Preparation by Direct Metal Exchange and Kinetic Study of Active Site Metal Substituted Class I and Class II *Clostridium histolyticum* Collagenases†

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ABSTRACT: Active site metal substitutions for both γ- and ζ-collagenases from *Clostridium histolyticum* have been made by direct metal exchange. The incubation of Co(II), Cu(II), Ni(II), Cd(II), and Hg(II) with these native collagenases results in changes in activity that parallel those observed for the reconstitution of the respective apoenzymes with these metal ions. For both collagenases, the exchange reactions with Co(II) and Cu(II) are complete within 1 min. However, the changes in activity observed on addition of Ni(II), Cd(II), and Hg(II) to γ-collagenase and Cd(II) and Hg(II) to ζ-collagenase are time dependent. The kinetic parameters k_{cat} and K_M have been determined for each of the active metallospecies. The substitution of the active-site metal ion in γ-collagenase results in changes in both k_{cat} and K_M, while the effect observed in ζ-collagenase is primarily on K_M. This suggests that there are differences in the mechanisms of these two collagenases, at least with respect to the role of the zinc ion in catalysis.

The preparation of two series of *Clostridium histolyticum* metallocollagenases, [(γ- or ζ-CHC)M(II)],[1] by reconstitution of the respective apoenzymes with several divalent metal ions has been described in the preceding paper (Angleton & Van Wart, 1988). These studies have provided important information about the activity of these metallocollagenases and about the strength and stoichiometry of metal ion binding in each active species. Unfortunately, the preparation of these active site metal substituted collagenases by this method is difficult, since scrupulously metal-free conditions must be used to prevent reactivation of the apocollagenases by adventitious metal ion contamination.

In order to simplify the preparation of these species and to facilitate a more detailed examination of their kinetic properties, the preparation of [(γ- or ζ-CHC)M(II)] by direct exchange of the active-site Zn(II) of the native collagenases with other M(II) is described herein. The conditions used to prepare these [(γ- or ζ-CHC)M(II)] entail the use of excess M(II), thus minimizing problems due to contamination by adventitious metal ions. This new procedure has permitted kinetic measurements to be carried out over a wide range of substrate concentrations and has enabled the effects of active site metal substitutions on the individual kinetic parameters k_{cat} and K_M to be evaluated. These data generally provide a basis for comparison with other, more intensively studied zinc metallohydrolases (Vallee & Galdes, 1984) and also provide mechanistic information on the role of the active-site metal in the binding and catalytic steps.

Materials and Methods

Materials. The purification of the collagenases (Bond & Van Wart, 1984a), the synthesis of FALGPA (Van Wart & Steinbrink, 1981), and the source of the metal ions and Hepes were the same as described in the previous paper (Angleton & Van Wart, 1988). Sodium barbital, Tricine, and Tris were all purchased from Sigma Chemical Co.

Metal-Free Procedures, Enzymatic Assays, and Metal-Exchange Experiments. Metal-free conditions were maintained in all experiments (Thiers, 1957). Assays for the hydrolysis of FALGPA were carried out spectrophotometrically (Van Wart & Steinbrink, 1981), and enzyme and substrate concentrations were determined as before (Angleton & Van Wart, 1988). Metal-exchange experiments were carried out by adding an excess of the metal ion of interest to an incubation tube containing the collagenase dissolved in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5 at 0 °C. The time dependence of metal exchange was determined by assaying aliquots of these mixtures at various time intervals in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5 at 25 °C, in the presence of the same concentration of metal ion that was present in the incubation tube. Following the determination of the time dependence, the effect of changes in metal ion concentration on activity was measured by varying the concentration of metal ion in the incubation tubes and assaying for activity after the exchange reaction was allowed to come to equilibrium. Assays were always carried out in the presence of the same concentration of metal ion used in the incubation. The assays for γ- and ζ-collagenases in all of these experiments were carried out at a FALGPA concentration of 0.1 mM.

