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EVIDENCE FOR MAMMALIAN COLLAGENASES AS ZINC ION METALLOENZYMES

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Summary

Collagenases (EC 3.4.24.3) from human skin, rat skin and rat uterus were inhibited by the chelating agents EDTA, 1,10-phenanthroline and tetraethylene pentamine in the presence of excess Ca^{2+}, suggesting that a second metal ion participates in the activity of the enzyme. Collagenase inhibition by 1,10-phenanthroline could be both prevented and reversed by a number of transition metal ions, specifically Zn^{2+}, Co^{2+}, Fe^{2+} and Cu^{2+}. However, Zn^{2+} is effective in five-fold lower molar concentrations ($1 \cdot 10^{-4}$ M) than the other ions. Furthermore, Zn^{2+} was the only ion tested able to prevent and reverse the inhibition of collagenase by EDTA in the presence of excess Ca^{2+}.

Atomic absorption analysis of purified collagenase for Zn^{2+} showed that Zn^{2+} was present in the enzyme preparations, and that the metal co-purifies with collagenase during column chromatography.

Introduction

A previous study from this laboratory on the metal requirements of mammalian collagenases (EC 3.4.24.3) [1] has shown that Ca^{2+} functions both as an enzyme activator and to stabilize the tertiary structure of the enzyme at physiological temperatures. The Ca^{2+} requirement is apparently extrinsic, since the metal is freely exchangeable with the environment.

During the course of these studies, the possibility was raised that these enzymes may contain a second metal more tightly bound within the molecule. The collagenase from *Clostridium histolyticum* has been shown to contain Zn^{2+} as an intrinsic metal in addition to requiring extrinsic Ca^{2+} [2]. The suggestion has been made that a transition metal requirement may exist for a number of vertebrate collagenases, including that from rabbit cornea [3].

Using collagenases from three different mammalian sources, the present study sought to define which divalent cations could prevent or reverse chelator-

dependent inhibition of enzyme activity. The results indicate that Zn^{2+} is the most likely of several transition metal ions tested to be the intrinsic metal in collagenases from human skin, rat skin and rat uterus. Furthermore, atomic absorption spectroscopy of a highly purified human skin collagenase confirms the presence of zinc.

Materials and Methods

Preparation and partial purification of collagenases. Human skin, rat skin and rat uterus collagenase were obtained from tissue culture medium using techniques previously described [1].

Tissue culture medium was lyophilized, dissolved in 0.05 M Tris, pH 7.5 containing 0.005 M Ca^{2+} and partially purified by salt fractionation with ammonium sulfate followed by gel filtration [4]. This method yielded approximately 30-fold purification. In the experiments with chelating agents reported here, only these semi-purified collagenase preparations were used, because sufficient quantities of enzyme purified to homogeneity were not available.

In preparing collagenase from human skin organ culture for atomic absorption spectroscopy, lyophilized culture medium was dissolved in 0.05 M Tris, pH 7.5, 0.005 M Ca^{2+} and fractionated with ammonium sulfate. The precipitate from 30—60% of saturation was chromatographed on CM-cellulose according to Stricklin et al. [5]. Collagenase was eluted with a 0—0.3 M NaCl gradient with the activity contained in a sharp peak at 0.15 M NaCl. At this stage the enzyme has been shown to be purified about 135-fold. Fractions containing collagenase were analyzed directly for zinc.

Atomic absorption Spectroscopy. The collagenase-containing peak from CM-cellulose chromatography was analyzed for Zn^{2+} at 213.9 nm on an Instrumentation Laboratories Model 251 Flame Atomic Absorption Spectrophotometer, using a No. 63141 hollow cathode lamp. In a modification of standard procedures made possible by improvements in this new photometer, samples as small as 100 μl could be employed (personal communication from J.Y. Hwong, Instrumentation Laboratory, Inc., Wilmington, Massachusetts), allowing reliable analysis of as little as 0.3 nmol of metal. Buffer blanks were run for all analyses.

Assay procedures. Collagenase activity was determined by the release of soluble peptides from [^{14}C]glycine-labeled reconstituted collagen fibrils [6,7]. All collagen preparations employed had specific activities of approximately 25000 cpm/mg. Incubation of the fibrils with 0.01% trypsin resulted in the release of less than 10% of the total radioactivity.

A typical reaction mixture consisted of 200 μg [^{14}C]glycine-labeled collagen fibrils (4500 cpm) in a gel volume of 50 μl, and 250—300 μl enzyme solution containing 5—20 μg protein. The samples were incubated for appropriate times at 37° C in a shaken water bath, and the reaction terminated by centrifugation at 12000 $\times g$ for 10 min. The entire supernatant was removed for counting. The volume of the collagen pellet after centrifugation is negligible.

