Zinc metalloenzyme properties of active and latent collagenase from rabbit bone

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1. Inhibition of collagenase from rabbit bone cultures by the chelating agents 1,10-phenanthroline and EDTA is almost completely reversed by Zn^{2+} ; other metal cations are less effective in reversing the inhibition. Optimal restoration of activity is achieved at Zn^{2+} concentrations below that of the chelator, but excess of Zn^{2+} is inhibitory. 2. Prolonged incubation of collagenase with either chelator causes irreversible inactivation. This inactivation is prevented by Zn^{2+} at the same concentrations needed to reverse the primary inhibition. 3. Collagenase incorporates ^{65}Zn by exchange when incubated with 1,10-phenanthroline and Zn^{2+} containing this radioactive isotope. The $^{65}\text{Zn}^{2+}$ can be removed from its binding site in collagenase by 1,10-phenanthroline or EDTA. Irreversible inactivation of collagenase by chelators destroys its ability to incorporate $^{65}\text{Zn}^{2+}$. 4. Latent collagenase, the inhibited form in which collagenase first appears in culture, behaves similarly to the active enzyme in $^{65}\text{Zn}^{2+}$ -exchange experiments, but is resistant to irreversible inactivation by chelators. 5. It is concluded that collagenase is a zinc metalloenzyme that forms an inactive and unstable apoenzyme on treatment with chelators. The bound inhibitor component of latent collagenase evidently stabilizes the apoenzyme.

Collagenases synthesized in culture by mammalian tissues display a dependence on metal-ion cofactors and a tendency to be secreted in a latent form (for a review see Harris & Cartwright, 1977). One essential metal ion is Ca^{2+} , required as an extrinsic activator and stabilizer of collagenase (Tokoro *et al.*, 1972; Seltzer *et al.*, 1976). Several mammalian collagenases also contain a firmly bound intrinsic metal-ion cofactor, evidently Zn^{2+} (Berman & Manabe, 1973; Seltzer *et al.*, 1977). Although it is not certain that the Zn^{2+} functions within the active site, this seems likely by analogy with other zinc metalloproteinases, carboxypeptidases A and B (Hartsuck & Lipscomb, 1971; Schmid & Herriott, 1976) and thermolysin (Matthews *et al.*, 1974), which have been analysed by X-ray crystallography.

Latency of collagenase appears to involve an inhibitory protein component that is removed to form the active enzyme, activation being accom-

panied by a decrease in molecular weight (Sellers *et al.*, 1977). Proteolytic enzymes have been most widely used for activation (Vaes, 1972; Werb *et al.*, 1977; Vaes *et al.*, 1978). However, some latent collagenases can be activated by non-proteolytic agents, e.g. chaotropic salts (Nagai *et al.*, 1975; Shinkai & Nagai, 1977) or organic mercurial compounds (Sellers *et al.*, 1977; Morales *et al.*, 1978), suggesting that the collagenase and inhibitor components, though forming a tight complex, might not be peptide-linked as in a proenzyme.

Collagenases are difficult to purify in sufficient quantities for physicochemical characterization. Gel-filtration chromatography has generally been possible, but seems to yield anomalous molecular weights with these enzymes (Sellers *et al.*, 1977; Stricklin *et al.*, 1978). Thus the chemistry of interaction between components of latent collagenases is poorly understood, and the probable zinc metalloenzyme character invites a new approach to eliciting structural details by investigating the properties of the essential Zn^{2+} in the active and latent forms. The collagenase of rabbit bone appeared to be suitable for a study of this kind, as previous work in this laboratory has shown it to be a metalloenzyme

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(Sellers *et al.*, 1978), occurring as a latent complex with an inhibitory protein synthesized in the early stages of growth in culture (Sellers & Reynolds, 1977; Sellers *et al.*, 1977). In the present paper we show that the essential metal ion in rabbit bone collagenase is Zn^{2+} , which can be detached by chelating agents to form an apoenzyme or exchanged for radioactive $^{65}\text{Zn}^{2+}$. We report on similarities and differences between active and latent collagenase in the enzyme– Zn^{2+} interaction and the stability of the apoenzyme.

Materials and methods

Materials

Chemicals were purchased from the following suppliers: $^{65}\text{ZnCl}_2$ ($2.6 \times 10^4 \text{ Ci/mol}$) and *N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate ($1.6 \times 10^6 \text{ Ci/mol}$) from The Radiochemical Centre (Amersham, Bucks., U.K.); Ultrogel AcA-54 from LKB Instruments (Croydon, Surrey, U.K.); Sephadex G-25 from Pharmacia (London W.5, U.K.); Chelex-100 from Bio-Rad Laboratories (St. Albans, Herts., U.K.); 1,10-phenanthroline and Tris (Trizma grade) from Sigma Chemical Co. (Poole, Dorset, U.K.); Picofluor liquid scintillant from Packard Instrument Co. (Wembley, Middx., U.K.); Brij-35 (polyoxyethylene lauryl ether) from BDH Chemicals (Poole, Dorset, U.K.); 4-aminophenylmercuric acetate from Aldrich Chemical Co. (Gillingham, Dorset, U.K.); EDTA (trisodium salt) from Hopkin and Williams (Chadwell Heath, Essex, U.K.). Salts of calcium, magnesium and transition metals were analytical-reagent-grade chlorides or sulphates. Solutions were prepared in double-glass-distilled water.

