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INACTIVATION OF HUMAN FIBROBLAST COLLAGENASE BY CHLOROACETYL N-HYDROXYPEPTIDE DERIVATIVES

TSAU-YEN LIN* and DAVID W. KUO

Department of Enzymology, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065 U.S.A.

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When human fibroblast collagenase was incubated with $ClCH_2CO$-(N-OH)Leu-Ala-Gly-NH_2 (2–5 mM) in Tris buffer, pH 7.4 at 25°C, a slow, time-dependent inhibition of the enzyme was observed. Dialysis against a buffer to remove free inhibitor did not reactivate the enzyme. A reversible competitive inhibitor, phthaloyl-Gly^P-Ile-Trp-NHBzl (50 μM) partially protected the enzyme from inactivation by the compound. From the concentration dependent rates of inactivation $K_i = 0.5 \pm 0.1$ mM and k_3, the rate constant for inactivation $= 3.4 \pm 0.3 \times 10^{-3}\,min^{-1}$ were determind. The inactivation followed the pH optimum (6.5–7.0) for the enzyme activity, suggesting direct involvement of the same active site residue(s). The reaction mode of the inhibitor may be analogous to that of the inactivation of *Pseudomonas aeruginosa* elastase [Nishino, N. and Powers, J. (1980) *J. Biol. Chem.*, **255**, 3482] in which the catalytic glutamate carboxyl was alkylated by the inhibitor after its binding to enzyme through the hydroxamic Zn^{2+} ligand. All carboxyl groups in the inactivated collagenase were modified with 0.1 M ethyl dimethylaminopropyl carbodiimide/0.5 M glycinamide in 4 M guanidine at pH 5. The inactivator-affected carboxyl group was then regenerated with 1 M imidazole at pH 8.9, 37°C for 12 h and the protein was radiolabeled with ^{3}H-glycine methyl ester and carbodiimide to incorporate 0.9 residue glycine per mol enzyme.

KEY WORDS: Collagenase, Chloracetyl N-hydroxypeptide derivatives, human fibroblast.

INTRODUCTION

An active site-directed inactivator of interstitial collagenase may be useful for the characterization of the enzyme catalytic center as well as aiding the design of potential therapeutics. As a member of the family of matrix metallo-proteinases that degrade extracellular matrix and basement membrane components, collagenase plays important roles in normal development processes and in pathological tissue damage.[1] Although development of mechanism-based inactivators for a zinc peptidase, such as (R)-2-Benzyl-5-cyano-4-oxopentanoic acid for carboxypeptidase A[2] and haloacetyl-N-hydroxy derivatives of peptides for neutral metalloproteinases of the thermolysin family[3,4], have been described, no inactivator for collagenase or other matrix metallo-

Abbreviations used: CAL, $ClCH_2CO$-(N-OH)Leu-Ala-Gly-NH_2; CAP, $ClCH_2CO$-*D,L*-(N-OH)Phe-Ala-Ala-NH_2; TAC, trypsin activated collagenase; TIMP, tissue inhibitor of metalloproteinase; TSC buffer, 20 mM Tris-HCl containing 5 mM $CaCl_2$ and 0.15 M NaCl, pH 7.4; HEPES, N-2-hydroxyethylpiperazine-N′-ethanesulfonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. -Gly^P-, The superscript^P denotes that the carboxyl group of glycine is replaced by a phosphonic acid residue.

*Correspondence

proteinases has been reported. The evidene indicated that inactivation of thermolysin[3] by $ClCH_2CO$-*D,L*-(N-OH)Leu-OCH_3 and of *Pseudomonas aeruginosa* elastase[4] by $ClCH_2CO$-(N-OH)Leu-Ala-Gly-NH_2 was due to alkylation of the active site glutamic acid residue after binding of the inhibitor to the active site through interaction of the Leu side chain with S'_1 subsite and the hydroxamic acid function group with the zinc atom.[3,5,6] We observed previously that $ClCH_2CO$-*D,L*-(N-OH)Leu-OCH_3 did not inactivate human neutrophil collagenase.[7]

In this study evidence was obtained that human fibroblast collagenase could be irreversibly inhibited by $ClCH_2CO$-(N-OH)Leu-Ala-Gly-NH_2. The reaction was slow but the inactivated enzyme appeared to be stable at pH 6.5 at least for two days. The structure of this inhibitor is homologous to a part of the sequence segment of the known cleavage site of Type I collagen by collagenase (-Pro-Gln-Gly-↓-Ile-Ala-Gly-Gln-)[8].

