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Preparation and Reconstitution with Divalent Metal Ions of Class I and Class II *Clostridium histolyticum* Apocollagenases†

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ABSTRACT: Both γ- and ζ-collagenases from *Clostridium histolyticum* are fully and reversibly inhibited by 1,10-phenanthroline at pH 7.5 in the presence of 10 mM $CaCl_2$ with K_I values of 0.11 and 0.040 mM, respectively. The inhibition is caused by removal of the single, active-site Zn(II) present in each of these enzymes. The nonchelating analogue 1,5-phenanthroline has no effect on the activity of either enzyme. Dialysis of the enzymes in the presence of 1,10-phenanthroline, followed by back dialysis against buffer containing no chelating agent, gives the respective apocollagenases. Both apoenzymes can be instantaneously and fully reactivated by the addition of 1 equiv of Zn(II). Variable amounts of activity are restored to both apocollagenases by Co(II) and Ni(II) and to γ-apocollagenase by Cu(II). The activity titration curve for γ-apocollagenase with Co(II) and Scatchard plots for the reconstitution of γ-apocollagenase with Cu(II) and Ni(II) and of ζ-apocollagenase with Ni(II) and Co(II) indicate that all activity changes are the result of binding of a single equivalent of these divalent metal ions at the active site of the collagenases. Cd(II) and Hg(II) do not restore measurable activity to either apoenzyme.

The culture filtrate of *Clostridium histolyticum* contains at least seven distinct collagenases (EC 3.4.24.3)[1] with molecular weights that vary from 68000 to 130000 (Bond & Van Wart, 1984a,b). These enzymes have been divided into two classes that differ with respect to their sequences, as determined by chromatographic analysis of their tryptic digests and cyanogen bromide reaction products (Bond & Van Wart, 1984c), their mode of attack on native collagen (French et al., 1987), their relative activities toward synthetic peptides (Steinbrink et al., 1985; Van Wart & Steinbrink, 1985; Mookhtiar et al., 1985), and their differential inhibition by substrate analogues (Mookhtiar et al., 1988). Early studies showed that these collagenases are reversibly inhibited by chelating agents and led to the suggestion that they are metalloproteinases (Seifter & Harper, 1971). Confirmation of this has been provided by direct metal determination using atomic absorption spectroscopy which has demonstrated that these collagenases contain 1 mol of zinc/mol of protein and variable amounts of calcium ions (Bond & Van Wart, 1984b). Both the zinc and calcium ions are required for activity (Bond et al., 1981). The zinc ion is believed to be present at the active site, and the calcium ions are believed to stabilize the tertiary structure of the protein, as observed for thermolysin (Latt et al., 1969; Feder et al., 1971).

Since these collagenases contain an active-site zinc ion as an intrinsic, functional constituent, it is of interest to examine the role of the zinc ion in catalysis. Studies of the effects of active-site metal substitutions on the activities of zinc metalloenzymes have historically been a fruitful means of exploring the functional role of zinc (Vallee & Galdes, 1984). In this study, the mechanism of inhibition of a representative class I (γ) and class II (ζ) collagenase by the transition metal chelating agent 1,10-phenanthroline has been investigated, and the respective apocollagenases have been prepared. These species have been reconstituted with various divalent metal ions, and the effects on peptidase activity have been measured. This represents the first intensive investigation of the role of the active-site metal ion for any collagenase and lays the basis for examining the kinetic properties of the resulting metallocollagenases in more detail (Angleton & Van Wart, 1988).

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; FALGPA, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine; *Clostridium histolyticum* collagenase is referred to as collagenase; the various metallocollagenases are designated [(CHC)M(II)], where the brackets denote the firm binding of the divalent transition metal ion, M(II), to the apoenzyme, CHC.

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Materials and Methods

Materials. Crude clostridial collagenase preparations were purchased from Sigma Chemical Co. and Advanced Biofactures Corp., and γ- and ζ-collagenases were isolated from them as described earlier (Bond & Van Wart, 1984a). Thermolysin was obtained from Sigma and recrystallized before use (Latt et al., 1969). FALGPA was synthesized by the method of Van Wart and Steinbrink (1981). Solutions of Zn(II), Cu(II), and Cd(II) were prepared from the Johnson-Matthey spectrographically pure sulfate salts, while Co(II), Ni(II), and Hg(II) solutions were prepared from the chloride salts. 1,5-Phenanthroline was purchased from ICN and 1,10-phenanthroline hydrochloride from Lancaster Synthesis. All buffers were purchased from Sigma. All other materials were of reagent grade or higher quality.

Metal-Free Procedures. Metal-free conditions were maintained in all experiments. Reagent grade water was prepared by passing doubly distilled water through a Millipore Milli-Q system (resistivity 18 MΩ cm^{-1}). All solutions, buffers, salts, and substrates were freed from adventitious metal ions by extraction with dithizone in carbon tetrachloride (Thiers, 1951). Metal-free hydrochloric acid was prepared by isothermal distillation. Plasticware and quartz spectrophotometer cells were rendered metal-free by treatment with dilute metal-free hydrochloric acid. Dialysis membranes were soaked in metal-free water at 70 °C for 30 min. This procedure was repeated three times, after which the membranes were stored in metal-free water at 4 °C.

