 46*, 1999.

Colowick, S. P., and Womack, F. C. (1969), *J. Biol. Chem. 244*, 774.

Darnall, D. W., and Birnbaum, E. R. (1970), *J. Biol. Chem. 245*, 6484.

Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4, Part 1*, 313.

Loyter, A., and Schramm, M. (1962), *Biochim. Biophys. Acta 65*, 200.

Reuben, J. (1971), *Biochemistry 10*, 2834.

Smolka, G. E., Birnbaum, E. R., and Darnall, D. W. (1971), *Biochemistry 10*, 4556.

Stein, E. A., Hsiu, J., and Fischer, E. H. (1964), *Biochemistry 3*, 56.

Whistler, R. L., and Durso, D. F. (1950), *J. Amer. Chem. Soc. 72*, 677.

Williams, R. J. P. (1970), *Quart. Rev., Chem. Soc. 24*, 331.

Wrigley, C. (1968), *LKB Instrum. J. 15*, 2.

Peptide Chloromethyl Ketones as Irreversible Inhibitors of Elastase†

Robert C. Thompson‡ and Elkan R. Blout*

ABSTRACT: Three peptide chloromethyl ketones have been prepared which irreversibly inhibit elastase. Amino acid analyses of the inactivated enzyme show that inhibition results from the alkylation of a histidine residue of the enzyme. The three peptide chloromethyl ketones differ markedly in their ability to inactivate the enzyme with the longer peptides reacting more rapidly. A parallel dependence of the ease of substrate hydrolysis on peptide chain length has been observed previously with amide substrates of elastase.

Elastase (EC 3.4.4.7) is a serine proteinase secreted by the pancreas in the form of a zymogen. Its catalytic mechanism, amino acid sequence, and tertiary structure show that it is a member of the same family of enzymes as chymotrypsin and trypsin. Nonetheless, certain properties distinguish elastase from the other enzymes of this family. One of the more puzzling features has been the failure, despite considerable effort (Kaplan *et al.*, 1970; Visser *et al.*, 1971, and unpublished results), to develop reagents which alkylate the histidine residue known to be at the active site of the enzyme (Shotton and Watson, 1970). Such alkylating reagents are available for both chymotrypsin and trypsin and have played an important role in implicating the active-site histidine residue in their catalytic mechanisms (Shaw, 1970).

Recently, it has become apparent that the catalytic efficiency of elastase is considerably enhanced by interaction with amino acid residues of the substrate N terminal to the scissile bond (Atlas *et al.*, 1970; Thompson and Blout, 1970, 1973b,c). The major increase in catalytic efficiency arises from the increased ability of the enzyme to transfer an acyl group of the substrate to the hydroxy group of serine-188. It appeared possible that the factors contributing to this increased ability to acylate serine-188 would also facilitate the alkylation of histidine-45 by the proper reagent. Other investigators have previously reported that peptide chloromethyl ketones can react more rapidly than tosylamino acid chloromethyl ketones with other, related, enzymes (Morihara and Oka, 1970; Powers and Wilcox, 1970). Consequently, we have prepared three peptide chloromethyl ketones and report here that these reagents inactivate elastase rapidly and irreversibly. Evidence is presented that the inactivation is the result of alkylation of an histidine residue.

Materials and Methods

Porcine pancreatic elastase (>99.8% pure) was purchased from Whatman Biochemicals, England.

Carbobenzoxyalanine and acetylproline were purchased from the Fox Chemical Co., Los Angeles.

N-Carbobenzoxyalanine chloromethyl ketone was prepared according to the method used by Shaw (1967) to prepare the phenylalanine analog: yield based on Z-Ala-OH, 38%; mp 87–88°, $[\alpha]_D^{25}$ −43.6° (*c* 2.8, MeOH). *Anal.* Calcd for $C_{12}H_{14}ClNO_3$: C, 56.4; H, 5.49; Cl, 13.9; N, 5.5. Found: C, 56.4; H, 5.5; Cl, 13.3; N, 5.6.

Alanine Chloromethyl Ketone Hydrobromide. Z-Ala-CMK[1] (1.0 g; 3.9 mmol) was dissolved in a saturated solution of hydrogen bromide in glacial acetic acid (2 ml). After 20 min, anhydrous ether (200 ml) was added and the mixture was shaken vigorously. The ether was decanted when clear, and

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received August 7, 1972.* This work was supported, in part, by U. S. Public Health Service Grants AM-07300 and AM-10794.

‡ Present address: Bacterial Physiology Unit, Harvard Medical School.