Collagenase

Rabbit bone culture medium (Sellers *et al.*, 1977) was fractionated (Cawston & Tyler, 1979) to give 200-fold purified collagenase, activated with 4-aminophenylmercuric acetate. The purified enzyme was free of other metalloproteinases produced by the cultures (Sellers *et al.*, 1978). Latent collagenase was fractionated only through the initial gel-filtration stage, a 10-fold purification, to avoid spontaneous activation during isolation (Cawston & Tyler, 1979).

Preincubation and assay of collagenase

Preincubations before assay or chromatography of collagenase were in a 0.1 M-Tris buffer adjusted with HCl at 20–25°C to a pH that would become 7.6 at 37°C, and contained 15 mM- CaCl_2 , 0.05% Brij-35 and 0.02% NaN_3 . Collagenase activity was measured on [^{14}C]acetylated rat skin collagen (Cawston & Barrett, 1979) by the release of soluble radioactive products from thermally reconstituted fibrils (Sellers & Reynolds, 1977). All incubation

mixtures contained 83 mM-Tris/HCl buffer, pH 7.6 (37°C), 67 mM- NaCl (200–400 mM when fractions from chromatography were assayed), 10 mM- CaCl_2 , 0.033% (w/v) Brij-35, 0.02% NaN_3 and ^{14}C -labelled collagen (100–110 μg). Samples were incubated in duplicate for 20 h; in a few cases where quadruplicate assays were run the standard deviation was 2.6% of the mean. For some assays, solutions were treated to remove possible traces of heavy-metal ions either by passage through a Chelex-100 column or by extraction with 0.01% dithizone in CCl_4 , but no significant differences were found in comparison with results obtained with buffer solutions that had not been subjected to rigorous purification.

$^{65}\text{Zn}^{2+}$ incorporation into collagenase by exchange

Collagenase was incubated for 2 h at 37°C with 0.5 mM-1,10-phenanthroline and 0.1 mM- $^{65}\text{Zn}^{2+}$ (specific radioactivity 1 mCi/ μmol) in the preincubation buffer described above. The incubation mixture (0.5 ml) was then dialysed against $2 \times 200 \text{ vol.}$ of the preincubation buffer alone for 24 h each at 2°C before chromatography.

Chromatography

Dialysed incubation mixtures (0.5–0.6 ml) from $^{65}\text{Zn}^{2+}$ -exchange experiments were chromatographed at 20–25°C on an Ultrogel AcA-54 column (30 cm $\times$ 1.5 cm); 2.05 ml fractions were collected at 10.5 ml/h. In experiments to measure loss of $^{65}\text{Zn}^{2+}$ from labelled collagenase, samples (0.5 ml) were chromatographed at 20–25°C on a Sephadex G-25 column (15 cm $\times$ 0.9 cm) at 15 ml/h, and 0.7 ml fractions were collected. The eluent in all experiments was 1 M- NaCl in the preincubation buffer described above.

Measurement of ^{65}Zn radioactivity and quantification of chromatography

Radioactivities in fractions from Ultrogel AcA-54 chromatography were measured by solid-scintillation counting in a Packard Prias γ -radiation counter. The efficiency for ^{65}Zn was only 3.5%, but samples could be recovered intact, thus allowing the radioactivity of entire 2.05 ml fractions to be counted. Liquid-scintillation counting of radioactivity was used for fractions from Sephadex G-25 chromatography, as the amounts of ^{65}Zn were much lower in these experiments. Samples (0.4 ml) were mixed with 0.4 ml of water and 5.0 ml of Picofluor scintillant; the ^{65}Zn radioactivity could then be counted with 44% efficiency by using settings recommended for ^3H on a Packard Prias β -radiation counter.

The background radioactivity count rate in chromatographic experiments was determined from the first five or six fractions, at least two clear fractions before the emergence of any material eluted at the void volume. The sum of background-

corrected radioactivity count rates for the five (Sephadex G-25) or six (Ultrogel AcA-54) fractions corresponding to maximum collagenase activity gave a measure of collagenase-bound ⁶⁵Zn²⁺.

Results

Inhibition of rabbit bone collagenase by chelators, and re-activation by metal ions

Lysis of collagen fibrils in the collagenase assay was blocked completely by either 1 mM-1,10-phenanthroline or 10 mM-EDTA, and partially by lower concentrations of these chelating agents (Table 1). In subsequent experiments 1,10-phenanthroline was used at 0.5 mM and EDTA at 5 mM. By thus allowing low but detectable extents of fibril lysis it was easier to compare the effectiveness of different ways of re-activating collagenase. Furthermore, it was desirable to maintain an excess of Ca²⁺ over EDTA to ensure that possible re-activation was not prevented by depletion of Ca²⁺.