The kinetic parameters k_{cat} and K_M for the native and metal-substituted collagenases were obtained from double-reciprocal plots of assays carried out at FALGPA concentrations that varied from 0.005 to 5 mM. The initial rates for assays at FALGPA concentrations less than 0.2 mM were measured at 324 nm, while those above this concentration were monitored at 350 nm. Assays of metal-substituted collagenases were carried out in an excess [5 μM for Cu(II); 1 mM for Co(II) and Ni(II)] of the respective metal ions to prevent the exchange of adventitious Zn(II) or other metal ions for the active-site metal.

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; 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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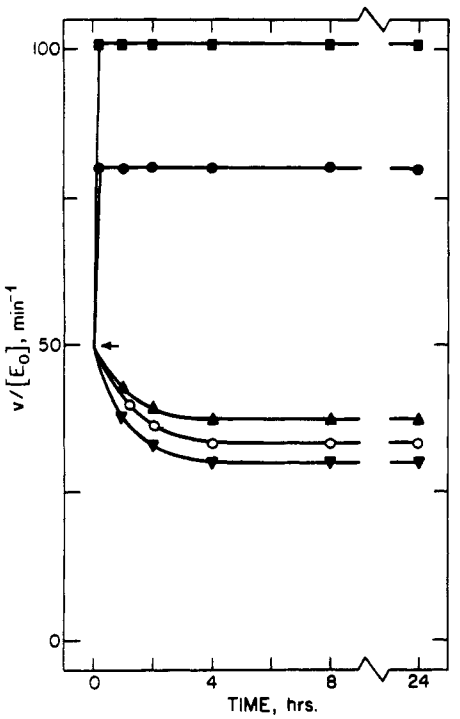

FIGURE 1: Variation in the activity of [(γ-CHC)Zn(II)] as a function of time after addition of (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II). Metal ion concentrations in both the incubation and assay solutions were 5 μM for Cu(II) and 0.1 mM for all others. Incubations were carried out at 0 °C at an enzyme concentration of 500 nM 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).

RESULTS

Effect of Divalent Metal Ions on Native Collagenases. In principle, active site metal substitutions in Zn(II) metalloenzymes can be effected by the direct incubation of the native enzyme with other M(II). The success of this approach depends upon the kinetics of Zn(II) dissociation, the relative affinities of Zn(II) and the other M(II) for the enzyme active site, and whether there are alternate binding sites for the new M(II). The data presented in the preceding paper (Angleton & Van Wart, 1988) suggest that the Zn(II) dissociation rate for both collagenases is fast and that several of the M(II) studied bind sufficiently tightly to the active site that, when added to the native enzymes in excess, they could displace Zn(II) by mass action. Thus, the direct exchange of several M(II) for Zn(II) should be possible for both γ- and ζ-collagenases, provided that the concentrations of the M(II) needed to displace Zn(II) are not so high that secondary binding sites are populated. The conditions to selectively effect these active site metal exchanges have been investigated empirically for each collagenase.

[(γ-CHC)Zn(II)] was incubated at 0 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, with the same five M(II) [Co(II), Ni(II), Cu(II), Cd(II), and Hg(II)] tested in the reconstitution experiments described earlier (Angleton & Van Wart, 1988). In addition, the effect of Zn(II) was also investigated, since at the end of each incubation experiment with the other M(II) variable quantities of Zn(II) are added to test the reversibility of the exchange and to assess whether the enzyme has been damaged by the M(II). The changes in activity of [(γ-CHC)Zn(II)] as a function of time after addition of a fixed concentration (5 μM for Cu(II) and 0.1 mM for all others) of each of these M(II) to separate incubation tubes are shown in Figure 1. Activity is increased by Cu(II) and Co(II) within 1 min, after which no further changes occur for 24 h. Ni(II), Cd(II), and Hg(II) all slowly and progressively lower activity, which levels off after 6 h.