[1] The abbreviation Ala-CMK is used to denote the chloromethyl ketone derived from alanine, *viz.*, 1-chloro-3-aminobutan-2-one.

the residue was washed with a further 200 ml of anhydrous ether. Crystallization from acetone–ethyl acetate gave white crystals 453 mg (57%), mp 90–93° dec. *Anal.* Calcd for $C_4H_6BrClNO$: C, 23.73; H, 4.48; Br, 39.5; Cl, 17.5; N, 6.92. Found: C, 24.1; H, 4.6; Br, 38.3; Cl, 16.8; N, 6.8.

Acetylprolylalanine Chloromethyl Ketone. Acetylproline (78 mg; 0.5 mmol) was dissolved in acetonitrile (10 ml) and stirred at −20°, while 0.055 ml of *N*-methylmorpholine (0.5 mmol) and 0.065 ml of isobutyl chloroformate (0.5 mmol) was added. After a further 10 min, 0.055 ml of *N*-methylmorpholine (0.5 mmol) was added, followed by a solution of 101 mg of alanine chloromethyl ketone hydrobromide (0.5 mmol). The mixture was allowed to warm to room temperature over a period of 1 hr and stirred a further 4 hr. The solvent was evaporated *in vacuo*, and the residue was applied to a 20 × 2 cm column of silica gel and eluted with chloroform–methanol (9:1). A white solid, (15 mg; 12%) mp 110–112°, was recovered after crystallization from ethyl acetate–ether: single spot by thin-layer chromatography R_F($CHCl_3$–MeOH,9:1) 0.8. *Anal.* Calcd for $C_{11}H_{17}ClN_2O_3$: C, 50.66; H, 6.57; N, 10.75. Found: C, 51.2; H, 6.6; N, 10.8.

Acetylalanylprolylalanine chloromethyl ketone was prepared similarly to Ac-Pro-Ala-CMK from Ac-Ala-Pro-OH (Thompson and Blout, 1973b) and HBr·H-Ala-CMK: yield, 78 mg (47%); mp 178–180°. *Anal.* Calcd for $C_{14}H_{22}ClN_3O_4$: C, 50.67; H, 6.68; N, 12.66. Found: C, 50.4; H, 6.6; N, 12.3.

Acetylprolylalanylprolylalanine chloromethyl ketone was prepared similarly to Ac-Pro-Ala-CMK from Ac-Pro-Ala-Pro-OH (Thompson and Blout, 1973b) and HBr·H-Ala-CMK: yield, 72 mg (42%); mp 150–153°. *Anal.* Calcd for $C_{19}H_{29}ClN_4O_5$: C, 53.19; H, 6.81; N, 13.06. Found: C, 53.0; H, 6.7; N, 12.8.

Irreversible inhibition of elastase by the chloromethyl ketones was carried out by incubating 200 μl of a 10^{-5} M solution of the enzyme with at least a 25-fold excess of inhibitor at 25° in a 50 mM phosphate buffer (pH 6.5). Aliquots (25 μl) were removed after 15, 30, 45, 60, 90, and 120 sec and diluted into 1 ml of ice-cold buffer. The concentration of active elastase was then determined by the *p*-nitrophenyl-*tert*-butyloxycarbonyl alaninate assay of Visser and Blout (1972). With Ac-Pro-Ala-Pro-Ala-CMK, a 19-fold excess of inhibitor was used and inhibition was about 80% complete in 60 sec.

Elastase, fully inhibited by Ac-Pro-Ala-Pro-Ala-CMK, was prepared by incubating a solution of 30 mg of elastase in 3 ml of pH 6.5 50 mM phosphate buffer with 2.6 mg of Ac-Pro-Ala-Pro-Ala-CMK at 0°. After 15 min, less than 0.1% active enzyme remained. After 1 hr at 25°, less than 0.02% active enzyme remained. The solution was dialyzed against three changes of 1000 ml of distilled demineralized water over a period of 24 hr at 4°. After centrifugation, the supernatant was lyophilized to give 16.5 mg of the modified protein.

Performic acid oxidation was by the method of Hirs (1967). Alkylated or native elastase (9 mg) was dissolved in a mixture of 0.5 ml of methanol and 2.5 ml of 97–99% formic acid. The protein solution at −5° was mixed with 5 ml of a mixture of 0.5 ml of 30% hydrogen peroxide and 9.5 ml of 97–99% formic acid which had stood previously for 2 hr at room temperature. After 8 hr at −5° the mixture was poured into 200 ml of ice–water and lyophilized. The lyophilized solid was suspended in 200 ml of ice–water and again lyophilized.