Enzymatic Assays. Routine FALGPA assays were carried out spectrophotometrically in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a substrate concentration of 0.1 or 1 mM by continuously monitoring the decrease in absorbance at 324 or 350 nm, respectively, after the addition of enzyme with a Varian Model 219 spectrophotometer (Van Wart & Steinbrink, 1981). The reaction temperature was maintained at 25 °C by using a thermostated cell and a Neslab Model RTE-4 circulator bath. The FALGPA concentration was determined spectrophotometrically by using ϵ_{305} = 24 700 M^{-1} cm^{-1}. Values of the initial rate (v) were calculated from the first 10% of hydrolysis by using $\Delta\epsilon_{324}$ = 2250 M^{-1} cm^{-1} or $\Delta\epsilon_{350}$ = 165 M^{-1} cm^{-1}. For assays carried out at a single substrate concentration, activity is expressed as $v/[E_0]$, where $[E_0]$ was determined spectrophotometrically by using $\epsilon_{280} = 1.19 \times 10^5$ and 1.55×10^5 M^{-1} cm^{-1} for γ- and ζ-collagenases, respectively (Bond & Van Wart, 1984b).

Inhibition Studies. Inhibition studies with 1,5- and 1,10-phenanthrolines were performed by incubating them with the collagenases at 0 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. For the assays, aliquots of the incubation mixtures were added to cuvettes containing 0.1 mM FALGPA in the same buffer containing the same concentration of chelating agent that was present in the incubation solution. Values of K_I, the concentration of inhibitor needed to achieve 50% inhibition, and n, the number of moles of ligand complexed per mole of enzyme, were obtained from the inhibition data by using

$$(v_0/v_I) - 1 = K_I I^n \quad (1)$$

by plotting ln $[(v_0/v_I) - 1]$ vs ln $(K_I I)$, where v_0 and v_I are the activities of the enzyme in the absence and presence of inhibitor, respectively (Kistiakowsky & Shaw, 1953).

Preparation of Apoenzymes. Aliquots (1 mL) of 8 and 1.2 μM γ- and ζ-collagenases, respectively, and 10 mg/mL thermolysin were placed in separate metal-free dialysis bags and dialyzed together against a 1000-fold excess of 5 mM Hepes, 1 M NaCl, and 10 mM $CaCl_2$, pH 7.5, containing 2 mM 1,10-phenanthroline at 4 °C for 24 h with two changes of this solution. This was followed by dialysis against three changes of a 1000-fold excess of this same buffer without the 1,10-phenanthroline at 4 °C for 24 h. This procedure yields apoenzyme preparations with less than 5 and 15% of the initial activity of γ- and ζ-collagenases, respectively. The accompanying thermolysin had less than 1% of its initial activity.

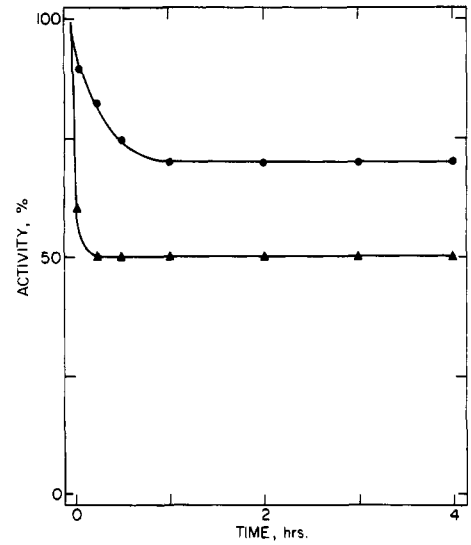

FIGURE 1: Time dependence of the inhibition of (●) γ- and (▲) ζ-collagenases by 0.075 and 0.040 mM 1,10-phenanthroline, respectively. Incubations were carried out at 0 °C at γ- and ζ-collagenase concentrations of 4 and 0.5 μM, respectively, in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 0.1 mM and at enzyme concentrations of 80 and 10 nM for γ- and ζ-collagenases, respectively.

Reconstitution Studies. To determine the time dependence of metal reconstitution of the apocollagenases, a 1:1 or 2:1 molar ratio of divalent metal ion to apoenzyme was prepared in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5 at 0 °C. Aliquots of the incubation mixtures were removed at various time intervals and assayed for activity at 25 °C in this same buffer. The concentration dependence of metal ion reconstitution was examined by adding variable quantities of the various metal ions to apoenzyme samples in separate incubation tubes. aliquots were removed from these tubes after a period of time, as determined by the time dependence studies, and assays were carried out as described above.

Results

Inhibition Studies. Both γ- and ζ-collagenases, denoted [(γ-CHC)Zn(II)] and [(ζ-CHC)Zn(II)], respectively, are inhibited by 1,10-phenanthroline. The time dependence of the inhibition of these two collagenases by 0.075 and 0.040 mM 1,10-phenanthroline, respectively, is shown in Figure 1. The activity of [(γ-CHC)Zn(II)] is reduced to 70% in 1 h and that of [(ζ-CHC)Zn(II)] to 50% in 0.5 h. The variation in the activities of both collagenases as a function of the concentrations of 1,5- and 1,10-phenanthrolines is shown in Figure 2. All assays were carried out after each reaction had reached equilibrium. The nonchelating analogue 1,5-phenanthroline has no effect on the activity of either enzyme at any concentration up to its solubility limit. However, both collagenases are completely inhibited by 1 mM 1,10-phenanthroline. This inhibition is fully reversed by the addition of 0.1 mM zinc ions (not shown).