Removal of chelators from enzyme solutions. Free 1,10-phenanthroline, tetraethylene pentamine and EDTA were removed by gel filtration on a 1.2 × 25 cm column of Sephadex G-25 (Pharmacia) equilibrated with 0.05 M Tris ·

HCl, pH 7.5. Since, 1,10-phenanthroline absorbs at 280 nm, and the effluent protein was monitored at the same wave length, it could be shown that the protein peak was completely separated from the inhibitor peak.

Precautions against ionic contamination. All solutions were prepared with doubly glass-distilled water to minimize extrinsic ion contamination. In order to eliminate any Zn^{2+} contamination, buffers were extracted with dithizone [8] and plastic was used instead of glass wherever possible. However, when these precautions were omitted from some experiments, results were identical, showing that Zn^{2+} contamination was negligible.

Highly purified solutions of the transition metals used, as the chloride salts, were obtained from St. Louis Testing Laboratories, St. Louis, Missouri. 1,10-Phenanthroline and EDTA were obtained from Sigma Chemical Co. Tetraethylene pentamine was from Eastman Chemicals.

Results

During the course of a study of the Ca^{2+} requirement of mammalian collagenases, it was found that EDTA inhibited these enzymes in the presence of a large molar excess of Ca^{2+} (Fig. 1). Such inhibition could have been due to a requirement for a second metal, since EDTA has much higher affinities for transition metal ions than for Ca^{2+}. Therefore, experiments were performed to test whether reagents which could chelate only transition metals would affect collagenase activity. 1,10-Phenanthroline and tetraethylene pentamine, both reagents which hve a high affinity for transition metal ions and no measurable affinity for Ca^{2+}, also inhibited human skin collagenase in the presence of excess Ca^{2+}; both inhibitors were maximally effective at concentrations of approximately 1 mM (Fig. 1). Virtually identical inhibition curves were ob-

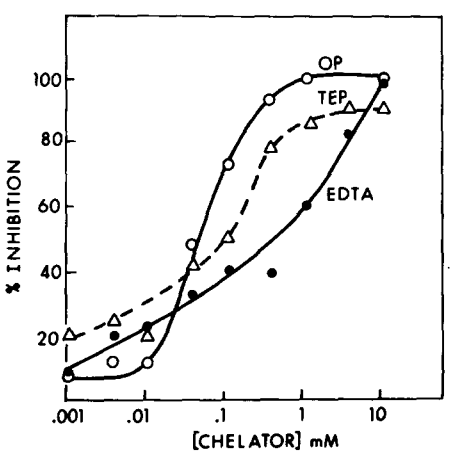

Fig. 1. Inhibition of human skin collagenase by chelating agents. Inhibitors were added at verying concentrations to reaction mixtures containing 0.05 M Tris, pH 7.5, 0.01 M Ca^{2+}, and 50 μl of [^{14}C]collagen in a substrate gel. 5—10 μg of semi-purified human skin collagenase initiated the reaction which was incubated at 37°C for 20 h. ○———○, 1,10-phenanthroline; ●———●, EDTA; △———△, tetraethylene pentamine.

tained when either rat skin or rat uterus collagenase was used as the enzyme source (data not shown).

A number of transition metal ions when added to the incubation mixture completely or partially prevented 1,10-phenanthroline inhibition of these collagenases. In preparations fully inhibited by $6.25 \cdot 10^{-4}$ M 1,10-phenanthroline, the prevention of inhibition was greatest with Zn^{2+} and was concentration dependent, but differed in extent depending upon the enzyme source. For example, $1.25 \cdot 10^{-4}$ M Zn^{2+} completely prevented the inhibition of human skin collagenase by this concentration of 1,10-phenanthroline, but was less than one-third as effective when the rat enzymes were employed. Irrespective of enzyme source, however, at $[Me^{2+}]$ below 10^{-4} M, Zn^{2+} was the only ion which was effective. Co^{2+} and Fe^{2+}, and in the case of rat uterus Cu^{2+}, were effective, but only where their molar concentrations approached that of 1,10-phenanthroline (data not shown). The ability to prevent inhibition was not related to the affinity of 1,10-phenanthroline to these ions in free solution [9]. Furthermore, transition metal ions added after a two hour preincubation at 37°C reversed 1,10-phenanthroline inhibition with the same relative effectiveness and at the same concentrations as they prevented activity loss. Prevention and reversal of tetraethylene pentamine inhibition by metal ions was qualitatively similar to that by 1,10-phenanthroline (not shown).