Approximately 1 mg of the lyophilized protein was dissolved in 1 ml of 6 M hydrochloric acid and hydrolyzed by heating to 110° for 24 hr in a sealed tube. Amino acid analyses were performed on a Beckman 120B analyzer.

TABLE I: Amino Acid Analyses of Performic Acid Oxidized, Alkylated, and Native Elastase.

	Oxidized Alkylated Elastase[a]	Oxidized Native Elastase[a]	Theoretical[b]
Cysteic acid	7.6	7.8	8
Asx	28.3	27.8	24
Thr	19.0	19.0	19
Ser	21.4	21.8	22
Glx	20.1	20.2	19
Pro	9.1	7.5	7
Gly	26.9	27.1	25
Ala	18.1	17.4	17
Val	21.8	21.4	27
τ-CM-His	0.5	0	0
Ile[c]	7.6	7.6	10
Leu[c]	18.2	17.9	18
Tyr[c]	9.4	9.6	11
Phe[c]	3.1	3.0	3
Lys[d]	3.2	3.1	3
His[d]	5.1	5.8	6
Arg[d]	12.0	12.0	12

[a] Average of four runs, based on Thr = 19.0, except where stated otherwise. [b] Based on amino acid sequence reported by Shotton and Hartley (1970). [c] Average of three runs, based on Thr = 19.0. [d] Average of two runs, based on Arg = 12.0.

Results

Elastase is irreversibly inhibited by all three peptide chloromethyl ketones described here. The nature of the inhibition reaction has been provisionally identified from amino acid analyses of the Ac-Pro-Ala-Pro-Ala-CMK-inhibited enzyme. Amino acid analyses of the performic acid oxidized, modified and native proteins are shown in Table I.

For the performic acid oxidized enzyme only the glycine and aspartic acid contents, which are high, and valine, isoleucine, and tyrosine contents which are low, differ significantly from the predicted values. The high content of aspartic acid probably arises as a result of the poor separation of this amino acid from the two residues of methionine sulfone known to be present. The low content of valine and isoleucine probably result from the short time allowed for protein hydrolysis; peptide bonds containing these amino acids are known to be refractory to acid-catalyzed hydrolysis.

The amino acid analysis of the performic acid oxidized, modified enzyme is very similar to that of the oxidized native enzyme except for its proline and histidine content. The modified enzyme appears to contain 1.6 ± 0.4 more proline residues and 0.7 ± 0.4 less histidine residue than the native protein. This is consistent with the covalent attachment of one molecule of inhibitor to an histidine residue of the enzyme, concomitant with the inhibition. The anticipated increase in the alanine content of the modified protein which should accompany this process is observed (+0.7 ± 1.0), but, as might be predicted, is within the anticipated error of analysis.

The most plausible reaction between a chloromethyl ketone and an N^a-acylhistidine, based both on model systems (Shaw and Ruscica, 1971) and on reactions observed in other en-

TABLE II: Kinetic Constants for Inhibition of Elastase by Peptide Chloromethyl Ketones.

Inhibitor	k_2/K_i (M^{-1} sec^{-1})	k (sec^{-1})	K (mM)
Ac -Pro-Ala-CMK[a]	0.05	0.002	40
Ac-Ala-Pro-Ala-CMK[a]	35	0.03	0.85
Ac-Pro-Ala-Pro-Ala-CMK[b]	260		

[a] Determined according to Kitz and Wilson (1962). [b] Determined from the rate of inhibition at [I] = 0.19 mM.

zymes (Shaw, 1970), is N-alkylation of the amino acid side chain. The product of such a reaction has been reported (Stevenson and Smillie, 1965; Petra *et al.*, 1965) to give rise to *N*-carboxymethylhistidine upon performic acid oxidation. Using a mixture of the isomeric *N*-carboxymethylhistidines prepared according to Crestfield *et al.* (1963), we were able to identify a trace amount of N^{τ}-carboxymethylhistidine (N^{3}-carboxymethylhistidine of Crestfield *et al.* (1963)) in the amino acid hydrolysate of the modified, performic acid oxidized protein. On the long column of the amino acid analyzer, the trace product chromatographed in the same position as synthetic N^{τ}-carboxymethylhistidine when eluting buffers of pH 3.10, 3.05, and 3.02 were used. In all three cases the trace amino acid appeared after valine, and would have been clearly separated from both half-cystine and methionine were these amino acids present.[2] Using the color value derived for glycine under the same conditions, the N^{τ}-carboxymethylhistidine content of the performic acid oxidized, modified enzyme would be about 0.5 ± 0.1 mol/mol. If performic acid oxidation of the ketone occurred with equal facility at either of the α-carbon atoms, the theoretical yield of N-$^{\tau}$carboxymethylhistidine could not exceed 50% and the modified enzyme's content of alkylated histidine would approach 1 mol/mol of enzyme. Taken together, the above results constitute evidence for the view that inactivation of the enzyme proceeds through the alkylation of a single histidine residue by the peptide chloromethyl ketone inhibitor.