Plots of ln $[(v_0/v_I) - 1]$ vs ln $(K_I I)$ for the inhibition of these two collagenases by 1,10-phenanthroline are shown in Figure

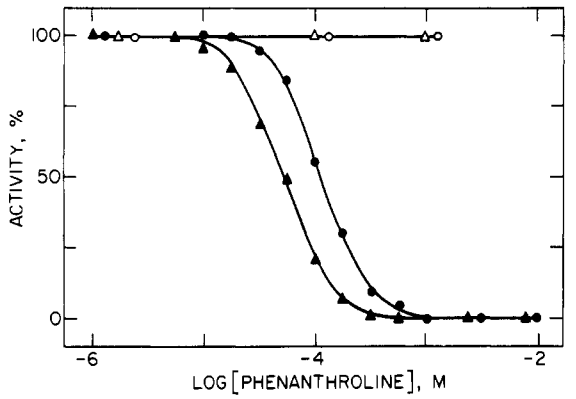

FIGURE 2: Effect of 1,10-phenanthroline (closed symbols) and 1,5-phenanthroline (open symbols) on the activity of (●,○) γ- and (▲,△) ζ-collagenases. Incubations were carried out at 0 °C at γ- and ζ-collagenase concentrations of 4 and 0.5 μM, respectively, in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 0.1 mM and at enzyme concentrations of 80 and 10 nM for γ- and ζ-collagenases, respectively.

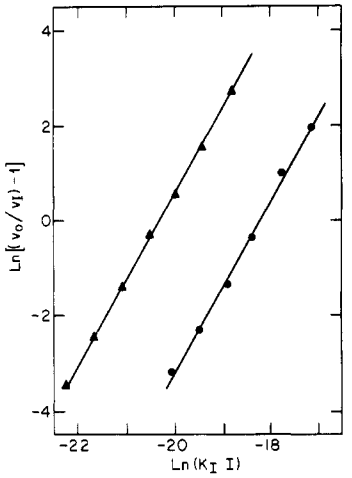

FIGURE 3: Plots of ln $[(v_0/v_I) - 1)]$ vs ln $(K_I I)$ for the data of Figure 2 to determine the values of n and pK_I for the inhibition of (●) γ- and (▲) ζ-collagenases by 1,10-phenanthroline.

3. From these plots, the K_I values for [(γ-CHC)Zn(II)] and [(ζ-CHC)Zn(II)] are 0.11 and 0.040 mM, respectively, and the values of n, the average number of complexed ligands per enzyme, is 1.8 for both collagenases. The values of pK_I and n found here for the inhibition of these collagenases by 1,10-phenanthroline are very similar to those for carboxypeptidase A (Coombs et al., 1962) and thermolysin (Holmquist & Vallee, 1974), as shown in Table I. The observations that the inhibition is time dependent and occurs with a value of n greater than unity indicate that inhibition occurs by removal of the active-site Zn(II) (Holmquist & Vallee, 1974).

Preparation and Reconstitution of Apocollagenases. The knowledge that 1,10-phenanthroline removes the active-site Zn(II) from these collagenases suggested that the apocollagenases could be prepared by dialysis against this chelating agent. When these dialyses were carried out as described under Materials and Methods, the respective apoenzymes were obtained. The inclusion of a concentrated solution of thermolysin in a separate dialysis bag facilitated the preparation of γ-CHC and ζ-CHC, since it complexed traces of contaminating metal ions during the back dialysis used to remove the 1,10-phenanthroline.

The time and metal ion concentration dependences of the restoration of activity to these apoenzymes after the addition of divalent metal ions were investigated. The metal ions tested

Table I: Comparison of the Values of pK_I and n for the Inhibition of γ- and ζ-Collagenases, Carboxypeptidase A, and Thermolysin by 1,10-Phenanthroline

enzyme	pK_I (M)	n
γ-collagenase	4.0	1.8
ζ-collagenase	4.4	1.8
carboxypeptidase A[a]	4.2	2.3
thermolysin[b]	4.4	1.8

[a] Coombs et al. (1962). [b] Holmquist and Vallee (1974).

