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METAL BINDING PEPTIDE INHIBITORS OF VERTEBRATE COLLAGENASE

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Summary. A series of metal binding peptide analogues of the C-terminal sequence of collagen adjacent to its known site of cleavage by vertebrate collagenases has been synthesized by solid phase methods and tested as inhibitors both of collagenolysis and of hydrolysis of the model substrate DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg by tadpole backskin collagenase. The peptide analogue inhibitors have the general structure R-Ala-Gly-Gln-D-Arg-NH_2. Using an HPLC assay system, I_{50} values of 70 μM, 50 μM and 10 μM were obtained for the three most potent inhibitors for which R = $HSCH(CH_2C_6H_5)CO$-, $HSCH_2CH(NH_2)CO$- and $HSCH(CH_2CH(CH_3)_2)CO$-, respectively, when the above octapeptide was used as substrate. In contrast, an I_{50} value of 3 mM was found for cysteine under the same conditions. The mercaptophenylalanyl derivative was also highly effective in inhibiting collagen degradation. Using calf skin collagen as substrate, inhibition was obtained at concentrations of peptide analogue below 10 μM.

Native triple helical collagen is highly resistant to proteolysis by enzymes other than collagenases. Vertebrate collagenases make a specific cleavage at the Gly-Ile bond located at position 775-776 in the α1 chain (and at the homologous Gly-Leu pair in the α2 chain) thereby producing the so called TC^A and TC^B fragments (1). Inreased collagenolytic activity has been implicated in several pathological states, including corneal ulceration, rheumatoid arthritis, periodontal disease and in invading tumors (2). Specific high-affinity collagenase inhibitors should be of value in arresting the degradation of collagen which accompanies these conditions and therefore may be therapeutically useful. Bacterial and vertebrate collagenases are inhibited by compounds which bind transition metal ions such as thiols and chelating agents (6). These and other studies (7) suggest that collagenases are zinc metalloproteases. It is reasonable to assume that the metal ion functions during catalysis as it does with other zinc peptidases by coordination to the carbonyl oxygen of the cleaved peptide bond (8).

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An approach based on this presumption has provided a rational basis for the design of potent inhibitors of several Zn-containing peptidases (9-12). In such inhibitors, a metal-coordinating ligand is coupled to a peptide carrier which has the same amino acid sequence as that existing in the natural substrate on the carboxyl side of the susceptible bond. The most effective of these inhibitors have been obtained when the coordinating group is located at a position stereochemically equivalent to that of the scissile carbonyl in the substrate. We report here the properties of some peptide analogues utilizing this approach. Sundeen and Dejneka (13) also recently reported the synthesis of collagenase inhibitors using this same rationale.

MATERIALS AND METHODS

Inhibitors - The details of the synthesis and characterization of the peptide derivatives will be reported elsewhere. In brief, solid phase peptide synthetic methods using benzhydrylamine resin and dicyclohexylcarbodiimide coupling procedures were utilized to produce the tetrapeptide carrier BOC-Ala-Gly-Gln-D-Arg-resin. Appropriate protected thiol acids were then coupled to the carrier to produce the desired inhibitor. The thiol-pentapeptide derivatives were deblocked and cleaved from the resin using anhydrous HF. The derivatives were subsequently oxidized to the corresponding disulfide and purified by gel filtration (Sephadex G-10) in 1 N acetic acid, followed by preparative reversed phase HPLC using 0.1% H_3PO_4/CH_3CN as the mobile phase. The purified peptides were treated with an anion exchange resin to remove phosphate and then were lyophilized. The amino acid composition of each purified peptide was determined. Prior to assay for inhibitory potency, the oxidized derivatives were reduced by either of two methods: reduction at pH 8 with dithiothreitol (DTT) followed by gel filtration at pH 6.5 to remove the oxidized DTT, or by addition of reduced lipoamide glass beads (Pierce Chemical Co., Rockford, IL), followed by centrifugation to remove the beads. In both cases, the -SH titer of each solution determined immediately before assay using the Ellman reaction (14). In addition, the purity of the stock reduced inhibitor solution was checked by reversed phase HPLC.

Collagenase - Collagenase was purified from tadpole backskin tissue culture medium as described by Nagai *et al.* (15) by ammonium sulfate precipitation and subsequent gel filtration on Sephadex G-200. The specific activity using the synthetic octapeptide substrate of Masui *et al.* (16) was 310 nmole/min/mg protein, and when using acid-soluble calf skin collagen (Sigma, St. Louis, MO) as substrate it was approximately 10 µg collagen degraded/min/mg protein.