For Ac-Pro-Ala-CMK and Ac-Ala-Pro-Ala-CMK it has been possible to study the relationship between the rate of enzyme inhibition and the inhibitor concentration. The inhibition is not simply first order with respect to inhibitor, but is subject to a saturation effect. This is consistent with the reversible formation of a noncovalent enzyme–inhibitor complex prior to the irreversible step of reaction, and supports the reaction scheme I, where EI and EI_{cov} denote the noncovalent

$$E + I \underset{K_i}{\rightleftharpoons} EI \xrightarrow{k_2} EI_{cov} \qquad (I)$$

and covalent complexes, respectively.

Of the various kinetic parameters characterizing this reaction, the quotient, k_2/K_i, is most easily determined and is a valid basis for comparing the efficiency of inhibition by the various chloromethyl ketones. For Ac-Pro-Ala-CMK and Ac-Ala-Pro-Ala-CMK this parameter could be determined from the slope of a plot of the reciprocal rate of inhibition *vs.* 1 inhibitor concentration (Kitz and Wilson, 1962). In the case of Ac-Pro-Ala-Pro-Ala-CMK, the inhibition was too rapid to follow at inhibitor concentrations higher than 0.2 mM. An approximate value of k_2/K_i was, therefore, obtained from the pseudobimolecular rate constant k_2' calculated for reaction II.

$$E + I \xrightarrow{k_2'} EI_{cov} \qquad (II)$$

From the equation $v = k_2'[E][I]$, k_2' will be a good approximation of k_2/K_i when $[I] \ll K_i$. It is our belief that this condition is satisfied for Ac-Pro-Ala-Pro-Ala-CMK at $I = 0.19$ mM, since the value of K_i for this compound can be estimated from the K_i of the analogous methyl ketone (1.5 mM, R. C. Thompson, to be published) to be about 1 mM.

The values of k_2/K_i presented in Table II show the increased efficiency of inhibition corresponding to the increased chain length of the inhibitor. This pattern of reactivity is strongly reminiscent of the increased efficiency of elastase-catalyzed hydrolysis of long peptide amides (Thompson and Blout, 1973b). The relationship between the hydrolysis and inhibition reactions will be discussed more fully below.

Also listed in Table II are the reciprocals of the axis intercepts, $1/K$ and $1/k$, of the plot of reciprocal rate of inhibition *vs.* reciprocal inhibitor concentration (Kitz and Wilson, 1962). The relationship of these intercepts to the fundamental kinetic parameters, K_i and k_2, will depend on the presence or absence of enzyme–peptide binding modes other than that leading to covalent-bond formation. For Ac-Ala-Pro-Ala-CMK, but not Ac-Pro-Ala-CMK, we can be sure that nonproductive modes will be weak compared to the productive one (Thompson and Blout, 1973a), and in this case, the intercept $1/K$, with the dimensions M, is equal to $1/K_i$ and the intercept $1/k$, with the dimensions sec, is equal to $1/k_2$. The value of K determined for this compound is comparable to the K_i determined for the analogous methyl ketone (4.8 mM) and K_m determined for the analogous amide (6.0 mM) (Thompson and Blout, 1973c).

Discussion

The irreversible inhibition of elastase by chloromethyl ketone analogs of substrates described above appears to proceed *via* a noncovalent enzyme–inhibitor complex of similar strength to comparable enzyme–substrate complexes. Binding, at least of the two longer inhibitors, should be restricted almost entirely to a mode analogous to the productive one of substrates (Thompson and Blout, 1973a). The covalent bond formed is therefore likely to be to the S_1 or S_1' subsite of the active center. Amino acid analyses of the irreversibly inhibited enzyme are consistent with the alkylation of one histidine residue per molecule of inhibitor reacted. Since His^{45} (analogous to His^{57} of α-chymotrypsin) is the only such residue in the immediate vicinity of S_1 and S_1' (Shotton *et al.*, 1972), it is the most likely candidate for reaction with the inhibitor. Confirmation of this hypothesis would complete the analogy between elastase inhibition by these chloromethyl ketones and the inhibition of α-chymotrypsin and trypsin by chloromethyl ketones structurally related to their substrates.