Table 1 also shows the relative abilities of various metal cations to prevent the inhibition when present in the incubation at a molar ratio of 1:5 relative to the chelating agent. It is apparent that Zn²⁺, which gave nearly complete restoration of activity, was far more effective than the other metal ions in preventing inhibition. When the metal ions were added at the higher molar ratio of 4:5 relative to chelator, collagenase activity was higher in most cases. Nevertheless the highest relative activities observed with any other metal ion (56% for Co²⁺ + 1,10-phenanthroline, and 60% for Co²⁺ + EDTA) were

still below that given by Zn²⁺ at the lower molar ratio.

Dependence of collagenase activity on concentration of Zn²⁺

Although Zn²⁺ at low concentration prevented inhibition of collagenase by 0.5 mM-1,10-phenanthroline or 5 mM-EDTA, it became inhibitory at higher concentration, despite the chelator still being in excess (Figs. 1a and 1b). Incubation mixtures containing chelator with a less than equimolar amount of Zn²⁺ comprise Zn²⁺-ion buffers for which the pZn (i.e. -log [free Zn²⁺]) can be calculated for the appropriate pH and total concentrations of chelator, Zn²⁺ and Ca²⁺ (Reilley, 1961; Perrin & Dempsey, 1974) by using the stability constants for the Zn²⁺-chelator complexes (O'Sullivan, 1968). Thus we also show in Figs. 1(a) and 1(b) calculated values of pZn to illustrate how collagenase activity correlates with this parameter.

With 1,10-phenanthroline, Zn²⁺ forms three complexes of different stoicheiometry (O'Sullivan, 1968), and hence pZn changes more sharply with changes in concentration of total zinc in solutions of 1,10-phenanthroline than in EDTA. This is probably why the range of concentrations of total zinc giving optimal collagenase activity is narrow for 1,10-phenanthroline (Fig. 1a) but comparatively wide for EDTA (Fig. 1b). The optimum pZn for collagenase appears to be about 1.5 units lower in the presence of 0.5 mM-1,10-phenanthroline than in the presence of 5 mM-EDTA. Such a shift could arise from an interaction of either chelator with the enzyme or

Table 1. *Inhibition of collagenase by chelators, and re-activation by metal cations*

Reaction mixtures were incubated for 20h at 35°C in the standard fibril assay (see the Materials and methods section). Collagenase activity is expressed as percentage of control (58% fibril lysis in the absence of added effectors). The concentration of added metal ions was 0.1 mM in experiments with 1,10-phenanthroline, and 1.0 mM in experiments with EDTA; Ca²⁺ was not included among the metal ions tested because all incubation mixtures contained 10 mM-CaCl₂.

Added metal ion	1,10-Phenanthroline		EDTA	
	Concn. of chelator (mM)	Collagenase activity (%)	Concn. of chelator (mM)	Collagenase activity (%)
None	0	100	0	100
None	0.2	11	2.0	37
None	0.5	2	5.0	24
None	1.0	0	10.0	0
Zn ²⁺	0.5	89 (85-104)*	5.0	97 (85-115)*
Co ²⁺	0.5	17	5.0	33
Ni ²⁺	0.5	16	5.0	26
Cu ²⁺	0.5	0	5.0	31
Fe ²⁺	0.5	6	5.0	21
Fe ³⁺	0.5	0	5.0	26
Mg ²⁺	0.5	1	5.0	29
Mn ²⁺	0.5	0	5.0	20

* Range for five series of experiments with different samples of collagenase.

Fig. 1. Effect of concentration of Zn^{2+} on activity of chelator-inhibited collagenase

Reaction mixtures were incubated for 20 h at $37^{\circ}C$ in the standard fibril assay (see the Materials and methods section). Collagenase activity is expressed as percentage of control (49% fibril lysis in the absence of added effectors). (a) 0.5 mM-1,10-phenanthroline present in incubation mixture; (b) 5 mM-EDTA present in incubation mixture. ●, Collagenase activity; ·····, pZn (calculated values of $-\log [free\ Zn^{2+}]$; see the text).

from a difference in the concentration of free Ca^{2+} , which would be lower in solutions containing 5 mM-EDTA. However, it is not certain that this apparent difference in pZn is significant, as the calculated values, based on stability constants measured at a different temperature ($25-30^{\circ}C$) and different buffer composition from the assay incubation mixtures, are only an approximate indication of the true pZn of the solutions.

In the absence of any added chelating agent, Zn^{2+} at concentrations of 0.33 mM and 1 mM respectively decreased the activity of collagenase to 28% and 6% of that of an untreated control. This inhibition by Zn^{2+} could be reversed by 1,10-phenanthroline or EDTA.