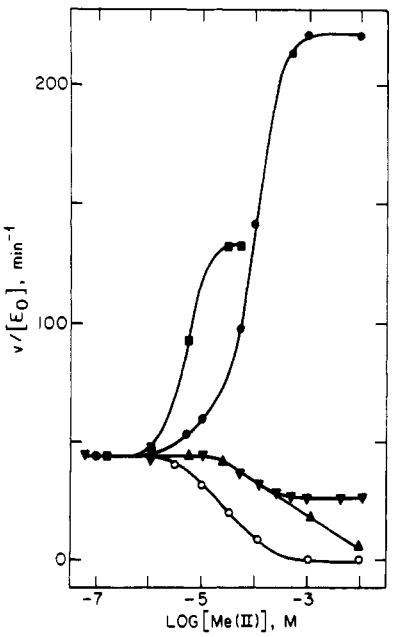

FIGURE 2: Effect of variable concentrations of (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II) on the activity of [(γ-CHC)Zn(II)]. Incubations were carried out at 0 °C at an enzyme concentration of 500 nM in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 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).

The changes in activity of [(γ-CHC)Zn(II)] as a function of the concentrations of Co(II), Cu(II), Ni(II), Cd(II), and Hg(II) have been measured after each of the reactions was allowed to reach equilibrium (Figure 2). As their concentrations are increased, Co(II) and Cu(II) raise and Ni(II) lowers the activity of the enzyme until it reaches a plateau at high [1 mM for Co(II) and Ni(II); 50 μM for Cu(II)] metal ion concentrations. The activities in the plateau regions are the same as those found when γ-CHC is reconstituted with small molar excesses of these metal ions (Angleton & Van Wart, 1988). Thus, addition of 1 mM $CoCl_2$, 50 μM $CuSO_4$, and 1 mM $NiCl_2$ results in species that have activities that are approximately 500, 300, and 60% that of [(γ-CHC)Zn(II)], respectively. These changes in activity are apparently due to replacement of the active-site Zn(II) by these ions. The addition of 1 μM Zn(II) to all of the incubation tubes returns the activity to that of [(γ-CHC)Zn(II)] (Table I).

Increasing concentrations of Cd(II) and Hg(II) monotonically decrease activity, resulting in total and almost total inhibition, respectively, at a final concentration of 10 mM. These changes are not totally reversed by 1 μM Zn(II) (Table I), and it is unclear whether the inhibition is due solely to binding of these ions at the active site or to other sites. It should be noted that Zn(II) also inhibits [(γ-CHC)Zn(II)] at concentrations above 1 μM. The inhibition is instantaneous, and the concentration dependence is summarized in Table I. As with thermolysin (Holmquist & Vallee, 1974), this inhibition is probably due to binding of a second Zn(II) to the protein.

The results shown in Figure 2 for Cu(II) are highly dependent on the buffer used for the incubations and assays. The choice of buffer is extremely important for these studies, since buffers are potential chelators and can decrease the free M(II)

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

M(II)	[M(II)] (mM)	$v/[E_0]$ (min^{-1})
Zn(II)	0	50
	0.001	48
	0.01	38
	0.10	25
	1.0	10
Co(II)	1.0	240
	1.0 (+1 μM $ZnSO_4$)	50
Ni(II)	1.0	30
	1.0 (+1 μM $ZnSO_4$)	50
Cu(II)	0.01	130
	0.01 (+1 μM $ZnSO_4$)	50
Cd(II)	1.0	0
	1.0 (+1 μM $ZnSO_4$)	12
Hg(II)	10	8
	10 (+1 μM $ZnSO_4$)	8

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

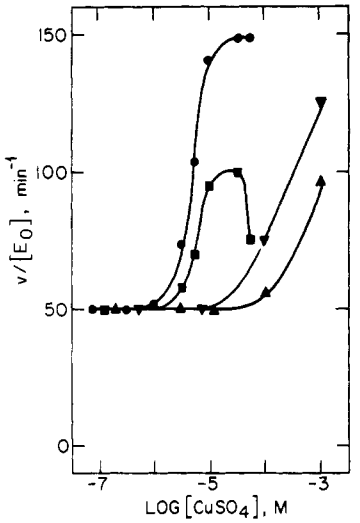

FIGURE 3: Effect of different buffers on the exchange of Cu(II) for Zn(II) in [(γ-CHC)Zn(II)] as measured by the variation in activity as a function of Cu(II) concentration. Assays were carried out in 5 mM (●) Hepes, (■) barbital, (▼) Tricine, and (▲) Tris at 25 °C in 0.4 M NaCl and 10 mM $CaCl_2$, pH 7.5, at a FALGPA concentration of 0.1 mM and at an enzyme concentration of 50 nM.

concentration (Good & Izawa, 1972). This is illustrated by the data shown in Figure 3 for the incubation of [(γ-CHC)-Zn(II)] with Cu(II) in four different buffers. Higher concentrations of Cu(II) are required to effect the exchange of Cu(II) for Zn(II), as the buffer is changed from Hepes to barbital to Tricine to Tris. Apparently, these buffers have markedly different affinities for Cu(II).