Assays - Collagenase activity was determined by two methods. The initial rate of hydrolysis by tadpole collagenase of the model substrate DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (U.S. Biochemicals, Cleveland, OH) to form the products DNP-Pro-Gln-Gly and Ile-Ala-Gly-Gln-D-Arg was measured by quantitative reversed phase HPLC (17). The substrate concentration was 0.6 mM, which had previously been determined to be the K_m value of the enzyme under the conditions of the assay at pH 6.5. The formation of the TC^A and TC^B (1) fragments of acid-soluble calf skin collagen was also assessed. The substrate and product proteins were separated by electrophoresis in sodium dodecylsulfate-polyacrylamide slab gels

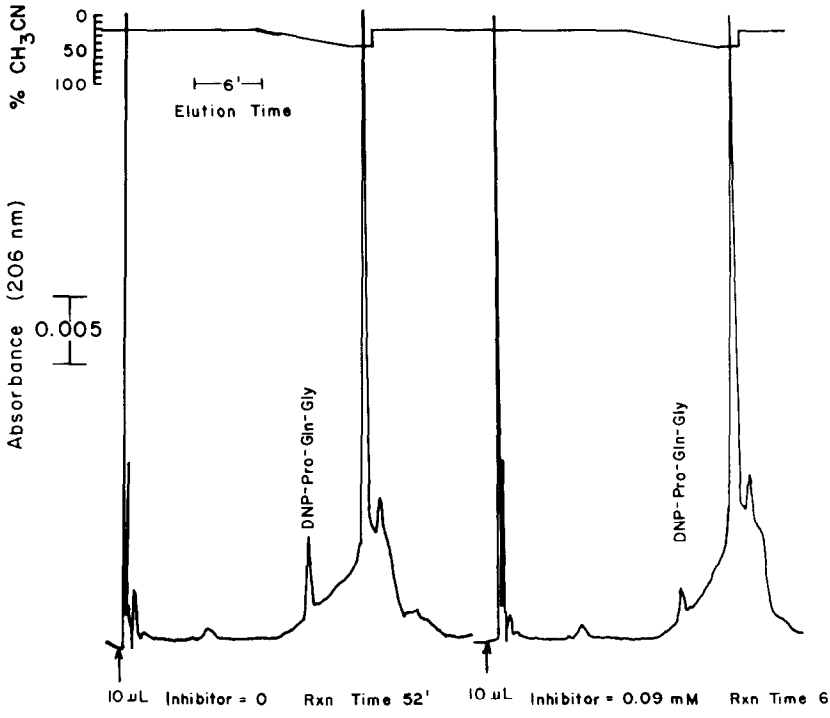

Fig. 1. Inhibition of hydrolysis of DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg catalyzed by tadpole collagenase. The separation of the DNP-tripeptide product from the DNP-octapeptide substrate by reversed phase HPLC using an 0.1% H_3PO_4/CH_3CN gradient and a C_{18} column is shown. The reaction mixture on the right contained 0.09 mM $HSCH(CH_2C_6H_5)CO$-Ala-Gly-Gln-D-Arg-NH_2. Ten µl of each reaction mixture was injected on the column at the point indicated by the arrows. The reaction times are shown at the bottom of the elution profiles.

using the system of Laemmli (18). Assays using the model peptide substrate were conducted at 37°C in 0.05 M bis-tris, 0.15 M NaCl, 5 mM $CaCl_2$, pH 6.5, while those with collagen were run under the same conditions except that the temperature was maintained at 25°C.

RESULTS

Synthetic Substrate - Fig. 1 illustrates the inhibition of collagenase-catalyzed cleavage of DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg at the Gly-Ile position by the mercaptophenylalanyl- derivative, $HSCH(CH_2-C_6H_5)CO$-Ala-Gly-Gln-D-Arg-NH_2. The height of the peak at 8.3 min (which is DNP-Pro-Gln-Gly based on co-chromatography with an authentic standard (17)) is clearly smaller when the inhibitor is included in the reaction mixture. In addition, the magnitude of the peak appearing early in the elution profile at 2.5 min (which is Ile-Ala-Gly-Gln-D-Arg, the other product) is also reduced in the reaction mixture which included