METHODS AND MATERIALS

Human procollagenase (M_r = 53,000, 3–4 μM; recombinant enzyme from CELL-TECH, Slough, UK, and human gingival fibroblast procollagenase from Dr. M. Lark, MSDRL, Rahway, NJ) was activated by treatment with trypsin (2 μM) for 20 min at room temperature[9] followed by addition of soluble soybean trypsin inhibitor (10 μM) or soybean trypsin inhibitor-Sepharose gel (Sigma; 50 μl) (TAC). The active collagenase was titrated with known amounts of TIMP.[10] TAC (10 μl, about 2 μM) in 20 mM Tris-HCl buffer, pH 7.4 containing 5 mM $CaCl_2$ and 150 mM NaCl (TSC) was mixed with $ClCH_2CO$-(N-OH)Leu-Ala-Gly-NH_2 [CAL, Protein Research Foundation, Osaka, Japan); 134 mM in dimethyl sulfoxide or methyl pyrrolidone] (2.5 mM) or $ClCH_2CO$-*D,L*-(N-OH)Phe-Ala-Ala-NH_2 (CAP, Enzyme System Products, Livermore, CA) (2.5 mM) at 25°C. A mixture of TAC and methyl pyrrolidone or Me_2SO served as a control. The activity of collagenase was measured with N-Ac-Pro-Leu-Gly-SCH(iBu)CO-Leu-GlyOEt (BACHEM Bioscience, Philadelphia, PA; 0.4 mM) as substrae[11] in the presence of 4,4′-dithiodipyridine (1 mM) in 50 mM HEPES buffer containing 10 mM $CaCl_2$ and 0.01% Brij 35, pH 6.50 or ^{14}C-methylated bovine skin collagen as substrate using the *p*-dioxane differential precipitation method.[12] Phthaloyl-Gly^p-Ile-Trp-NHBzl, a slow binding competitive inhibitor of collagenase ($K_{i\ initial\ complex}$ = 25 nM)[10,13] was obtained from Elastin Products Co., Owensville, MO. For modification of carboxyl groups with a water-soluble carbodiimide, the conditions were those described by Carraway and Koshland.[14] 1-Ethyl-3-(3-dimethylaminipropyl) carbodiimide (EDC) was obtained from Pierce, Rockford, IL. The protein concentration was determined by absorbance at 275 nm or with amino acid analysis of the acid hydrolysate.

RESULTS AND DISCUSSION

Human fibroblast collagenase underwent a slow, time-dependent inactivation by reaction with a chloroacetyl-N-hydroxy tripeptides typified by $ClCH_2CO$-(N-OH)Leu-Ala-GlyNH_2(CAL). When collagenase (TAC) (2 μM) was mixed with the reagent (2.5 mM) at 25°C, time-dependent inhibition of the enzyme activity was observed on both thioesterolysis and collagenolysis. A pseudo first order rate constant of inhibition was about $2.1 \times 10^{-3}\,min^{-1}$. CAP was not an effective inhibitor (Figure 1). With higher concentrations of CAL combined with a higher reaction temperature the rate of inhibition increased: e.g., with 5.7 mM CAL the k_{obs} was $1.2 \times 10^{-2}\,min^{-1}$ at 22°C and $5.8 \times 10^{-2}min^{-1}$ at 37°C.