Reversible and irreversible inhibition of collagenase by chelators

Preincubation of collagenase with 1,10-phenanthroline or EDTA before addition of Zn^{2+} de-

Fig. 2. Reversible and irreversible inhibition of collagenase by 1,10-phenanthroline

Incubation mixtures prepared as for the standard fibril assay were incubated at $37^{\circ}C$. Collagenase was added at 0 h to start the reaction; effectors (final concentrations in the incubation mixture given below) were added at the times shown below. The values in parentheses are rates of fibril lysis measured from the linear regression slopes of the corresponding sets of data and expressed as μg of collagen digested/h. ●, Control, no added effectors (9.5); ■, 0.5 mM-1,10-phenanthroline at 0 h (0.5); ▲, 0.5 mM-1,10-phenanthroline + 0.1 mM- Zn^{2+} both at 0 h (8.7); ▼, 0.5 mM-1,10-phenanthroline at 0 h, 0.1 mM- Zn^{2+} at 6 h (3.7, measured from time of addition of Zn^{2+}).

creased the extent to which inhibition could be reversed. Fig. 2 shows results with 1,10-phenanthroline as inhibitor. More collagenase was used than in the standard (20 h) fibril assay, thereby giving measurable lysis within 1–2 h. After incubation for 6 h at $37^{\circ}C$ with 0.5 mM-1,10-phenanthroline, during which time collagenase was strongly inhibited, activity was restored by adding Zn^{2+} in optimal amounts (i.e. to give a concentration of 0.1 mM). However, the rate of fibril lysis by collagenase re-activated in this way was less than when Zn^{2+} was added together with the chelator at the start of incubation (Fig. 2). Results were similar for inhibition by 5 mM-EDTA with regeneration of activity at 1 mM- Zn^{2+} . The rates of fibril lysis, expressed as μg of collagen digested/h, calculated by linear regression on data spaced at the same time intervals as those in Fig. 2, were: control, 9.5; EDTA added, 2.6; EDTA + Zn^{2+} added together at zero time, 9.3; Zn^{2+} added 6 h after EDTA and rate measured from time of Zn^{2+} addition, 6.1. We conclude that both 1,10-phenanthroline and EDTA

produce not only a rapid inhibition, reversible by Zn^{2+} , but also a slower, irreversible, inactivation.

The irreversible inactivation displayed first-order kinetics, giving linear semi-logarithmic plots of residual collagenase activity against time of preincubation with either chelator. This was established by stopping the inactivation after various times of preincubation by addition of Zn^{2+} to make its final concentration 0.1 mM in solutions containing 0.5 mM-1,10-phenanthroline or 1 mM in solutions containing 5 mM-EDTA. These concentrations of Zn^{2+} and chelator were then maintained in 20 h assay incubations. In preincubations with 0.5 mM-1,10-phenanthroline, collagenase was 50% inactivated in 4 h at 37°C or 15 h at 25°C, but retained 82% of its activity after 72 h at 2°C. When 0.1 mM- Zn^{2+} was present in the preincubation with 1,10-phenanthroline there was negligible irreversible loss of activity in 72 h even at 25°C or 37°C. With 5 mM-EDTA, 50% inactivation of collagenase occurred in 6 h at 37°C or in 40 h at 25°C. No loss of activity was observed in 72 h at 2°C, nor if 1 mM- Zn^{2+} was present with the EDTA in preincubations at 25°C or 37°C. Thus Zn^{2+} , at concentrations that can prevent reversible inhibition by 1,10-phenanthroline or EDTA, also completely protects collagenase against irreversible inhibition by these chelators. The stability of collagenase was even enhanced in comparison with that of a control sample, which when preincubated in the absence of added chelators or metal ions retained 78% of its activity after 72 h at 37°C and 88% after 72 h at 25°C.

The possibility of proteolytic degradation being the cause of irreversible inactivation was investigated by ^{125}I -labelling a sample of collagenase that had been inactivated by incubation at 37°C for 24 h with 0.5 mM-1,10-phenanthroline. Labelling was with *N*-succinimidyl-3-(4-hydroxy-5- ^{125}I iodophenyl)-propionate as described by Cawston & Tyler (1979), after dialysis against the cacodylate buffer described by these authors. (Tris interferes with the iodination.) An active collagenase control sample was similarly labelled. The two samples when examined by electrophoresis and radioautography (Cawston & Tyler, 1979) showed similar amounts of protein-bound ^{125}I , indicating no proteolytic degradation of the inactivated sample.

Incorporation of $^{65}\text{Zn}^{2+}$ into collagenase

The interaction of Zn^{2+} with collagenase was investigated by incubating the enzyme with 1,10-phenanthroline and radioactive $^{65}\text{Zn}^{2+}$, then dialysing and chromatographing on Ultrogel AcA-54 (see the Materials and methods section). Fig. 3 shows that ^{65}Zn radioactivity was eluted at the same point as collagenase activity, demonstrating that Zn^{2+} from the solution had bound to collagenase. The only other detectable ^{65}Zn was eluted after the column volume and was probably a small amount of Zn^{2+} complex of 1,10-phenanthroline not removed by dialysis.