The effects of divalent metal ions on [(ζ-CHC)Zn(II)] have been investigated by the same procedures described above for [(γ-CHC)Zn(II)]. The time dependence of the activity changes observed on incubation of the enzyme with a fixed concentration of each divalent metal ion [5 μM for Cu(II) and 0.1 mM for all others] is shown in Figure 4. Cu(II), Cd(II), and Hg(II) all lower the activity of the enzyme. The inhibition observed with Cu(II) is complete within 1 min, while 2 h of incubation are required for Cd(II) and Hg(II) to exert their full effect. Co(II) instantaneously stimulates the enzyme, while Ni(II) *appears* to have no effect.

The variations in the activity of [(ζ-CHC)Zn(II)] when it is incubated with variable concentrations of these five M(II) and allowed to reach equilibrium are plotted in Figure 5. Co(II) causes an increase in activity that levels off at a concentration of approximately 3 mM. This activation can be

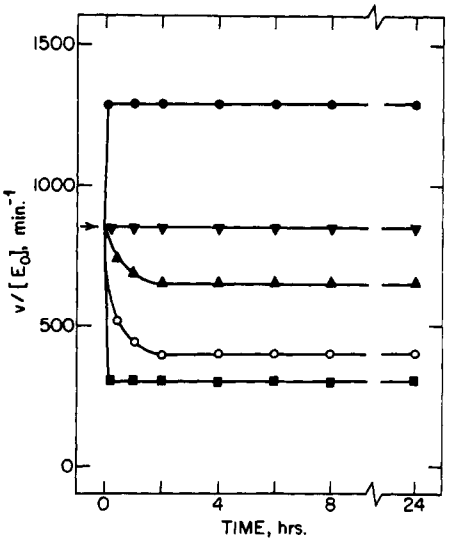

FIGURE 4: Variation in the activity of [(ζ-CHC)Zn(II)] as a function of time after addition of (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II). Metal ion concentrations in both the incubation and assay solutions were 5 μM for Cu(II) and 0.1 mM for all others. Incubations were carried out at 0 °C at an enzyme concentration of 500 nM 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 10 nM.

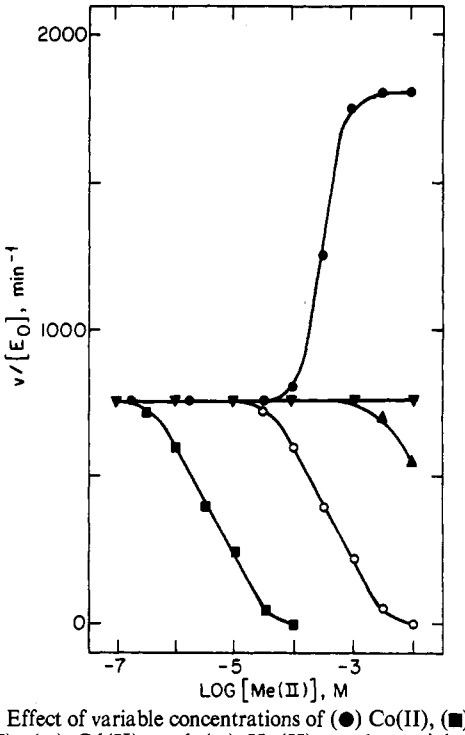

FIGURE 5: Effect of variable concentrations of (●) Co(II), (■) Cu(II), (▼) Ni(II), (○) Cd(II), and (▲) Hg(II) on the activity of [(ζ-CHC)Zn(II)]. Incubations were carried out at 0 °C at an enzyme concentration of 500 nM in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Assays were carried out at 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 10 nM.

completely reversed by the addition of 10 μM Zn(II), which has a minimal effect on [(ζ-CHC)Zn(II)] (Table II). The addition of Ni(II) produces no apparent change in activity. This is consistent with the earlier results (Angleton & Van Wart, 1988) for the reconstitution of ζ-CHC with Ni(II) which, although carried out at a different FALGPA concentration, indicate that there is little difference in the activities of [(ζ-CHC)Zn(II)] and [(ζ-CHC)Ni(II)].

