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Inhibition of Mammalian Collagenases by Thiol-Containing Peptides

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The following thiol-containing peptide analogues of the carboxyl side of the collagenase-sensitive bond of collagen were synthesized and tested as inhibitors of collagenases partially purified from homogenates of rabbit V-2 tumor and culture medium of pig synovium: $HSCH_2CH(CH_3)CO$-Ala-OEt (I), $HSCH_2CH(CH_2Ph)CO$-Ala-OEt (II), $HSCH_2CH[CH_2CH(CH_3)_2]CO$-Ala-OEt (III); $HSCH_2CH$-$[CH_2CH(CH_3)_2]CO$-Ala-Gly-OEt (IV); $HSCH_2CH[CH_2CH(CH_3)_2]CO$-Ala-Gly-Gln (V). The compounds are listed in order of their inhibitory potency when assayed with nonfibrillar-acid-soluble calf skin collagen at pH 7.6, 35°C. The best inhibitor (III) gave 50% inhibition between 1 and 4 μM. II was a competitive inhibitor with a K_i value of 75 μM. The enzymes preferred an isobutyl side chain at the 2-carbon position, and, where tested (III, IV), did not discriminate strongly between stereoisomers at the chiral 2-carbon. Increasing the length of the inhibitor did not markedly increase potency.

Key words: inhibitors, collagen breakdown, cleavage site analogues

Mammalian collagenases are zinc endoproteinases that initiate the degradation of triple helical collagen by making a single cleavage in each of the three collagen chains at a point three quarters from the amino terminal end, resulting in the production of two fragments, referred to as TC^A and TC^B [1]. In type I collagen, the amino acid sequence flanking the bond cleaved by tadpole [2] or rabbit V-2 tumor [3] collagenase in the α1(I) chain has been reported to be -Pro-Gln-Gly-↓-Leu-Ala-Gly-Gln-Arg- (the arrow indicates the point of cleavage). In the α2(I) chain, the -Leu- at the S_1'[1] position in the substrate is replaced by -Ile- [1].

Collagenases are elaborated in vivo as a result of physiological processes such as resorbing uterus [4] and in pathological processes such as corneal ulceration [5], rheumatoid arthritis [6], periodontal disease [7], and tumor metastasis [8]. Synthetic

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[1]The nomenclature is that of Schechter and Berger [26] in which P designates residues of the substrate and S designates subsites of the active site of the enzyme. Primed letters designate residues or subsites to the carboxyl side of the cleaved peptide bond.

collagenase inhibitors should be of value in studying these processes. An approach to this goal is synthesis of peptide analogues of the collagen cleavage site in which a metal-binding functionality replaces the scissile carbonyl of the substrate. When combined with the appropriate peptide, this strategy has yielded specific, high-affinity inhibitors of several zinc-dependent metallopeptidases, including angiotensin-converting enzyme [9,10], enkephalinase [11], and thermolysin [12]. We extended this approach to include inhibitors of tadpole backskin collagenase [13]. This report deals with the preferred specificity of the mammalian enzyme for inhibitor side chains at the S_1' and additional sites at the extended active site of mammalian collagenases.

MATERIALS AND METHODS

Inhibitors

D-(-)-S-Acetyl-β-mercaptoisobutyric acid was obtained from Chemical Dynamics (South Plainfield, NJ). (±)-2-[(Acetylthio)methyl]-4-methylpentanoic acid and (±)-2-[(acetylthio)methyl]-3-phenylpropanoic acid were synthesized by the procedure of Sundeen and Dejneka [14]. The latter two compounds were obtained as racemic mixtures. Peptides and peptide analogues were synthesized in solution by using active esters or dicyclohexylcarbodiimide methodologies [15]. All amino acids were of the L-configuration. Intermediates and final products were purified to apparent homogeneity by C_{18} reversed-phase HPLC with aqueous 0.1% trifluoroacetic acid/CH_3CN gradients. Diasteromeric pairs were resolved by HPLC. The acetyl-protecting group was removed at the last step by treatment with dilute NH_4OH in nitrogen-flushed methanol. The peptides were characterized by amino acid analysis after acid hydrolysis. Thiol titers were determined by using Ellman's reagent [16].