In contrast to 1,10-phenanthroline inhibition, Zn^{2+} was the only ion of those tested which could prevent or reverse inhibition of EDTA in the presence of excess Ca^{2+} (Table I). Low concentrations of Zn^{2+} ($6.25 \cdot 10^{-4}$ M) prevented any inhibition by $3 \cdot 10^{-3}$ M EDTA of all three collagenases. Furthermore, after a two-hour, 37°C incubation of these enzymes with EDTA, addition of the same low concentrations of Zn^{2+} totally reversed the inhibition. Co^{2+}, Fe^{2+} and Cu^{2+} were tested at concentrations varying from $6 \cdot 10^{-5}$ M to $1.25 \cdot 10^{-3}$ M, and were completely ineffective at preventing or reversing EDTA inhibition. Because Zn^{2+} was most effective at $6.25 \cdot 10^{-4}$ M in these experiments, all of the values shown in Table I are for metal ions added to a concentration of $6.25 \cdot 10^{-4}$ M. In some cases the addition of metal ions to EDTA-inhibited preparations apparently caused slight further inhibition. The reason for this phenomenon is unknown but may be related to the finding that certain metalloenzymes can be inhibited by high concentrations of Zn^{2+} [10].

Since the experiments with chelating inhibitors pointed to Zn^{2+} as the intrinsic metal ion in mammalian collagenases, a highly purified preparation of collagenase from human skin organ culture was subjected to zinc analysis by atomic absorption spectroscopy. As shown in Fig. 2, the peak of Zn^{2+} concentration coincided exactly with the peak of enzymatic activity eluted from CM-cellulose. The ratio of collagenase activity to Zn^{2+} content was constant across the collagenase peak.

The difference in specificity between EDTA and 1,10-phenanthroline led to the hypothesis that although both chelated an intrinsic metal ion in the presence of excess Ca^{2+}, the means of inhibition may be different. That is, one chelator may bind to the metal in the enzyme, forming an enzyme · metal · chelator complex, while the other may remove the intrinsic metal from the active site, forming an apo-enzyme.

One possible means of distinguishing between these alternatives is simply to

TABLE I

PREVENTION AND REVERSAL OF COLLAGENASE INHIBITION BY $3 \cdot 10^{-3}$ M EDTA

Semi-purified collagenase, 5—10 μg/tube was assayed with 25 μl of [^{14}C]collagen in a substrate gel for 20 h. Metals were added to the mixture immediately or after a 2 h preincubation at 37°C. Zn^{2+}, Fe^{2+}, Co^{2+}, and Cu^{2+} were tested at concentrations from $6.25 \cdot 10^{-5}$ M to $1.25 \cdot 10^{-3}$ M. The results given in this table are for [Me^{2+}] at $6.25 \cdot 10^{-4}$ M. Because [Ca^{2+}] always exceeded [EDTA] by $5 \cdot 10^{-3}$ M inhibition was not complete at $3 \cdot 10^{-3}$ M EDTA. % Restoration is calculated as the number of counts restored to partially inhibited enzyme by a given Me^{2+} concentration as a percent of the zero time activity for each enzyme minus the inhibited blank.

Enzyme source	Additions		Restoration of activity			
	Zero time (prevention)	After 2 h at 37°C (reversal)	Zero time (prevention)		After 2 h at 37°C (reversal)	
			cpm	% restoration	cpm	% Restoration
Human skin	None		1450			
	EDTA		835			
	EDTA + Zn^{2+}		1416	97		
	EDTA + Co^{2+}		816	0		
	EDTA + Fe^{2+}		704	0		
	EDTA + Cu^{2+}		722	0		
	None	None			1384	
	EDTA	Zn^{2+}			1380	100
	EDTA	Co^{2+}			809	0
	EDTA	Fe^{2+}			574	0
	EDTA	Cu^{2+}			601	0
Rat skin	None		1240			
	EDTA		787			
	EDTA + Zn^{2+}		1296	104		
	EDTA + Co^{2+}		830	0		
	EDTA + Fe^{2+}		703	0		
	EDTA + Cu^{2+}		541	0		
	None	None			1240	
	EDTA	Zn^{2+}			1298	100
	EDTA	Co^{2+}			602	0
	EDTA	Fe^{2+}			594	0
	EDTA	Cu^{2+}			570	0
Rat uterus	None		846			
	EDTA		420			
	EDTA + Zn^{2+}		933	110		
	EDTA + Co^{2+}		508	18		
	EDTA + Fe^{2+}		307	0		
	EDTA + Cu^{2+}		513	20		
	None	None			846	
	EDTA	Zn^{2+}			863	102
	EDTA	Co^{2+}			433	1
	EDTA	Fe^{2+}			414	0
	EDTA	Cu^{2+}			428	0

dilute inhibited preparations and assay for restoration of activity. A typical experiment is given in Table II, showing that 1,10-phenanthroline inhibition of human skin collagenase was reversible by dilution, but EDTA inhibition was not.