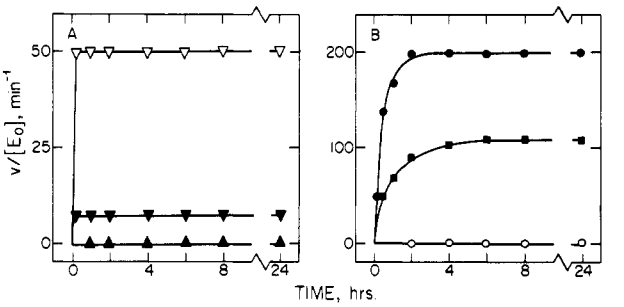

FIGURE 4: Time dependence of the reconstitution of γ-CHC by (▽) Zn(II), (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II). Incubations were carried out at 0 °C at a γ-CHC concentration of 5 μM in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 0.1 mM and at enzyme concentrations of 50 nM for Zn(II), Co(II), and Cu(II) and 100 nM for Ni(II), Cd(II), and Hg(II). A 1:1 molar ratio of metal ion to γ-CHC was used for Zn(II) and Co(II), and a 2:1 molar ratio was used for all others.

were Zn(II), Co(II), Cu(II), Ni(II), Cd(II), and Hg(II). Mixtures containing 1:1 or 2:1 molar ratios of metal ion to γ-CHC were incubated at 0 °C and assayed as a function of time at 25 °C at a FALGPA concentration of 0.1 mM. The addition of Zn(II) and Ni(II) results in restoration of activity in less than 1 min (Figure 4A). Co(II) and Cu(II) also restore activity, but the activity does not level off for 2 and 6 h, respectively (Figure 4B). Neither Cd(II) nor Hg(II) restores activity over 24 h of incubation (Figure 4A,B).

The dependence of the reconstituted activity on the ratio of divalent metal ion to γ-CHC was studied next. Aliquots of incubation mixtures containing metal ion to γ-CHC ratios ranging from substoichiometric to large excesses were removed after equilibrium was reached and assayed at 25 °C at a FALGPA concentration of 0.1 mM. The aliquot size was chosen so that the enzyme concentration in the assays was 50 nM for the titrations with Zn(II), Co(II), and Cu(II) and 100 nM for those with Ni(II), Cd(II), and Hg(II). The results of these titrations are shown in Figure 5, where the $v/[E_0]$ values have been adjusted to subtract the residual 5% activity remaining in the apoenzyme. For the Zn(II) and Co(II) titrations, maximal activity is reached at a 1:1 molar ratio of metal to apoenzyme (Figure 5A). The linear increases in activity that level off sharply after the addition of 1 equiv of metal ion indicate that these metal ions are tightly bound. The 1:1 stoichiometry found for Zn(II) is consistent with the results of atomic absorption measurements on native γ-collagenase which show that it contains 1 mol of Zn(II)/mol of protein (Bond & Van Wart, 1984b). The $v/[E_0]$ value for the Zn(II)-reconstituted apoenzyme is identical with that of native [(γ-CHC)Zn(II)], indicating that full reconstitution has been achieved.

The titration curves for Cu(II) and Ni(II), on the other hand, show that 4- and 10-fold molar excesses of metal ion over γ-CHC, respectively, are required to achieve full activity (Figure 5B). The values of $v/[E_0]$ for γ-CHC fully recon-

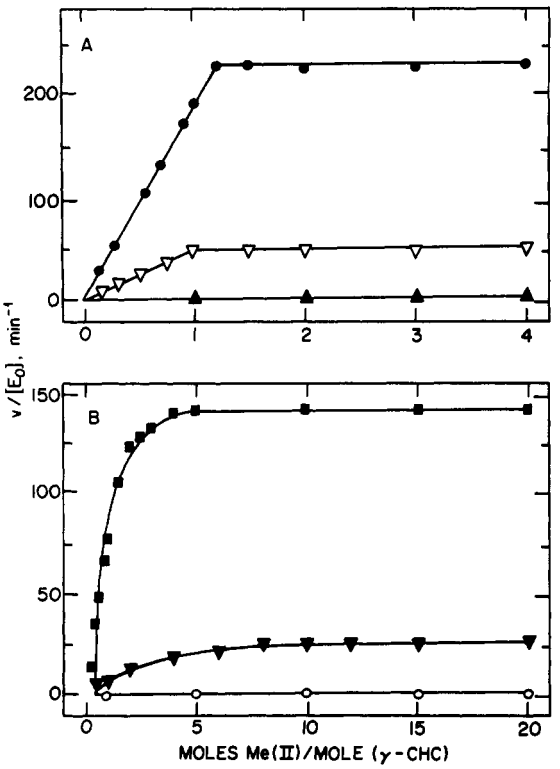

FIGURE 5: Reconstitution of γ-CHC by (▽) Zn(II), (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II). Incubations were carried out at 0 °C at a γ-collagenase concentration of 5 μM in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 0.1 mM and at enzyme concentrations of 50 nM for Zn(II), Co(II), and Cu(II) and 100 nM for Ni(II), Cd(II), and Hg(II).

stituted with Co(II), Cu(II), and Ni(II) measured at a FALGPA concentration of 0.1 mM are 500, 300, and 60%, respectively, that of [(γ-CHC)Zn(II)]. The effect of the addition of variable quantities of these metal ions on the activity of γ-CHC is summarized in Table II.