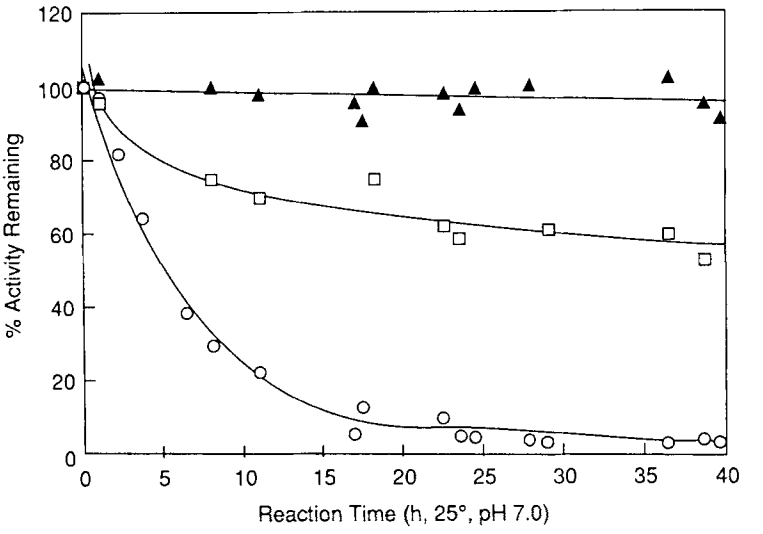

FIGURE 1 Inhibition of collagenase activity by chloroacetyl peptides. TAC (about 2 μM) in TSC buffer, pH 7.4 was mixed with 2.5 mM CAL or 2.5 mM CAP at 25°C. A mixture of TAC and 2 μl methyl pyrrolidone served as a control. At various time intervals 0.5 μl aliquots of the incubation mixture were taken and the residual enzyme activity on N-Ac-Pro-Leu-Gly-SCH(iBu)CO-Leu-GlyOEt was measured. No inhibitor (▲ ▲); CAL (○ ○); CAP (□ □). Under the reaction condition the inhibitors were shown to be stable; more than 95% of the reagents were recovered as native by HPLC analysis (0–80% acetonitrile gradient in 0.1% trifluoroacetic acid).

After completion of the reaction at 25°C 5 μl aliquots of the incubation mixture of TAC and CAL were dialyzed against 50 mM HEPES buffer containing 10 mM $CaCl_2$, pH 6.5 overnight at 5°C. The CAL-inhibited TAC showed less than 5% of the native activity. As a reference, a mixture of TAC (2 μM) and phthaloyl-Gly^p-Ile-Trp-NHBzl (50 μM) showing no detectable thioesterolytic activity, was treated in a similar manner. Another set of reaction mixtures was subjected to treatment with 1 M NH_2OH at pH 7 for 2 h or with 1 M imidazole at pH 8.9 for 12 h at 37°C, and dialyzed against TSC buffer containing 0.5 mM zinc acetate at 4°C. The reaction mixtures were washed three times with HEPES buffer on a Millipore Ultrafree MC filtration unit before determination of the activity (Table 1). The results indicated that, (a) dialysis to remove the free inhibitor did not regenerte the enzyme activity from CAL-inhibited TAC even though the phosphonamidate effected inhibition was completely reversible, (b) the treatment with imidazole at pH 8.9 regenerated the enzyme activity from CAL-inhibited TAC suggesting the formation of a carboxyl ester in the modification reaction; a slight activity shown after NH_2OH treatment was probably due to the results of the hydrolysis of the affected carboxyl ester and did not imply a functional role of a hydroxamic acid residue formed; no reactivation could be achieved by merely treatment with a chaotropic agent (4 M urea or guanidine) and dialysis against buffer, (c) the mode of inactivation may be analogous to the reaction of CAL on *P. aeruginosa* elastase and is the result of alkylation of the catalytic carboxyl side chain.[4] On the basis of chloroacetyl-N-hydroxy peptide inactivation of thermolysin and *P. aeruginosa* elastase in which the site of modification was established by X-ray diffraction analysis,[5,6] the CAL-modified site in collagenase could be the glutamate residue

TABLE I
Inhibition and Reactivation of Collagenase

Treatment	Relative enzyme activity
Collagenase, dialyzed against TSC buffer containing 0.5 mM Zn acetate	100[a]
Collagenase inhibited with CAL and dialyzed	4.5 ± 1.5
Collagenase inhibited with PhtN-Gly^{P}-Ile-Trp-NHBzl and dialyzed	98.1 ± 3.1
Collagenase treated with 1 M NH_2OH for 2 h at 37°C and dialyzed	96.2 ± 5.2
Collagenase inhibited with CAL, treated with 1 M NH_2OH, pH 7.0 for 2 h at 37°C and dialyzed	6.6 ± 1.7
Collagenase in 1 M imidazole, pH 8.9 for 12 h at 37°C, dialyzed	82.0 ± 4.8
Collagenase inhibited with CAL, incubated in 1 M imidazole, pH 8.9, for 12 h at 37°C and dialyzed	71.4 ± 2.0