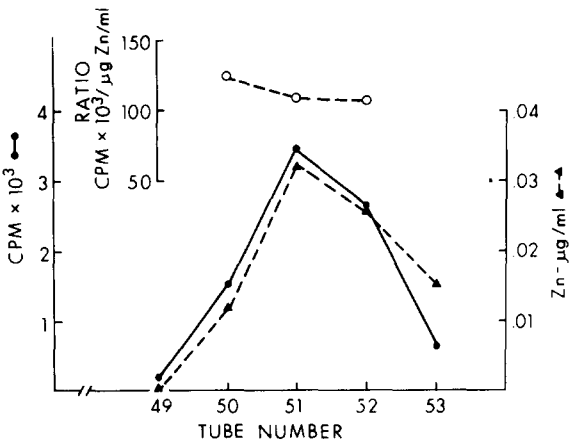

Fig. 2. Atomic absorption spectroscopy analysis for zinc in the collagenase peak from CM-cellulose chromatography. The cpm in the figure are those resulting from assay of 25 µl of each fraction with 125 µl of 0.05 M Tris, pH 7.5, 0.005 M Ca^{2+} for 1 h at 37°C. The µg/ml Zn^{2+} were calculated from a standard cruve, and the appropriate blanks subtracted.

Removal of free inhibitor on Sephadex G-25 again showed differences between 1,10-phenanthroline and EDTA inhibition. Although several pre-treatments of the collagenases with 1,10-phenanthroline and tetraethylene pentamine were tried, including preincubations in $6 \cdot 10^{-4}$ M 1,10-phenanthroline at 37°C and dialysis against 0.01 M 1,10-phenanthroline and tetraethylene pentamine at 4°C overnight, subsequent removal of free chelator always restored full activity.

When, however, the collagenases were inhibited by EDTA, removal of free

TABLE II

EFFECT OF DILUTION ON INHIBITION OF HUMAN SKIN COLLAGENASE BY 1,10-PHENANTHROLINE AND EDTA

Semi-purified preparations of collagenase were incubated with $6 \cdot 10^{-4}$ M 1,10-phenanthroline or $4.6 \cdot 10^{-3}$ M EDTA for 30 min at 37°C. Aliquots were then diluted to the indicated concentrations and assayed for 20 h at 37°C. Calculated values are those expected if the amount of enzyme in the diluted aliquot were fully reactivated at the final inhibitor concentration.

Experiment	cpm observed	cpm calculated if reversible	cpm calculated if not reversible
I. Inhibition by $6 \cdot 10^{-4}$ M 1,10-phenanthroline			
Total activity	1286		
Inhibited	71		
Diluted 1 : 3	255	321	71
Diluted 1 : 10	792	964	71
II. Inhibition by $4.6 \cdot 10^{-3}$ M EDTA			
Total activity	2525		
Inhibited	624		
Diluted 1 : 10	747	1185	624

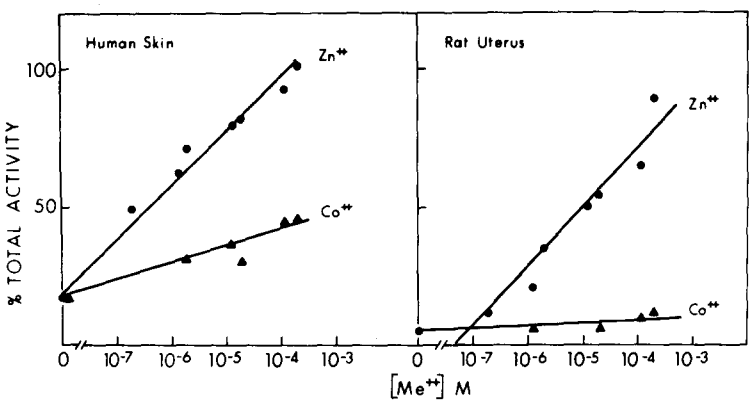

Fig. 3. Restoration by metal ions of activity from EDTA-inhibited preparations of human skin collagenase and rat uterus collagenase. Approximataly 200 μg of enzyme protein was preincubated at 0°C for 15 min with $6 \cdot 10^{-3}$ M EDTA, $5 \cdot 10^{-4}$ M Ca^{2+} before passing through a 28 X 1.2 cm column of Sephadex G-25 equilibrated with 0.05 M Tris, pH 7.5. Ca^{2+} was added to the column eluate to give $10.5 \cdot 10^{-3}$ M Ca^{2+}. Aliquots of the eluate were then incubated with 50 μl gels of [^{14}C]collagen and Me^{2+} at the indicated concentrations for 20 h at 37°C.