Collagenases

Details of collagenase purification will be reported elsewhere. Briefly, the method of Cawston and Murphy [17] was followed with the exception that the final zinc-chelate chromatographic step was omitted. Fresh pig synovium was obtained from a local slaughterhouse. Synovial membranes were removed and cultured in serum-free Dulbecco's modified Eagle's medium with antibiotics (Gibco, Grand Island, NY). Conditioned medium was pooled and frozen until use. The fraction precipitating between 20 and 60% of saturation with ammonium sulfate was collected. This fraction was activated for 90 min at 37°C with 4-aminophenylmercuric acetate and chromatographed on Bio-Gel A-0.5 (Bio-Rad, Richmond, CA). The active fractions were then chromatographed on DEAE-cellulose (Whatman DE-52, Whatman Lab Sales, Hillsboro, OR) and followed by heparin-Sepharose (Sigma Chemical Co., St. Louis, MO). The K_m and V_{max} (determined as described below in the presence of 0.25 M glucose) was 5.6 ± μM and 11.2 ± 2 nmol collagen degraded/μg protein/hr. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) [18] followed by silver staining showed the presence of a major and three minor bands.

V-2 tumor was grown subcutaneously in rabbits [19]; when the tumors reached 2–3 cm in size, the rabbit was killed and the were tumors excised and then homogenized as described by Biswas et al [20]. The homogenate was then subjected to the steps outlined above for the pig synovial enzyme except that the activation step was omitted since preliminary experiments showed that it was unnecessary. Several active

fractions were obtained. The K_m and V_{max} for the fraction used in these experiments was 4.8 ± 0.7 μM and 0.1 ± 0.01 nmol collagen degraded/μg protein/hr. The preparation which was purified about 40-fold was heterogeneous on SDS-PAGE.

Collagenase Assays

Collagenase was assayed in 0.05 M Tris-HCl, 0.2 M NaCl, 0.25 M glucose, pH 7.6 at 35°C, containing 10% dimethylsulfoxide. Glucose was included to prevent fibril formation [21] and dimethylsulfoxide was present since the stock solutions of the inhibitors were dissolved in it. Undergraded collagen and degraded collagen were resolved in 7.5% polyacrylamide gels [18], stained with 1% Coomassie Blue R-250 in i-propanol-acetic acid-water (100/40/300, v/v). After destaining, the percentage of collagen degraded was estimated by scanning densitometry and integration of the peak areas of the α1, α2, $\alpha1^{TCA}$ and $\alpha2^{TCA}$ chains [22].

RESULTS

The electrophoretograms in Figure 1A and B illustrate qualitatively the inhibition by two diastereomers of $HSCH_2CH[CH_2CH(CH_3)_2]CO$-Ala-Gly-OEt of collagen degradation catalyzed by synovial and V-2 tumor collagenases. It is evident from these data that both diastereomers at concentrations between 1 μM and 100 μM inhibit formation of the TC^A and TC^B fragments of acid-soluble calf skin collagen.

In order to obtain a more quantitative estimate of the inhibitory potency of these compounds, the percentage degradation of the of α1(I) and α2(I) chains to form the corresponding TC^A fragments was determined by scanning densitometry of the gels shown in Figure 1A and B. The results of this determination are summarized in Figure 2. The data show that diastereomer 1 ($IC_{50} \simeq 4$ μM) was somewhat more effective in inhibiting collagenase action by enzymes from both sources than was diastereomer 2 ($IC_{50} \simeq 10$ μM). These values are maximal IC_{50} values since there may have been some autooxidation of the inhibitor during the assay. The enzymes from the two sources did not differ significantly in their sensitivity to this inhibitor.