In Fig. 3 the main radioactivity peak represents $^{65}\text{Zn}^{2+}$ remaining bound to collagenase after dialysis

Fig. 3. *Ultrogel AcA-54 chromatography of $^{65}\text{Zn}^{2+}$ -collagenase*

Collagenase was incubated with 1,10-phenanthroline and Zn^{2+} (containing $^{65}\text{Zn}^{2+}$), dialysed and chromatographed (sample volume 0.5 ml) on an Ultrogel AcA-54 column (30 cm $\times$ 1.5 cm) as described in the Materials and methods section. ^{65}Zn radioactivity was measured by γ -radiation (solid-scintillation) counting on entire 2.05 ml fractions. Collagenase activity was measured on 60 μl samples incubated for 20 h at 35°C in the standard fibril assay. ▲, ^{65}Zn radioactivity; ●, collagenase activity. The elution volumes of the following marker substances are shown: V_0 , Blue Dextran; 45 K, ovalbumin; 25 K, soya-bean trypsin inhibitor; V_t , potassium ferricyanide; Zn(OP)_3 , 1,10-phenanthroline containing Zn^{2+} (3:1 molar ratio).

followed by chromatography with no $^{65}\text{Zn}^{2+}$ in the eluting buffer. This suggests that the $^{65}\text{Zn}^{2+}$ was incorporated by exchange into a high-affinity binding site for Zn^{2+} in the enzyme, rather than non-specifically bound to exposed residues such as histidine. The necessity for the native structure of collagenase to be intact to retain affinity for Zn^{2+} was confirmed by measuring the incorporation of $^{65}\text{Zn}^{2+}$ into samples of the enzyme that had been preincubated at 37°C with 0.5 mM-1,10-phenanthroline. Total irreversible inactivation by preincubation resulted in total absence of protein-bound $^{65}\text{Zn}^{2+}$ from the eluate from the gel column in an experiment analogous to that giving the results shown in Fig. 3. In three samples that had undergone partial irreversible inactivation, intermediate amounts of $^{65}\text{Zn}^{2+}$ were detected at the elution volume of collagenase. These samples, which retained 82%, 47% and 4% of the activity of an untreated collagenase control of the same concentration, showed extents of collagenase-bound $^{65}\text{Zn}^{2+}$ relative to the control of 99%, 17% and 10% respectively. Although the correlation is only approximate, discrepancies probably reflect the difficulty of quantifying collagenase-bound $^{65}\text{Zn}^{2+}$, as incomplete recovery of sample and partial dissociation of enzyme-bound Zn^{2+} might occur during dialysis and chromatography. We conclude therefore that, during preincubation with 1,10-phenanthroline, binding sites for Zn^{2+} in collagenase are destroyed at the same time as functional active sites, and that in the exchange incubations $^{65}\text{Zn}^{2+}$ is incorporated into these binding sites.

An incubation time of 2 h for $^{65}\text{Zn}^{2+}$ exchange proved to be optimal. By comparison, a 1 h incubation gave only about 60% of the amount of collagenase-bound $^{65}\text{Zn}^{2+}$ in the column eluate, whereas increasing the incubation time to 24 h gave no greater incorporation of $^{65}\text{Zn}^{2+}$ than at 2 h but caused substantial loss of collagenase activity. We believe that this slow inactivation of collagenase without corresponding loss of $^{65}\text{Zn}^{2+}$ -binding properties was caused by Cu^{2+} , as analytical information from the suppliers of the $^{65}\text{ZnCl}_2$ showed the principal contaminant to be Cu^{2+} present at a molar ratio of 1:10 relative to Zn^{2+} . Furthermore, in an experiment where $^{65}\text{Zn}^{2+}$ was omitted but 0.01 mM- Cu^{2+} was included in an incubation with 0.5 mM-1,10-phenanthroline + 0.1 mM- Zn^{2+} , collagenase was totally inactivated in 24 h at 37°C .

Detachment of $^{65}\text{Zn}^{2+}$ from labelled collagenase

The extent to which $^{65}\text{Zn}^{2+}$ became detached from labelled collagenase under various conditions was studied by using pooled eluate fractions containing the highest concentrations of $^{65}\text{Zn}^{2+}$ -collagenase from Ultrogel AcA-54 chromatography. Samples were dialysed against the preincubation buffer

described above (containing no $^{65}\text{Zn}^{2+}$) at 2°C , incubated under the chosen conditions, then immediately chromatographed on Sephadex G-25 (see the Materials and methods section). Collagenase-bound $^{65}\text{Zn}^{2+}$ was eluted at the void volume whereas $^{65}\text{Zn}^{2+}$ that had become detached was eluted at or after the total column volume. As shown in Table 2, inhibitory concentrations of chelators caused rapid loss of $^{65}\text{Zn}^{2+}$ from labelled collagenase at 37°C , 0.5 mM-1,10-phenanthroline acting faster than 5 mM-EDTA. Even at $0-2^\circ\text{C}$ the effect of 1,10-phenanthroline was significant after 24 h. The appreciable loss of $^{65}\text{Zn}^{2+}$ in 1 h at 37°C without added chelators possibly represents exchange with traces of Zn^{2+} in the buffer, rather than dissociation, because, as described above, collagenase loses activity only very slowly at 37°C in the absence of chelators.