[a]Under the condition for the measurement of activity the concentration of zinc ion was less than 1.0 μM.

in the putative zinc binding site^{15}-His^{218}-Glu-Leu-Gly-His^{222}. It follows that the spatial arrangement of the critical carboxyl side chain relative to the catalytic zinc atom in collagenase should be similar to that of thermolysin family metalloproteinase.

The pH-dependence of inactivation of collagenase by CAL followed closely by the pH-dependence of thioesterolytic activity of the enzyme. Both the maximal enzyme activity and the inactivation rate were in the range of 6.5–7.0 (Figure 2). The phosphonamidate inhibitor, Phthaloyl-Gly^{P}-Ile-Trp-NHBzl partially protected the enzyme from inactivation by CAL. When TAC (3.6 μM) was treated with CAL (2.5 mM) at pH 7.0 in TSC buffer at 22°C for 960 min and the activity was determined after the reaction mixtures were dialyzed against 50 mM HEPES buffer, pH 6.5,

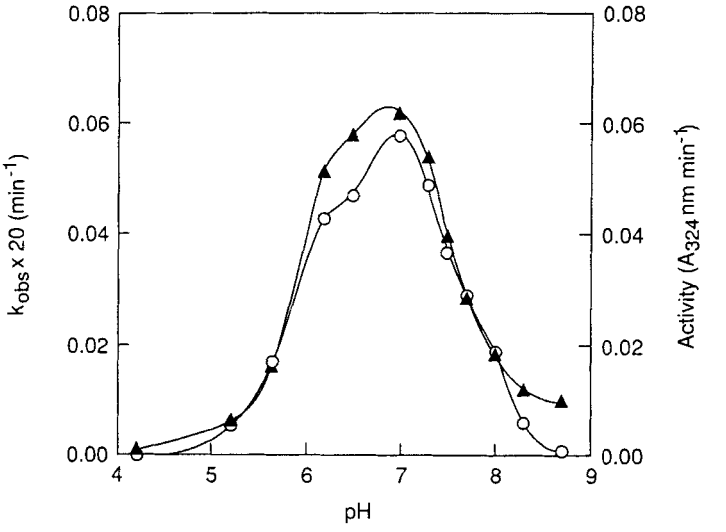

FIGURE 2 The pH dependence of collagenase thioesterolytic activity (▲ ▲) and of the inactivation of collagenase by CAL (○ ○). For the measurement of enzyme activity the concentration of TAC was about 1.5 nM. In the inactivation experiment the concentration of TAC and of CAL was about 3.6 μM and 2.5 mM, respectively. The residual activity of the reaction mixture was followed for 16 h at 22°C. 0.1 M acetate buffer was used for pH 4–6; Tris buffer for pH 6–7.5; borate buffer for pH 8–9.