Several other thiol peptide derivatives were also synthesized as a means of probing the specificity of the two collagenases for residues on the carboxyl side of the cleavage point. Approximate inhibitory potencies of these compounds, determined by scanning densitometry as illustrated above, are summarized in Table I. With the approximate nature of these values in mind, it may be noted first from that neither enzyme seems to discriminate markedly between diastereomeric pairs. With compounds III–V, the difference in inhibitory potency was less than tenfold. Second, the two collagenases apparently prefer bulkier hydrophobic groups at the site immediately adjacent to the scissile peptide bond. For example, the isobutyl side chain of the S_1' residue in III was better than that of the benzyl side chain of II or the methyl of I. Third, extending the peptide to encompass more potential interactions with the enzyme did not result in more potent inhibitors. Addition of -Gly-OEt (IV) or -Gly-Gln (V) provided little additional binding energy, although V is not strictly comparable with IV since the carboxyl is not blocked. Indeed, studies with N-carboxyalkyl dipeptides [23] indicate that analogues with a blocked carboxyl are better inhibitors. Finally, the inhibitors described here exhibit nearly equal potencies when assayed with collagenases from two different sources, although the pig synovial enzyme was

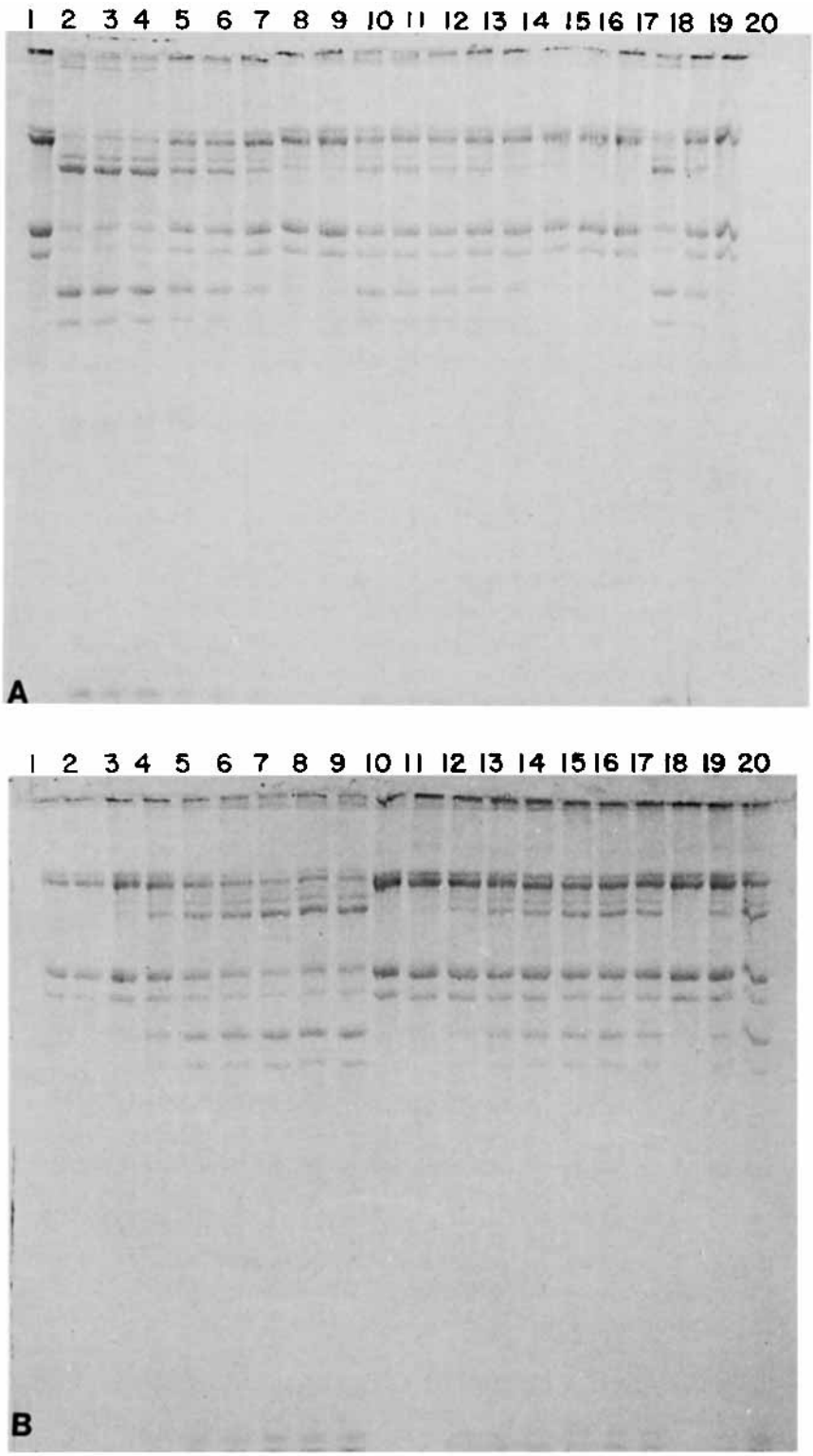
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B

Fig. 1.