Experiments on latent collagenase

We conducted on latent collagenase experiments analogous to those on the active enzyme, though requiring assay incubations both with and without the inclusion of 0.67 mM-4-aminophenylmercuric acetate.

Inhibition by chelators. For latent enzyme assayed in the presence of 4-aminophenylmercuric acetate the activity was decreased by 1,10-phenanthroline (0.5 mM) and EDTA (5 mM) to 0% and 22% respectively relative to a control without added chelators. Comparison with the results on active collagenase (Table 1) thus shows that 4-aminophenylmercuric acetate does not interfere with the inhibitory action of chelators.

Re-activation by Zn^{2+} . Latent enzyme remained latent (i.e. displayed no activity in the absence of 4-aminophenylmercuric acetate) when Zn^{2+} was present at 0.1 mM with 0.5 mM-1,10-phenanthroline or at 1 mM with 5 mM-EDTA. However, 4-amino-

Table 2. Detachment of $^{65}\text{Zn}^{2+}$ from $^{65}\text{Zn}^{2+}$ -collagenase by various treatments

Samples were incubated in Tris buffer, pH 7.6 (see the Materials and methods section), then immediately chromatographed on a Sephadex G-25 column (15 cm $\times$ 0.9 cm). Loss of $^{65}\text{Zn}^{2+}$ from collagenase was taken as the decrease in radioactivity eluted at the void volume (see the text).

Conditions of incubation	Loss of $^{65}\text{Zn}^{2+}$ (%)
Control, (no incubation)	0
1 h, 37°C , no added effectors	20
1 h, 37°C , 0.5 mM-1,10-phenanthroline	68
2 h, 37°C , 0.5 mM-1,10-phenanthroline	95
2 h, 37°C , 5 mM-EDTA	70
24 h, $0-2^\circ\text{C}$, no added effectors	0
24 h, $0-2^\circ\text{C}$, 0.5 mM-1,10-phenanthroline	33

phenylmercuric acetate restored the activities to 80% and 81% of the control respectively. Hence Zn^{2+} and chelators do not interfere with the action of 4-aminophenylmercuric acetate.

Irreversible inactivation. Latent collagenase was much more resistant than the active form to irreversible inactivation by chelators. The following incubations failed to bring about any irreversible inactivation of latent enzyme: 72 h at 25°C or 24 h at 37°C with 0.5 mM-1,10-phenanthroline; 72 h at 25°C or 37°C with 5 mM-EDTA. After each of the above treatments latent collagenase, when assayed in the presence of 4-aminophenylmercuric acetate and optimal concentrations of Zn^{2+} , was as active as a control sample stored at 2°C without added chelators. The only incubation to produce irreversible inactivation was 72 h at 37°C with 0.5 mM-1,10-phenanthroline, which decreased the activity to 36% of the control.

Incorporation of $^{65}\text{Zn}^{2+}$ by exchange. $^{65}\text{Zn}^{2+}$ became bound to latent collagenase under the exchange incubation conditions described above. The elution profile on Ultrogel AcA-54 showed a ^{65}Zn peak coincident with that of latent collagenase activity. The elution volume was slightly less than that observed in experiments with active collagenase, consistent with the higher molecular weight of the latent form (Sellers *et al.*, 1977). The latent collagenase sample was only partially purified, and hence protein-bound $^{65}\text{Zn}^{2+}$ also appeared in some fractions containing no latent collagenase activity. There was slight overlap between the latent $^{65}\text{Zn}^{2+}$ -collagenase peak and $^{65}\text{Zn}^{2+}$ bound to a species of higher molecular weight, probably gelatinase (Sellers *et al.*, 1978), which introduced some uncertainty into quantification of $^{65}\text{Zn}^{2+}$ bound to latent collagenase. Nevertheless the ratio of ^{65}Zn radioactivity to collagenase activity (with 4-aminophenylmercuric acetate present) at the peak appeared similar to that found in experiments with active enzyme.

Detachment of $^{65}\text{Zn}^{2+}$ from labelled latent enzyme. Latent $^{65}\text{Zn}^{2+}$ -collagenase incubated for 2 h at 37°C with 0.5 mM-1,10-phenanthroline, then chromatographed on Sephadex G-25, showed 96% loss of $^{65}\text{Zn}^{2+}$. From the close similarity in results for latent and active enzyme in this and the $^{65}\text{Zn}^{2+}$ -incorporation experiments, we conclude that the Zn^{2+} -binding sites in latent and active forms of collagenase must be similar.