The efficiency of elastase inhibition depends strongly on the chain length of the inhibitor. Thus the ratio k_2/K_i for inhibition, like the analogous parameter for substrate hydrolysis (k_{cat}/K_m), is partly determined by enzyme–peptide contacts remote from the site of reaction. In the case of substrates it has been demonstrated that the rate constant for the acylation

[2] Half-cystine and methionine will be oxidized to cysteic acid and methionine sulfone by the performic acid reagent (Hirs, 1967).

step of reaction is dependent on remote enzyme–substrate contacts (Thompson and Blout, 1970, 1973b). The data presented here would be consistent with these contacts similarly affecting the rate constant for irreversible inhibition (k_2).

The development of chloromethyl ketone inhibitors of elastase points up both the enzyme's similarities and differences to the other serine proteinases. Like the other enzymes, the target for these alkylating agents appears to be the catalytically important residue His-45. Unlike the related enzymes, elastase appears to be alkylated only by *peptide* chloromethyl ketones. The peptide groups of elastase substrates and inhibitors, therefore, appear to be necessary for enzyme recognition of such compounds.

Acknowledgment

We thank Elizabeth Turner for technical assistance with the amino acid analyses.

References

Atlas, D., Levit, S., Schechter, I., and Berger, A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 281.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 2413.

Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.

Kaplan, H., Symonds, V. B., Dugas, H., and Whitaker, D. R. (1970), *Can. J. Biochem. 48*, 649.

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

Morihara, K., and Oka, T. (1970), *Arch. Biochem. Biophys. 138*, 526.

Petra, P., Cohen, W., and Shaw, E. (1965), *Biochem. Biophys. Res. Commun. 21*, 612.

Powers, J. C., and Wilcox, P. E. (1970), *J. Amer. Chem. Soc. 92*, 1782.

Shaw, E. (1967), *Methods Enzymol. 11*, 677.

Shaw, E. (1970), *Enzymes 1*, 91.

Shaw, E., and Ruscica, J. (1971), *Arch. Biochem. Biophys. 145*, 484.

Shotton, D. M., and Hartley, B. S. (1970), *Nature (London) 225*, 802.

Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225*, 811.

Shotton, D. M., White, N. J., and Watson, H. C. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 91.

Stevenson, K. J., and Smillie, C. B. (1965), *J. Mol. Biol. 12*, 937.

Thompson, R. C., and Blout, E. R. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1734.

Thompson, R. C., and Blout, E. R. (1973a), *Biochemistry 12*, 51.

Thompson, R. C., and Blout, E. R. (1973b), *Biochemistry 12*, 57.

Thompson, R. C., and Blout, E. R. (1973c), *Biochemistry 12*, 66.

Visser, L., and Blout, E. R. (1972), *Biochim. Biophys. Acta 268*, 257.

Visser, L., Sigman, D. S., and Blout, E. R. (1971), *Biochemistry 10*, 735.

Use of Peptide Aldehydes to Generate Transition-State Analogs of Elastase†

Robert C. Thompson‡

ABSTRACT: Two peptide aldehydes analogous to amide substrates of elastase have been synthesized. The aldehydes bind to the enzyme up to 5000-fold more tightly than the substrates. The elastase–aldehyde complexes appear to be good analogs of the transition-state complex for elastase-catalyzed amide hydrolysis.

A great deal of information about the specificity and catalytic mechanism of enzymes has been obtained by the study of complexes formed between the enzyme and substrates, or substrate analogs. In most cases the complexes have been analogous to the low-energy Michaelis complexes formed by initial reversible absorption of substrate to the enzyme. In many ways a more interesting species is the transition-state complex, that complex of highest energy on the pathway from the Michaelis complex to the reaction products. Differences between the solution and enzyme-catalyzed reactions are likely to be most pronounced in the structure of the substrate in their respective transition states. The nature of that difference should therefore be extremely relevant to defining the catalytic power of the enzyme.

The lifetime of the transition-state complex will be of the order of the vibrational period of a covalent bond (about 10^{-13} sec), precluding its direct study. However, certain analogs of the substrate, which form stable complexes with the enzyme, might mimic the true transition-state complex sufficiently closely to reveal something of the structure of the transient species. Transition-state theory predicts the enzyme–substrate binding in the true transition-state complex will be considerably stronger than that in the Michaelis complex from which it is formed (for a review, see Lienhard *et al.*, 1972, and Wolfenden, 1972). It is therefore likely that any analog which mimics relevant features of the transition-state

† From the Department of Biological Chemistry, Harvard Medical School, Eoston, Massachusetts 02115. *Received August 7, 1972.* This work was supported, in part, by U. S. Public Health Service Grants AM-07300 and AM-10794.

‡ Present address: Bacterial Physiology Unit, Harvard Medical School.