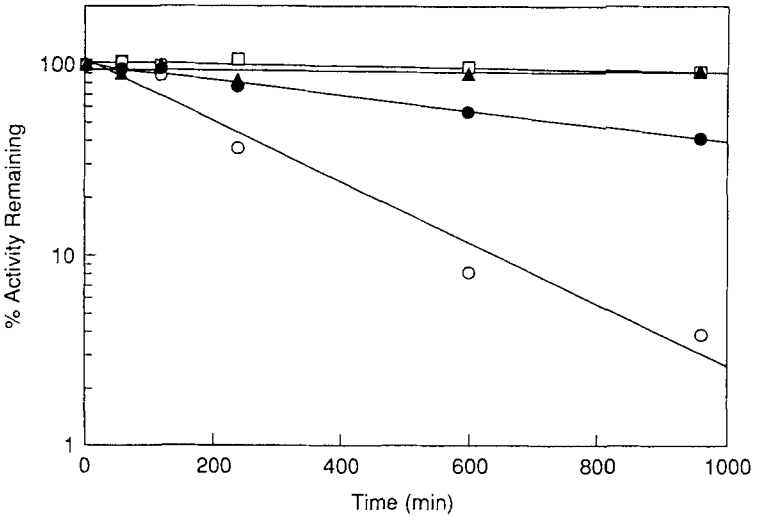

FIGURE 3 The effect of Phthaloyl-Gly^p-Ile-Trp-NHBzl on the inactivation of collagenase by CAL. TAC (3.6 μM) was treated with CAL (2.5 mM) in TSC buffer, pH 7.2 at 22°C. The enzyme activity was determined after the aliquots of reaction mixtures at the defined time periods were quenched by dilution and dialyzed against 50 mM HEPES buffer, pH 6.5, containing 10 mM $CaCl_2$ at 4°C for 16 h. TAC alone (□ □); enzyme + phosphonamidate inhibitor (50 μM) (▲ ▲); enzyme + CAL (○ ○) and enzyme + phosphonamidate inhibitor (50 μM) + CAL (● ●).

containing 10 mM $CaCl_2$ at 5°C for 16 h, about 92% activity was lost (k_{obs}, 2.1×10^{-3} min^{-1}). Including 50 μM of the phosphonamidate inhibitor in the reaction mixture significantly retarded the inactivation by CAL ($k_{obs} < 5 \times 10^{-4}$ min^{-1}) (Figure 3). An even higher extent of protection was effected by 100 μM inhibitor. The nearly identical pH-dependence of inactivation and of the enzyme activity as well as the protection of enzyme from inactivation by a competitive inhibitor suggest the catalytically critical nature of the carboxyl group modified.

By analogy with the inactivation of thermolysin[3] by $ClCH_2CO$-*D,L*-(N-OH)Leu-OCH_3 the possible reaction scheme of inactivation of collagenase by CAL is an active site-directed esterification of a cataytically critical carboxyl group: $E\text{-}CO_2^- + ClCH_2CO\text{-}(N\text{-}OH)Leu\text{-}Ala\text{-}Gly\text{-}NH_2 \rightarrow E\text{-}CO_2^- \cdot ClCH_2CO\text{-}(N\text{-}OH)Leu\text{-}Ala\text{-}Gly\text{-}NH_2 \rightarrow E\text{-}CO_2\text{-}CH_2CO\text{-}(N\text{-}OH)Leu\text{-}Ala\text{-}Gly\text{-}NH_2$. Thus, the kinetics of the inactivation may be described by the two stage reaction mechanism representing the active site-directed irreversible inhibition of an enzyme,[16]

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} E\text{-}I'; \text{ and, } 1/k_{obs} = K_i/k_3\,[I] + 1/k_3.$$

From the concentration-dependent rates of inhibition, k_{obs}, of the enzyme by the haloacyl peptide, a K_i value, the apparent dissociation constant for the complex of collagenase and CAL, and a k_3 vlaue, the rate constant for inactivation, were determined as 0.47 ± 0.14 mM and $3.4 \pm 0.33 \times 10^{-3}$ min^{-1}, respectively (Figure 4).

In Table II the characteristics of known inactivation of metalloproteinases by various chloroacetyl N-hydroxy peptides is shown. A k_3/K_i of 0.1 M^{-1}s^{-1} for CAL inactivation of collagenase is about 400 fold less than that determined for the

TABLE II
Inactivation of metalloproteinases by chloroacetyl peptide derivatives

Compound	Human Fibroblast Collagenase			*Pseudomonas aeruginosa* Elastase[¶]			Thermolysin[§]		
	k_3 (min^{-1})	K_i (mM)	k_3/K_i ($M^{-1}s^{-1}$)	k_3 (min^{-1})	K_i (mM)	k_3/K_i ($M^{-1}s^{-1}$)	k_3 (min^{-1})	K_i (mM)	k_3/K_i ($M^{-1}s^{-1}$)
$ClCH_2CO$-N(OH)Leu-Ala-Gly-NH_2	0.0034	0.47	0.1	0.0072	1.3	0.09	4.8	2	40
$ClCH_2CO$-N(OH)Phe-Ala-Ala-NH_2	0.0005	2.5	0.004						
$ClCH_2CO$-N(OH)Leu-OCH_3		NI			NI		0.045	7.5	1.0