Discussion

Chelating agents can inhibit a metalloenzyme either by binding to the enzyme through the metal ion to form an inactive complex, or by detaching the functional metal ion to produce an inactive apoenzyme (Vallee, 1960). Our results suggest that rabbit bone collagenase forms an apoenzyme on

treatment with either 1,10-phenanthroline or EDTA, and that the functional metal ion removed is Zn^{2+} . The first line of evidence for this is the protection provided against the chelators by low concentrations of Zn^{2+} . Activity is almost completely restored despite the presence of excess free chelator at concentrations shown to be strongly inhibitory in the absence of added Zn^{2+} . Thus Zn^{2+} apparently re-activates collagenase, not by removal of chelator, but by maintaining a sufficient concentration of free Zn^{2+} to ensure that the dissociation equilibrium favours the Zn^{2+} -containing holoenzyme rather than the inactive apoenzyme. Further support for reversible dissociation of Zn^{2+} from collagenase is provided by the $^{65}\text{Zn}^{2+}$ -exchange experiments demonstrating that collagenase readily incorporates $^{65}\text{Zn}^{2+}$ from the incubation medium. The subsequent rapid removal by chelators of collagenase-bound $^{65}\text{Zn}^{2+}$ also indicates that these agents inhibit by forming an apocollagenase.

Purified collagenase from rabbit bone could not be obtained in sufficient amounts to enable analysis for zinc by atomic absorption spectrophotometry. Nevertheless our results provide strong evidence that Zn^{2+} is the essential metal ion in the rabbit bone enzyme, because restoration of activity to collagenase inhibited by chelators is almost complete at appropriate concentrations of added Zn^{2+} . Various other metal ions give comparatively feeble re-activation. Furthermore, although it is not known whether the binding site for Zn^{2+} in collagenase is at the active site, the two must be closely related because brief incubation with chelators causes both inhibition and loss of bound Zn^{2+} , and in prolonged incubations irreversible inactivation is accompanied by destruction of binding sites for Zn^{2+} . Presumably once the essential Zn^{2+} is detached a slow irreversible conformational change affects the ligands at its binding site. Our results indicate that latent collagenase is stabilized by its bound inhibitor protein against this conformational change.

Fig. 4 shows a speculative model to account for the following properties of active and latent collagenase observed in the present study. (1) Collagenase contains an essential Zn^{2+} ion that becomes detached by chelators to produce an inactive apoenzyme. (2) Collagenase is inhibited by excess of Zn^{2+} , suggesting a second binding site for Zn^{2+} , in this case an inhibitory site. Whether this inhibitory site exists in latent collagenase is unknown because assays require its conversion into the active form. (3) Apocollagenase is unstable and undergoes slow transition to a form that we could not re-activate and that we termed irreversibly inactivated. (4) The Zn^{2+} in latent collagenase can also be detached or exchanged for $^{65}\text{Zn}^{2+}$; thus a latent apocollagenase can be formed. (5) Latent apocollagenase is much more resistant than apocollagenase to irreversible

Fig. 4. Model for effects of chelators and Zn^{2+} on rabbit bone collagenase

inactivation, indicating that the inhibitory portion plays a stabilizing role.

Other mammalian collagenases have been shown to be zinc metalloproteinases (Berman & Manabe, 1973; Seltzer *et al.*, 1977), displaying inhibition by chelators and re-activation by Zn^{2+} similar to the effects reported in the present paper. However, irreversible inactivation of collagenase by incubation with chelating agents in the presence of excess of Ca^{2+} has not previously been reported. Conclusions about the mechanism of inhibition by chelating agents of collagenases from rabbit cornea (Berman & Manabe, 1973), human skin, rat skin and rat uterus (Seltzer *et al.*, 1977) were that EDTA treatment produced apocollagenase but that 1,10-phenanthroline inhibited by binding to the enzyme. Our findings on the effect of 1,10-phenanthroline on rabbit bone collagenase, however, resemble those obtained by Feder *et al.* (1971) on thermolysin. The preparation of a collagenase incorporating $^{65}Zn^{2+}$ has been achieved previously by growing rabbit corneal cultures in the presence of $^{65}Zn^{2+}$ (Berman & Manabe, 1973). Although this was a convincing demonstration of the presence of Zn^{2+} in native collagenase, the method has the disadvantage that, if purification is subsequently attempted, any exchangeable or dissociable $^{65}Zn^{2+}$ will be largely lost. Incorporation of $^{65}Zn^{2+}$ into the purified enzyme by a simple exchange incubation such as we have used may therefore have useful applications in studies of other collagenases and zinc metallo-enzymes.