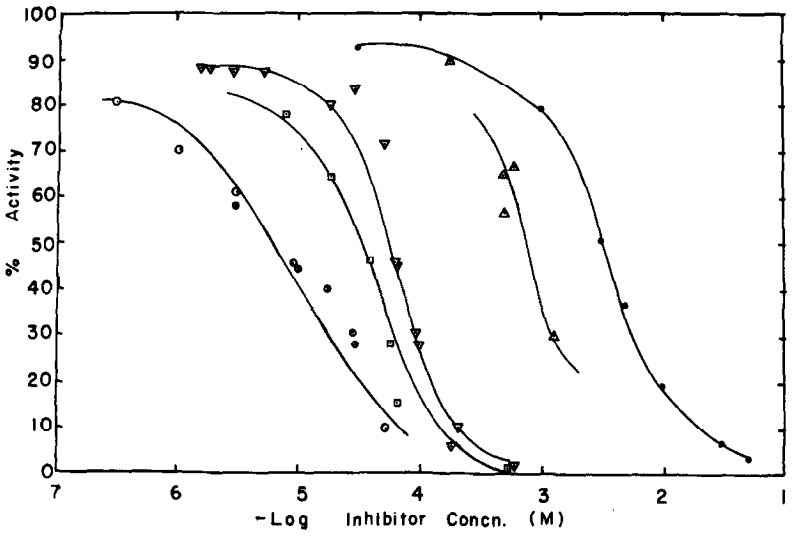

Fig. 2. Comparison of thiol peptide inhibitors with cysteine using octapeptide substrate and tadpole collagenase. Conditions: 0.6 mM DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg, 0.05 M bis-tris, 0.16 M NaCl, 5 mM $CaCl_2$, pH 6.5, 37°C, 80 µg collagenase. The inhibitors are: (●), cysteine; (△), $HSCH_2CH_2CO$-R; (▽), $HSCH(CH_2C_6H_5)CO$-R; (⊡) $HSCH_2CH(NH_2)CO$-R; (●) $HSCH(CH_2CH(CH_3)_2)CO$-R, where R = -Ala-Gly-Gln-D-Arg-NH_2.

the inhibitor. Similar results were obtained with cysteine, as well as the other peptide-thiol compounds.

Fig. 2 shows a plot of I_{50} values as a function of inhibitor concentration for several of the peptide analogues tested. It is evident from this data that each of the thiol-peptides is more effective than cysteine in inhibiting hydrolsis of the octapeptide collagen analogue. The concentrations of these compounds required to give 50% inhibition in this model assay are summarized in Table I.

Natural Substrate - Fig. 3 depicts an assay system in which the mercaptophenylalanyl-tetrapeptide was tested for inhibition of triple helical collagen degradation by tadpole collagenase. It is clear that the peptide analogue is a highly effective inhibitor of the formation of the TC^A fragment of collagen. Although we do present quantitative data here, it is evident from the figure (compare lanes 3 and 4) that in the presence of 700 µM inhibitor, almost no degradation of the collagen is evident over the 6 hr incubation period. Indeed,

TABLE I

INHIBITORS OF TADPOLE COLLAGENASE

COMPOUND	I_{50}(mM)*
H_2N-CH(CH_2SH)-COOH	3
H_2N-CH(CH_2SH)-CO-Ala-Gly-Gln-D-Arg-NH_2	0.05
Ac-NH-CH(CH_2SH)-CO-Ala-Gly-Gln-D-Arg-NH_2	0.07
HS-CH_2CH_2CO-Ala-Gly-Gln-D-Arg-NH_2	1
HS-CH($CH_2C_6H_5$)-CO-Ala-Gly-Gln-D-Arg-NH_2	0.07
HS-CH($CH_2CH(CH_3)_2$)-CO-Ala-Gly-Gln-D-Arg-NH_2	0.01

*I_{50} is the concentration of inhibitor required for 50% inhibition of hydrolysis of the octapeptide substrate. Conditions: 0.05 M bis-tris, 0.16 M NaCl, 5 mM $CaCl_2$, 0.6 mM DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg, pH 6.5, 37°C.

concentrations of inhibitor as low as 7 μM showed evidence of inhibition (compare lanes 8 and 7).