chelator resulted in an inactive enzyme. Full activity could then be restored by addition of low concentrations of Zn^{2+} as shown in Fig. 3. Interestingly, only in the case of human skin collagenase did the addition of Co^{2+} restore some enzyme activity in the absence of free EDTA; Fe^{2+} or Cu^{2+} were completely ineffective in these experiments.

Discussion

These studies indicate that collagenases from human skin, rat skin and rat uterus are members of a family of neutral proteases which contain an intrinsic Zn^{2+} in addition to the extrinsic Ca^{2+} [1] required for stabilization. The best characterized of these non-serine proteases are the bacterial endopeptidases such as those from *Bacillus subtilis*, *Bacillus cereus* and thermolysin from *Bacillus thermo-proteolyticus* [11—14]. In one mammalian system, collagenase from rabbit cornea, Berman and Manabe [3] have presented evidence that intrinsic Zn^{2+} is present. Their conclusion is based upon Zn^{2+} reversal of 1,10-phenanthroline and EDTA inhibition. In addition, they found that corneal organ cultures grown in the presence of ^{65}Zn incorporated the isotope into a number of extracellular proteins. Gel filtration of these proteins revealed that ^{65}Zn was present in the area in which collagenase chromatographed.

Our conclusion that the collagenases examined here contain Zn^{2+} rather than another transition metal is based on the finding that this ion was best able to reverse the inhibition of activity by transition metal chelators. In all cases, Zn^{2+} was more effective in reversing inhibition than other ions tested, in spite of the fact that the free-solution affinity of Zn^{2+} for 1,10-phenanthroline and EDTA is low, relative to the other ions. For example, the affinity of 1,10-phenanthroline and Co^{2+} ($pK_1 = 7.02$) is more than five-fold higher than for Zn^{2+} [9], yet Zn^{2+} was approximately 6 times more effective than Co^{2+} in reversing the 1,10-phenanthroline inhibition of all three collagenases studied. Similarly, the affin-

ity of Cu^{2+} is more than 100 times higher than Zn^{2+}, yet Cu^{2+} was completely unable to prevent or reverse 1,10-phenanthroline inhibition of two of the enzymes. Again, although the affinity of Co^{2+} for EDTA is approximately equal to that of Zn^{2+}, and that of Cu^{2+} is 100 times higher, only Zn^{2+} was able to reverse inhibition by this chelator. Of course, caution must always be exercised in drawing firm conclusions based on reversal of inhibition, especially in impure enzyme preparations; it is always possible that free-solution affinities are not applicable to metal ions within a protein molecule. Nevertheless, it appears unlikely that three different inhibitors could all be subject to an identical artifact.

The conclusion that Zn^{2+} is present as a co-factor in mammalian collagenases is reinforced by studies in which enzyme preparations were assayed directly for zinc. As shown in Fig. 2, when human skin collagenase was subjected to chromatography on CM-cellulose, and the highly purified enzyme analyzed by atomic absorption spectroscopy, Zn^{2+} was found to co-chromatograph with the enzyme. In addition, the ratio of Zn^{2+} to activity was constant across the enzyme peak, a further indication of the coincidence of metal ion and collagenase. Further studies to determine exact stoichiometry in homogeneous preparations are in progress.

The results of the dilution and gel filtration experiments suggest the possibility that 1,10-phenanthroline and probably tetraethylene pentamine inhibit mammalian collagenase by binding to the Me^{2+} at the active site, while EDTA might remove the Me^{2+} resulting in the formation of an apo-enzyme. Results from dilution experiments must be interpreted with much caution [15]. Nevertheless, if it is true that treatment of collagenases with EDTA creates an apoenzyme, then the activity of human skin collagenases with added Co^{2+} shown in Fig. 3 could mean that a Co^{2+} analog of the enzyme has been produced. If so, it would be most interesting to investigate the kinetics of such an enzyme, since Co^{2+} analogs of other Zn^{2+}-containing neutral proteases have yielded interesting enzymological data [16]. In addition, the fact that only the collagenase from human skin appears to be able to use Co^{2+} in its active site emphasizes, as has immunologic evidence [17], that species differences can exist between collagenases.

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