Cd(II) and Hg(II) fail to restore activity to γ-CHC at any of the concentrations studied (Figure 5, Table II). Indirect evidence that these metal ions do, in fact, bind to the active site is provided from metal competition experiments (Table II). Addition of 10- and 100-fold molar excesses of Cd(II) to γ-CHC followed by 1 equiv of Zn(II) results in restoration of only 86 and 70%, respectively, of the activity of [(γ-CHC)Zn(II)]. In fact, a 10-fold excess of Zn(II) is required in the presence of a 100-fold excess of Cd(II) to obtain the full activity of [(γ-CHC)Zn(II)]. Thus, Cd(II) binds to γ-CHC, and Zn(II) competes with Cd(II) for this binding site, which is probably the active site. A similar, though weaker, competition is observed for Hg(II). Thus, it is likely that Cd(II) and Hg(II) bind weakly to the active site of γ-CHC but do not restore activity under the assay conditions used here.

The effects of time and metal ion concentration on the reconstitution of ζ-CHC by these metal ions are characteristically different. Assays were carried out at a FALGPA concentration of 1 mM so that higher enzyme concentrations could be employed, thus minimizing problems with adventitious metal ion contamination from the buffer in these assays. Only Zn(II), Ni(II), and Co(II) restore measurable activity to ζ-CHC. The reconstitution of ζ-CHC by Zn(II) is complete within 1 min (Figure 6A), which is the same behavior observed for γ-CHC. In contrast to γ-CHC, however, the restoration of activity to ζ-CHC by Co(II) is rapid, while Ni(II) restores maximal activity only after 4 h.

Table II: Effect of Divalent Metal Ions on the Activity of γ-CHC[a]

M(II)	$[E_0]$ (μM)	mol of M(II)/ mol of γ-CHC	$v/[E_0]$ (min^{-1})
Zn(II)	0.05	1	49
		10	49
		100	45
Co(II)	0.05	1	240
		10	240
		100	240
Cu(II)	0.05	1	85
		10	130
		100	130
Ni(II)	0.10	1	4
		10	24
		100	24
Cd(II)	0.10	1 (+0.1 μM $ZnSO_4$)	0 (50)
		10 (+0.1 μM $ZnSO_4$)	0 (43)
		100 (+0.1 μM $ZnSO_4$)	0 (35)
		1 (+1 μM $ZnSO_4$)	0 (50)
		10 (+1 μM $ZnSO_4$)	0 (50)
		100 (+1 μM $ZnSO_4$)	0 (50)
Hg(II)	0.10	1 (+0.1 μM $ZnSO_4$)	0 (50)
		10 (+0.1 μM $ZnSO_4$)	0 (50)
		100 (+0.1 μM $ZnSO_4$)	0 (40)
		1 (+1 μM $ZnSO_4$)	0 (50)
		10 (+1 μM $ZnSO_4$)	0 (50)
		100 (+1 μM $ZnSO_4$)	0 (50)

[a] All samples were assayed in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, 25 °C, at a FALGPA concentration of 0.1 mM.

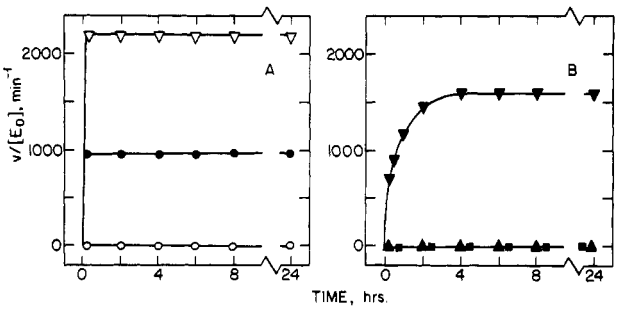

FIGURE 6: Time dependence of the reconstitution of ζ-CHC by (▽) Zn(II), (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II). Incubations were carried out at 0 °C at a ζ-collagenase concentration of 0.5 μM in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 1 mM and at enzyme concentrations of 50 nM for Zn(II), Co(II), and Cu(II) and 100 nM for Ni(II), Cd(II), and Hg(II). A 1:1 molar ratio of metal ion to ζ-CHC was used for Zn(II) and Co(II), and a 2:1 molar ratio was used for all others.

The variation in activity as a function of the molar ratio of these metal ions to ζ-CHC is shown in Figure 7, where the 15% residual activity of the apoenzyme has again been subtracted from the $v/[E_0]$ values. The titration curve for Zn(II) indicates tight binding with a 1:1 stoichiometry and restoration of the full activity of native [(ζ-CHC)Zn(II)]. As with γ-CHC, Ni(II) binds more weakly than Zn(II) with at least a 5-fold molar excess required for full activity. In contrast to γ-CHC, a 10-fold molar excess of Co(II) is required for full activity. Under these assay conditions, the activities of ζ-CHC fully reconstituted with Co(II) and Ni(II) are 120 and 110%, respectively, that of [(ζ-CHC)Zn(II)].