DISCUSSION

The most important conclusion to be drawn from these data is that coupling of the metal-coordinating thiol group to a peptide carrier whose sequence of amino acids is the same as that on the carboxyl side of the cleaved peptide bond of collagen results in a collagenase inhibitor of enhanced potency. Comparison of the I_{50} values in Table I for cysteine and the peptide Cys-Ala-Gly-Gln-D-Arg-NH_2 shows that coupling of the amino acid to a specific tetrapeptide carrier reduces concentration of inhibitor required to achieve 50% inhibition by 40-fold. Since shorter peptide derivatives have not been tested in this system, we cannot pre-

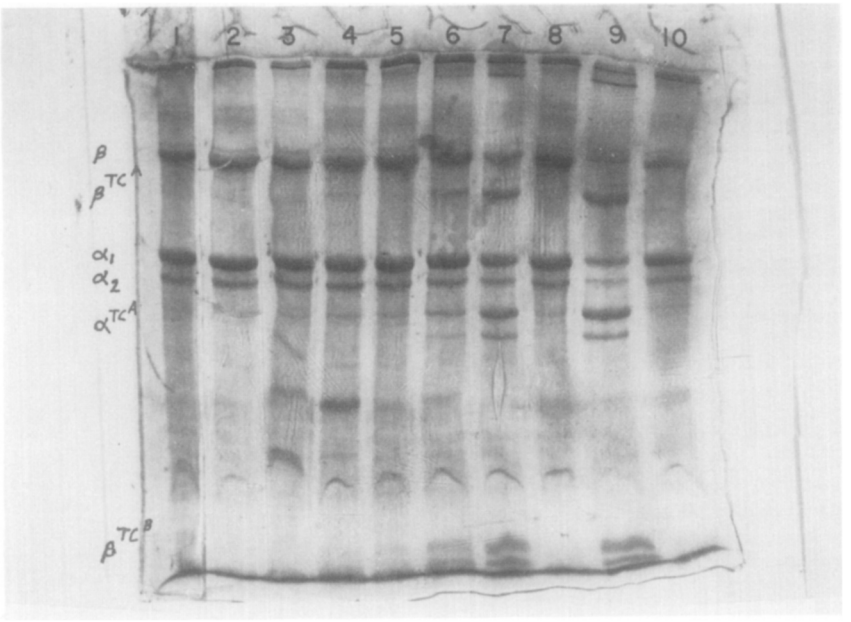

Fig. 3. Effect of $HSCH(CH_2C_6H_5)CO$-Ala-Gly-Gln-D-Arg-NH_2 on collagen cleavage by tadpole collagenase. Acid soluble calf skin collagen (4 mg/ml) was incubated at pH 6.5, 25°C, with 0.16 mg/ml purified tadpole backskin collagenase solution in a total volume of 0.2 ml in the presence and absence of inhibitor. After 6 hrs, the reaction was quenched by boiling in sample buffer (18) and the reaction products separated by electrophoresis in sodium dodecylsulfate-polyacrylamide (6%) slab gels. Each lane contained 40 µg collagen or collagen degradation products. The contents of each lane are indicated below:

LANE (left to right)	1	2	3	4	5	6	7	8	9	10
Reaction time (hr)	0	6	0	6	0	6	6	0	6	0
Inhibitor Concentration (µM)	0	0	700	700	70	70	7	7	0.7	0.7
Presence of Collagenase	-	-	+	+	+	+	+	+	+	+

sently tell whether the tetrapeptide carrier is the optimum length, or whether a shorter carrier would also be as effective.

The second important conclusion is the stabilizing effect of the hydrophobic side chain in the thiol-containing residue. For example, in changing from a hydrogen atom of the mercaptopropionyl-derivative to the benzyl group of the mercaptophenylalanyl-compound, a 20-fold increase in inhibitory potency was realized. Further alteration to the isobutyl group (the mercaptoisoleucyl-

derivative) provided nearly a 100-fold increase in inhibitory power compared to the mercaptopropionyl-peptide.

The most potent thiol peptide inhibitors of angiotensin converting enzyme (9) and thermolysin (10) are active in the nanomolar range. Our results demonstrate that combining the thiol group with an appropriate C-terminal sequence related to the collagen susbtrate results in an improvement of 300-fold in inhibitory potency compared with the simple thiol. Further gains in inhibitory potency may be expected by optimizing the position of the coordinating group and by altering or adding amino acid residues in order to more closely mimic the collagenase sensitive region of collagen. Efforts are currently in progress to synthesize amino terminal sequences which contain metal binding groups, as well as metal complexing peptides which combine both amino- and carboxyl-terminal sequences analogous to those flanking the sensitive Gly-Ile(Leu) bond in the natural substrate (19).

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