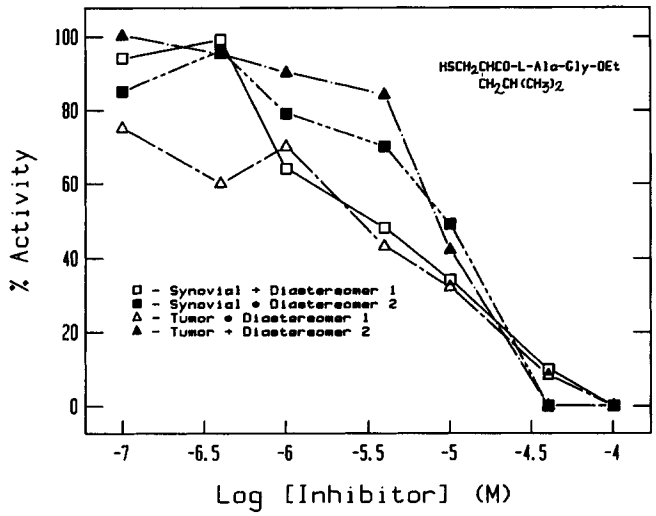

Fig. 2. Estimation of inhibitory potency of $HSCH_2CH[CH_2CH(CH_3)_2]$-CO-Ala-Gly-OEt-1 and $HSCH_2$-$CH[CH_2CH(CH_3)_2]$CO-Ala-Gly-OEt-2 determined from the experiment shown in Figure 1.

TABLE I. Inhibitory Potencies of Thiol Peptides*

		IC_{50} (μM) Collagenase	
		Synovial	V-2 Tumor
I	$HSCH_2CH(CH_3)$CO-Ala-OEt	400	1,000–4,000
II	$HSCH_2CH(CH_2Ph)$CO-Ala-OEt-1[a]	100	100
III	$HSCH_2CH[CH_2CH(CH_3)_2]$CO-Ala-OEt-1	4–10	40
	$HSCH_2CH[CH_2CH(CH_3)_2]$CO-Ala-OEt-2	4–10	40
IV	$HSCH_2CH[CH_2CH(CH_3)_2]$CO-Ala-Gly-OEt-1	1–4	4–10
	$HSCH_2CH[CH_2CH(CH_3)_2]$CO-AlA-Gly-OEt-2	4–10	4–10
V	$HSCH_2CH[CH_2CH(CH_3)_2]$CO-Ala-Gly-Gln-1	4–10	10–40
	$HSCH_2CH[CH_2CH(CH_3)_2]$CO-Ala-Gly-Gln-2	4–10	10–40

*IC_{50} values were estimated after subjecting reaction mixtures to SDS-PAGE as shown in Figure 1 and staining with Coomassie Blue R-250 followed by determining the percentage of $[\alpha 1(I)]_2\alpha 2(I)$ converted to TC^A fragments by densitometry. Where two values are given, IC_{50} is between the two.
[a]The numbers following a particular compound indicate the relative elution time from a C_{18} reversed-phase column for the particular diastereomer.

Fig. 1. Separation of products of collagenase action on collagen by SDS-PAGE. Acid-soluble calf skin collagen (0.25 mg/ml) was incubated at 35°C for 1 hr with 0.04 μg pig synovial collagenase or 0.3 μg V-2 tumor collagenase in the presence of varying concentrations of $HSCH_2CH[CH_2CH(CH_3)_2]$CO- Ala-Gly-OEt (diastereomer 1 at the left) or $HSCH_2CH[CH_2$-$CH(CH_3)_2]$CO-Ala-Gly-OEt (diastereomer 2 at the right). The total reaction volume was 20 μl. In photograph A, lane 1 contained collagen without enzyme; lanes 2–8 contained synovial collagenase plus 0, 0.1, 0.4, 1, 4, 10, 40, and 100 μM inhibitor, respectively; lanes 10–17 contained V-2 collagenase plus 0, 0.1, 0.4, 1, 4, 10, 40, and 100 μM inhibitor. In photograph B, lane 1 contained collagen without enzyme; lanes 2–8 contained synovial collagenase plus 100, 40, 10, 4, 1, 0.4, and 0.1 μM inhibitor; lane 9 contained no inhibitor; lanes 10–16 contained V-2 collagenase plus 100, 40, 10, 4, 1, 0.4 and 0.1 μM inhibitor. Lane 17 contained no inhibitor. In both gels A and B, lanes 18–20 contained compounds unrelated to this report.