The most striking difference between the two collagenases is found in the Cu(II)-reconstitution experiments. While Cu(II) restores the activity of γ-CHC to 3 times the level of [(γ-CHC)Zn(II)] at a FALGPA concentration of 0.1 mM, Cu(II) does not restore any activity to ζ-CHC. Competition experiments between Cu(II) and Zn(II) suggest that Cu(II) does indeed bind to the active site of ζ-CHC. For example, if a stoichiometric amount of Cu(II) is first added to ζ-CHC,

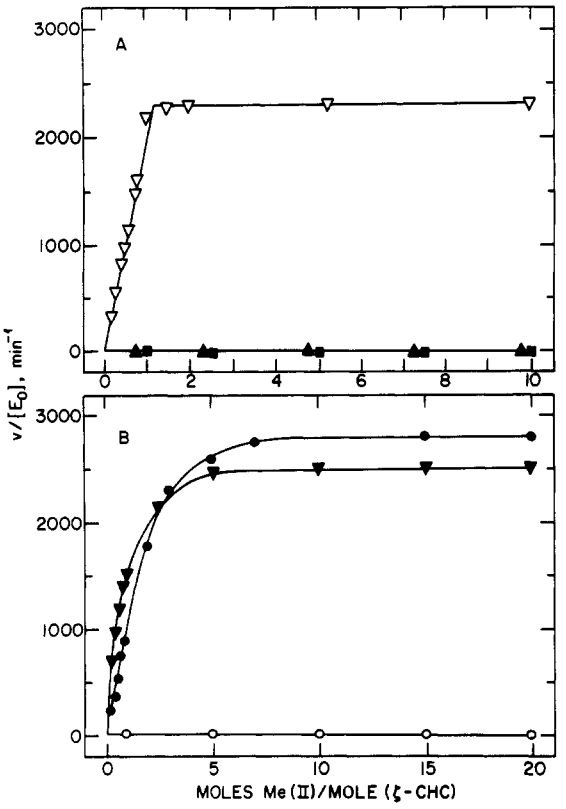

FIGURE 7: Reconstitution of ζ-CHC by (▽) Zn(II), (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II). Incubations were carried out at 0 °C at a ζ-collagenase concentration of 0.5 μM in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 1 mM and at enzyme concentrations of 50 nM for Zn(II) and Co(II) and 100 nM for Cu(II), Ni(II), Cd(II), and Hg(II).

full reconstitution can still be achieved with a stoichiometric amount of Zn(II) (Table III). However, if 10- and 100-fold excesses of Cu(II) are first added, 1 equiv of Zn(II) restores only 87 and 35% of activity, respectively, and 10 equiv of Zn(II) are required for attainment of the activity of [(ζ-CHC)Zn(II)]. This suggests that Cu(II) binds at the active site to form an inactive species. The results for Cd(II) and Hg(II) are similar but show that they compete much more weakly with Zn(II). The effect of variable concentrations of all six divalent metal ions on the activity of ζ-CHC is summarized in Table III.

Stoichiometry of Metal Binding in Active Metallocollagenases. The titration curves for γ-CHC with Zn(II) and Co(II) and for ζ-CHC with Zn(II) all clearly demonstrate tight binding at a 1:1 ratio, establishing that [(γ-CHC)Zn(II)], [(γ-CHC)Co(II)], and [(ζ-CHC)Zn(II)] are the species that have been prepared. Thus, there is a single tight binding site located at the active center of these collagenases whose occupancy by these metal ions confers activity to the apoenzyme. The parabolic shaped saturation curves for the reconstitution of γ-CHC by Cu(II) and Ni(II) (Figure 5B) and of ζ-CHC by Co(II) and Ni(II) (Figure 7B) are almost certainly the result of weak binding to this same site. If this is the case, then the number of moles of M(II) bound per mole of apocollagenase, ν, at any M(II) concentration will equal the fractional activity

$$\nu = \{v/[E_0]\}/\{v/[E_0]\}_{full} \quad (2)$$

where $v/[E_0]$ is the activity at any given [M(II)] and $\{v/[E_0]\}_{full}$ is the limiting activity after saturation. Such a binding equilibrium can be characterized as one with identical, independent sites and is described by the relationship (Cantor & Schimmel, 1980)

$$\nu/[M(II)] = nK - \nu K \quad (3)$$

where [M(II)] is the free metal ion concentration, K is the association constant for the binding, and n is the number of binding sites per collagenase.

If the assumptions delineated above are correct, then Scatchard plots of $\nu/[M(II)]$ vs ν constructed from the data in Figures 5B and 7B should be linear with a ν intercept of 1 and a slope of $-K$. If two or more nonidentical binding sites influence activity, these plots should be nonlinear. Scatchard plots for the reconstituton of γ-CHC with Ni(II) and Cu(II) and of ζ-CHC with Co(II) and Ni(II) calculated by using eq 2 and assuming a 1:1 complex are shown in Figure 8. For the titration of γ-CHC with Cu(II), the free [Cu(II)] was calculated by correcting for losses due to the formation of a small amount of $Cu(OH)_2$ at pH 7.5. All of the Scatchard plots are linear with ν intercepts of 1.05, 1.15, 1.10, and 1.10, and K values of 1.5×10^6, 4.7×10^7, 2.6×10^7, and 1.0×10^7 M^{-1} for the binding of Ni(II) and Cu(II) to γ-CHC and of Ni(II) and Co(II) to ζ-CHC, respectively. These results are summarized in Table IV. Thus, the Scatchard plots are consistent with the binding of a single atom of M(II) to each of the active metallocollagenases.