[¶]From Rasnick, D. and Powers, J. C.[3]
[§]From Nishino, N. and Powers, J. C.[4]
NI, no inhibition

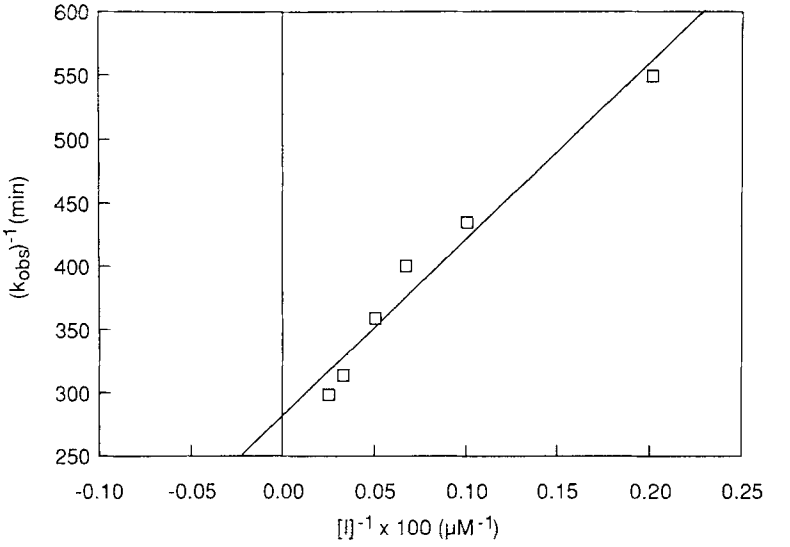

FIGURE 4 The concentration-dependent rates of inhibition of collagenase by CAL. The time-dependent inhibition of TAC (about 3.6 μM) in 10 μl of TSC buffer, pH 7.4 by mixing with 0, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mM of CAL at 25°C was followed.

inactivation of thermolysin. Nevertheless, this is the first time such an inactivation has been observed on a phosphoramidon-insensitive matrix metalloproteinase. The relative ineffectiveness of CAL as an inactivator of collagenase as compared with the potency of the haloacetyl N-peptide derivative on thermolysin may be associated with a suboptimal fit of the structure of CAL to the active site of collagenase. A N-terminal elongated analog of CAL with a structure such as ClCH(R)CO-(N-OH)-peptide which could occupy S-subsites by virtue of the R-group may facilitate the interaction.

The CAL-inhibited enzyme was subjected to carboxyl modification by EDC and glycine amide in the presence of 4 M guanidine hydrochloride at pH 5. The reagent was removed by dialysis against 1 mM acetic acid at 4°C. The product was then incubated at 37°C for 12 h in 1 M imidazole, pH 8.9 to regenerate the carboxyl group modified by CAL. The material was exhaustively dialyzed against 1 mM acetic acid at 4°C to remove the reagent. The lyophilized protein was then subjected to reaction with EDC (0.1 M) and 0.3 M ^{3}H-glycine methyl ester at pH 5.0 for 4 h at room temperature. The reagent was removed by dialysis against 1 mM acetic acid and lyophilized. About 0.9 $\pm$ 0.1 mol of ^{3}H-glycine was incorporated per mol proenzyme of a molecular weight 53,000 based on the concentration of protein determined by UV absorption and amino acid analysis. During the activation of the latent collagenase with trypsin and the course of inactivation of the active enzyme considerable coversion of 42,000 dalton- enzyme to 29,000- dalton active species occurred as judged by SDS-PAGE analysis of proteins. The extent of modification by CAL of each active enzyme species has not been determined. The labeling of collagenase before reaction with CAL or CAL-inactivated enzyme which was not subjected to imidazole treatment was about 0.1 $\pm$ 0.08 mol glycine incorporated per mol protein. The results support the assumption that CAL quantitatively modifies a critical carboxyl group

and can be used to label the active site. We are currently pursuing the use of a radioactive inactivator to facilitate the titration of active collagenase and the identification of the site of modification at the level of the primary structure.

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