Reversible and irreversible phases during inhibition by chelating agents have also been reported for the zinc metalloenzyme alkaline phosphatase of kidney (Ackermann & Ahlers, 1976). For this enzyme, however, higher concentrations of chelator

rather than longer incubations were required for the inhibition to become irreversible. These data led to the proposal that alkaline phosphatase contains two essential Zn^{2+} ions per molecule, one necessary for catalytic activity and the other, more firmly bound, necessary for structural integrity. Although we cannot exclude a similar structure for rabbit bone collagenase, all of our results can be interpreted in terms of the proposed model involving only one essential Zn^{2+} ion. We have not attempted to determine, from the $^{65}Zn^{2+}$ -exchange experiments, the number of Zn^{2+} ions incorporated per molecule of collagenase, because the concentration of protein in the Ultrogel AcA-54 eluate was too low to be determined, and we could not be sure of quantitative recovery of collagenase-bound $^{65}Zn^{2+}$ in the dialysis and chromatography. Furthermore, this type of experiment would not detect any collagenase-bound Zn^{2+} that does not exchange with $^{65}Zn^{2+}$ under the conditions of incubation.

In conclusion, two aspects of our results may be particularly relevant to the degradative activity of collagenase in connective tissues. Firstly, the critical dependence of collagenase activity on the concentration of free Zn^{2+} , expressed as pZn in Fig. 1, suggests that extracellular pZn could be a factor modulating collagenase activity. Secondly, the stabilizing effect of the bound inhibitor component on apocollagenase suggests that latency of collagenase, in addition to preventing uncontrolled collagen breakdown, could help to protect potential collagenase activity from conditions of low Zn^{2+} concentration.

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References

- Ackermann, B. P. & Ahlers, J. (1976) *Biochem. J.* **153**, 151–157
- Berman, M. B. & Manabe, R. (1973) *Ann. Ophthalmol.* **5**, 1193–1209
- Cawston, T. E. & Barrett, A. J. (1979) *Anal. Biochem.* **99**, 340–345
- Cawston, T. E. & Tyler, J. A. (1979) *Biochem. J.* **183**, 647–656
- Feder, J., Garrett, L. R. & Kochavi, D. (1971) *Biochim. Biophys. Acta* **235**, 370–377
- Harris, E. D. & Cartwright, E. C. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 249–284, North-Holland, Amsterdam
- Hartsuck, J. A. & Lipscomb, W. N. (1971) *Enzymes 3rd Ed.* **3**, 1–56

- Matthews, B. W., Weaver, L. H. & Kester, W. R. (1974) *J. Biol. Chem.* **249**, 8030–8044
- Morales, T. I., Woessner, J. F., Howell, D. S., Marsh, J. M. & Lemaire, W. J. (1978) *Biochim. Biophys. Acta* **524**, 428–434
- Nagai, Y., Hori, H., Kawamoto, T. & Komiya, M. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 171–187, North-Holland, Amsterdam
- O'Sullivan, W. J. (1968) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), 2nd ed., pp. 423–429, Clarendon Press, Oxford
- Perrin, D. D. & Dempsey, B. (1974) *Buffers for pH and Metal Ion Control*, pp. 94–108, Chapman and Hall, London
- Reilley, C. N. (1961) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **20**, 22–32
- Schmid, M. F. & Herriott, J. R. (1976) *J. Mol. Biol.* **103**, 175–190
- Sellers, A. & Reynolds, J. J. (1977) *Biochem. J.* **167**, 353–360
- Sellers, A., Cartwright, E., Murphy, G. & Reynolds, J. J. (1977) *Biochem. J.* **163**, 303–307
- Sellers, A., Reynolds, J. J. & Meikle, M. C. (1978) *Biochem. J.* **171**, 493–496
- Seltzer, J. L., Welgus, H. G., Jeffrey, J. J. & Eisen, A. Z. (1976) *Arch. Biochem. Biophys.* **173**, 355–361
- Seltzer, J. L., Jeffrey, J. J. & Eisen, A. Z. (1977) *Biochim. Biophys. Acta* **485**, 179–187
- Shinkai, H. & Nagai, Y. (1977) *J. Biochem. (Tokyo)* **81**, 1261–1268
- Stricklin, G. P., Eisen, A. Z., Bauer, E. A. & Jeffrey, J. J. (1978) *Biochemistry* **17**, 2331–2337
- Tokoro, Y., Eisen, A. Z. & Jeffrey, J. J. (1972) *Biochim. Biophys. Acta* **258**, 289–302
- Vaes, G. (1972) *Biochem. J.* **126**, 275–289
- Vaes, G., Eeckhout, Y., Lenaers-Claeys, G., François-Gillet, C. & Druetz, J. E. (1978) *Biochem. J.* **172**, 261–274
- Vallee, B. L. (1960) *Enzymes 2nd Ed.* **3B**, 225–276
- Werb, Z., Mainardi, C. L., Vater, C. A. & Harris, E. D. (1977) *N. Engl. J. Med.* **296**, 1017–1023