Table III: Effect of Divalent Metal Ions on the Activity of ζ-CHC[a]

M(II)	$[E_0]$ (μM)	mol of M(II)/ mol of ζ-CHC	$v/[E_0]$ (min^{-1})
Zn(II)	0.05	1	2300
		10	2300
		100	1800
Co(II)	0.05	1	940
		10	2800
		100	2800
Ni(II)	0.05	1	1500
		10	2500
		100	2500
Cu(II)	0.10	1 (+0.1 μM $ZnSO_4$)	0 (2300)
		10 (+0.1 μM $ZnSO_4$)	0 (2000)
		100 (+0.1 μM $ZnSO_4$)	0 (800)
		1 (+1 μM $ZnSO_4$)	0 (2300)
		10 (+1 μM $ZnSO_4$)	0 (2300)
		100 (+1 μM $ZnSO_4$)	0 (2000)
Cd(II)	0.10	1 (+0.1 μM $ZnSO_4$)	0 (2300)
		10 (+0.1 μM $ZnSO_4$)	0 (2300)
		100 (+0.1 μM $ZnSO_4$)	0 (2000)
		1 (+1 μM $ZnSO_4$)	0 (2300)
		10 (+1 μM $ZnSO_4$)	0 (2300)
		100 (+1 μM $ZnSO_4$)	0 (2300)
Hg(II)	0.10	1 (+0.1 μM $ZnSO_4$)	0 (2300)
		100 (+0.1 μM $ZnSO_4$)	0 (2300)
		10 000 (+0.1 μM $ZnSO_4$)	0 (2000)
		1 (+1 μM $ZnSO_4$)	0 (2300)
		100 (+1 μM $ZnSO_4$)	0 (2300)
		10 000 (+1 μM $ZnSO_4$)	0 (2300)

[a] All samples were assayed in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, 25 °C, at a FALGPA concentration of 1 mM.

Table IV: Values of K and n Obtained from Scatchard Plots for the Binding of M(II) to γ- or ζ-CHC

enzyme	M(II)	n	log K (M^{-1})
γ-CHC	Ni(II)	1.05	6.2
	Cu(II)	1.15	7.7
ζ-CHC	Ni(II)	1.10	7.4
	Co(II)	1.10	7.0

Discussion

Both [(γ-CHC)Zn(II)] and [(ζ-CHC)Zn(II)] are completely inhibited by 1,10-phenanthroline. Metal chelating

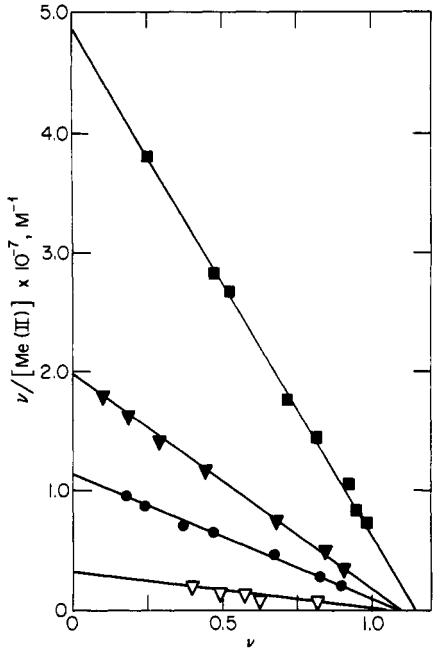

FIGURE 8: Scatchard plots for the binding of Ni(II) to (▽) γ-CHC and (▼) ζ-CHC, of Co(II) to (●) ζ-CHC and of Cu(II) to (■) γ-CHC.

agents can inhibit metalloproteinases by two different mechanisms. The first involves the *removal* of the intrinsic metal, M, from the metalloenzyme [(E)M], by n mol of inhibitor, I, to give a metal–inhibitor complex, $[M(I)_n]$, and an apoenzyme, (E):

$$[(E)M] + nI \rightleftharpoons (E) + [M(I)_n] \quad (4)$$

This mechanism has been observed for the inhibition of carboxypeptidase A (Coombs et al., 1962), leucine aminopeptidase (Himmelhoch, 1969), thermolysin (Holmquist & Vallee, 1974), and *Escherichia coli* alkaline phosphatase (Plocke & Vallee, 1962) by 1,10-phenanthroline. An alternative mechanism involves the formation of a long-lived, mixed complex between the enzyme, metal, and inhibitor:

$$[(E)M] + nI \rightleftharpoons [(E)M(I)_n] \quad (5)$$

This has been observed for the interaction of horse liver alcohol dehydrogenase with 1,10-phenanthroline (Drum & Vallee, 1970; Vallee & Coombs, 1959; Vallee et al., 1959). It is also possible that a short-lived ternary complex is first formed, followed by the slower removal of the metal ion, as observed for angiotensin converting enzyme (Kleeman et al., 1986). If the mechanism described in eq 4 is operative, n is expected to range between 2 and 3 for a bidentate chelating agent, since Zn(II) forms complexes that are 4–6 coordinate. If the dissociation of the active-site metal ion is slow, the inhibition produced by this mechanism will be time dependent. For the mechanism in eq 5, n is expected to be unity, since the active-site metal ion is itself usually coordinated to the protein at a minimum of three sites, leaving room for only one molecule of bidentate chelating agent. Since the rate of association of chelating agent with enzyme is expected to be fast, inhibition by this mechanism is usually instantaneous on the time scale of enzyme assays.