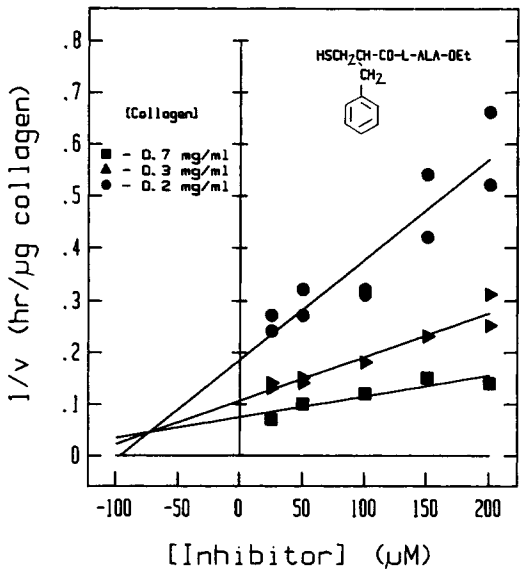

Fig. 3. Dixon plot showing kinetics of degradation of acid-soluble calf skin collagen by pig synovial collagenase in the presence of varying amounts of $HSCH_2CH(CH_2Ph)CO$-Ala-OEt-1. The conditions of the experiment were the same as those given in Figure 1 except that the incubation was for 2 hr. Initial collagen concentrations were (■), 0.7 mg/ml; (▲), 0.3 mg/ml and (●), 0.2 mg/ml.

somewhat more sensitive to $HSCH_2CH[CH_2CH(CH_3)_2]CO$-Ala-OEt and $HSCH_2$-$CH(CH_3)CO$-Ala-OEt than the V-2 enzyme.

Inhibition of pig synovial collagenase by increasing concentrations of $HSCH_2$-$CH(CH_2Ph)CO$-Ala-OEt at three different substrate concentrations was determined in order to ascertain the mode of inhibition by the thiol peptide analogues. The results are shown in the Dixon plot of Figure 3. Inhibition was competitive with nonfibrillar collagen as substrate; the K_i value estimated from these data is approximately 75 μM.

DISCUSSION

These experiments show that collagenases partially purified from two mammalian sources, pig synovium culture medium and rabbit V-2 tumor, are competitively inhibited by thiol-containing peptide analogues of the carboxyl side of the collagenase-sensitive bond in type I collagen. The enzymes prefer a hydrophobic group at the S_1' site, with a isoleucyl analogue preferred over the corresponding phenylalanyl and alanyl analogues. Extension of the peptide chain to encompass the S_2' site provides minimal additional potency (< tenfold). The apparent preference of mammalian collagenases for inhibitors with hydrophobic side chains is in agreement with the recent studies of Weingarten et al [24], who found that human skin fibroblast collagenase demonstrates a preference for lipophilic synthetic peptide and peptide ester substrates.

We have also shown that N-carboxymethyl peptide analogues of the type $HOOCCH(CH_3)$-[NH]-Leu-Ala-NH_2 inhibit the synovial and V-2 tumor collagenases with IC_{50} values in the millimolar range [23]. These analogues are therefore much less effective inhibitors than the analogous thiols reported here. Recently, however, a

more potent N-carboxyalkyl peptide derivative, N-[3-N-(benzyloxycarbonyl) amino-1-(R)-carboxypropyl]-L-leucyl-O-methyl-L-tyrosine N-methylamide, has been reported as a collagenase inhibitor [25]. An IC_{50} value of 0.8 μM was found with human rheumatoid synovial collagenase. Thus, it is possible prepare potent carboxyalkyl inhibitors of collagenase as well as thiols provided hydrophobic side chains are present.

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