The data presented for the inhibition of [(γ-CHC)Zn(II)] and [(ζ-CHC)Zn(II)] by 1,10-phenanthroline support the mechanism given by eq 4. First, the nonchelating analogue 1,5-phenanthroline fails to inhibit either enzyme. This indicates that the inhibition observed with 1,10-phenanthroline is due to its metal chelating capacity and not to a nonspecific affinity for the active site. Second, the time dependence observed for inhibition is consistent with a rate-limiting step involving the dissociation of the active-site zinc ion from the enzymes. Finally, the values of n found for the inhibition of these collagenases are greater than unity, as expected for the mechanism of eq 4.

The removal of the active-site zinc atom by 1,10-phenanthroline has enabled us to prepare stable, inactive apoenzymes by dialysis against this chelator. These apocollagenases are stable over a period of at least 4 weeks at 4 °C, as evidenced by our ability to restore full activity by addition of 1 equiv of Zn(II). The apocollagenases can also be reconstituted with other divalent metal ions to restore activity. However, the kinetics of reactivation, the level of activity restored and the strength of binding vary widely for each metal ion. γ-CHC and ζ-CHC are both reactivated by Zn(II), Co(II), and Ni(II), and γ-CHC is also reactivated by Cu(II). Neither Cd(II) nor Hg(II) restores activity to either apocollagenase, nor does Cu(II) reactivate ζ-CHC. Zn(II) and Ni(II) instantaneously reactivate γ-CHC, and Zn(II) and Co(II) have the same effect on ζ-CHC. The reactivation of γ-CHC by Co(II) and Cu(II) and of ζ-CHC by Ni(II) is complete only after 2–6 h of incubation. This suggests that the metal-binding ligands of the collagenases must undergo a reorientation to adapt to these metal ions.

The curves showing the restoration of activity on addition of variable quantities of metal ion for these two collagenases provide information about the strength and stoichiometry of metal binding. The sharp, biphasic titration curves for γ-CHC with Zn(II) and Co(II) and for ζ-CHC with Zn(II) establish that these metal ions bind very tightly at a 1:1 molar ratio of metal ion to apoenzyme. The parabolic titration curves for the reconstitution of γ-CHC with Ni(II) and Cu(II) and of ζ-CHC with Ni(II) and Co(II) are indicative of weaker binding with several-fold molar excesses of metal ions required to achieve full activity. Scatchard plots made from these reconstitution curves are consistent with reactivation being the result of the binding of a single metal ion to each apocollagenase. The Scatchard plots also provide estimates of the K_M values for these enzymes. The values shown in Table IV are similar to those for other metal-substituted metalloproteinases, such as carboxypeptidase A, where $K_{Co} = 1 \times 10^7$ M^{-1} and $K_{Ni} = 1.6 \times 10^8$ M^{-1} (Coombs et al., 1962). Collectively, these observations indicate that the reconstitution of γ-CHC with Zn(II), Co(II), Cu(II), and Ni(II) produces the active metallospecies [(γ-CHC)Zn(II)], [(γ-CHC)Co(II)], [(γ-CHC)Cu(II)], and [(γ-CHC)Ni(II)]. ζ-CHC can likewise be reconstituted with Zn(II), Co(II), and Ni(II) to produce the active metallospecies [(ζ-CHC)Zn(II)], [(ζ-CHC)Co(II)], and [(ζ-CHC)Ni(II)].

The failure of Cd(II) and Hg(II) to reactivate both apoenzymes and of Cu(II) to reactivate ζ-CHC is probably not due to the failure of these metal ions to bind at the active site. Competition experiments between Hg(II) or Cd(II) and Zn(II) for both apocollagenases indicate that the former metal ions compete weakly with Zn(II) to prevent restoration of activity. Similar competition experiments between Cu(II) and Zn(II) for ζ-CHC suggest that Cu(II) also binds to this apoenzyme. These results suggest that [(ζ-CHC)Cu(II)], [(γ- or ζ-CHC)Cd(II)], and [(γ- or ζ-CHC)Hg(II)] are formed in the reconstitution experiments, but are inactive. However, the possibility that the inactivity is the result of the binding of these metal ions at inhibitory sites distinct from the active site cannot be ruled out. This issue can be resolved when sufficient quantities of these enzymes become available to measure the

concentrations of these bound metal ions by atomic absorption spectroscopy.

The results presented here show that active, divalent metal ion substituted class I and II *C. histolyticum* collagenases can be prepared by reconstitution of the respective apocollagenases. The activity changes observed here were measured at a single but different substrate concentration for each class of collagenase. In the following paper (Angleton & Van Wart, 1988), it is shown that these same metallocollagenases can be prepared by direct exchange of the Zn(II) at the active site of the native enzymes with these metal ions. This observation greatly facilitates more detailed studies of the effects of these metal ion substitutions on the kinetics of hydrolysis of substrates.

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Registry No. Cu, 7440-50-8; Cd, 7440-43-9; Co, 7440-48-4; Ni, 7440-02-0; Hg, 7439-97-6; collagenase, 9001-12-1; 1,10-phenanthroline, 66-71-7.

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