Table II: Effect of Divalent Metal Ions on the Activity of [(ζ-CHC)Zn(II)][a]

M(II)	[M(II)] (mM)	$v/[E_0]$ (min^{-1})
Zn(II)	0	750
	0.001	730
	0.01	700
	0.10	380
	1.0	225
Co(II)	3.0	1800
	3.0 (+1 μM $ZnSO_4$)	750
Ni(II)	10	750
	10 (+1 μM $ZnSO_4$)	600
Cu(II)	0.01	230
	0.01 (+1 μM $ZnSO_4$)	600
Cd(II)	10	0
	10 (+1 μM $ZnSO_4$)	600
Hg(II)	10	600
	10 (+1 μM $ZnSO_4$)	730

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

Cu(II) and Cd(II) inhibit [(ζ-CHC)Zn(II)] completely at concentrations of 0.1 and 10 mM, respectively. In both cases, inhibition is only partially reversed by 10 μM Zn(II) (Table II). Hg(II) inhibits partially as its concentration is increased from 1 to 10 mM, and this inhibition is fully reversed by 10 μM Zn(II). [(ζ-CHC)Zn(II)] is also inhibited instantaneously by Zn(II) at concentrations above 1 μM (Table II). The magnitude of the inhibition is similar to that for [(γ-CHC)-Zn(II)].

Determination of Kinetic Parameters for Metallocollagenases. In order to fully assess the effect of active site metal substitutions on the activity of γ- and ζ-collagenases, the individual kinetic parameters k_{cat} and K_M have been determined for all of the active metallospecies. Double-reciprocal plots have been obtained by measuring the initial rate, v, as a function of the FALGPA concentration at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. All of the metallospecies were prepared by equilibration of the native enzymes with an excess of the appropriate metal ion. The metal ion concentrations used were 3 mM $CoCl_2$, 1 mM $NiCl_2$, and 50 μM $CuSO_4$ and were selected on the basis of the results of the metal-exchange experiments. The same concentrations of these metal ions were included in the assays to prevent adventitious metal ions from displacing the active-site metal.

Double-reciprocal plots for [(γ-CHC)M(II)], where M(II) = Zn(II), Co(II), Ni(II), and Cu(II), are shown in Figure 6. Similar plots (not shown) were obtained for [(ζ-CHC)M(II)] where M(II) = Zn(II), Co(II), and Ni(II). All seven of these plots are linear, indicating that all of the reactions obey Michaelis–Menten kinetics and exhibit no kinetic anomalies. Thus, the parameters k_{cat} and K_M have been estimated from the y and x intercepts of these plots, respectively, and the values obtained are summarized in Table III.

For γ-collagenase, substitution of Co(II) and Ni(II) for Zn(II) decreases K_M approximately 2-fold, while substitution by Cu(II) lowers K_M by 30-fold. For ζ-collagenase, substitution of Co(II) for Zn(II) decreases K_M approximately 3-fold, while the Ni(II) enzyme has the same K_M value as the Zn(II) enzyme. The k_{cat} values for the [(γ-CHC)M(II)] vary widely, ranging from 150 min^{-1} for Ni(II) to 1300 min^{-1} for Co(II). For [(ζ-CHC)Me(II)], the k_{cat} values vary little and fall in the 3000–3400-min^{-1} range. The values of k_{cat}/K_M for [(γ-CHC)Me(II)] vary widely and follow the order Cu(II) > Co(II) > Zn(II) > Ni(II). For [(ζ-CHC)M(II)], the overall order of specificity is Co(II) > Ni(II) > Zn(II). The most striking difference between the two enzymes, of course, is the observation that [(ζ-CHC)Cu(II)] is inactive.

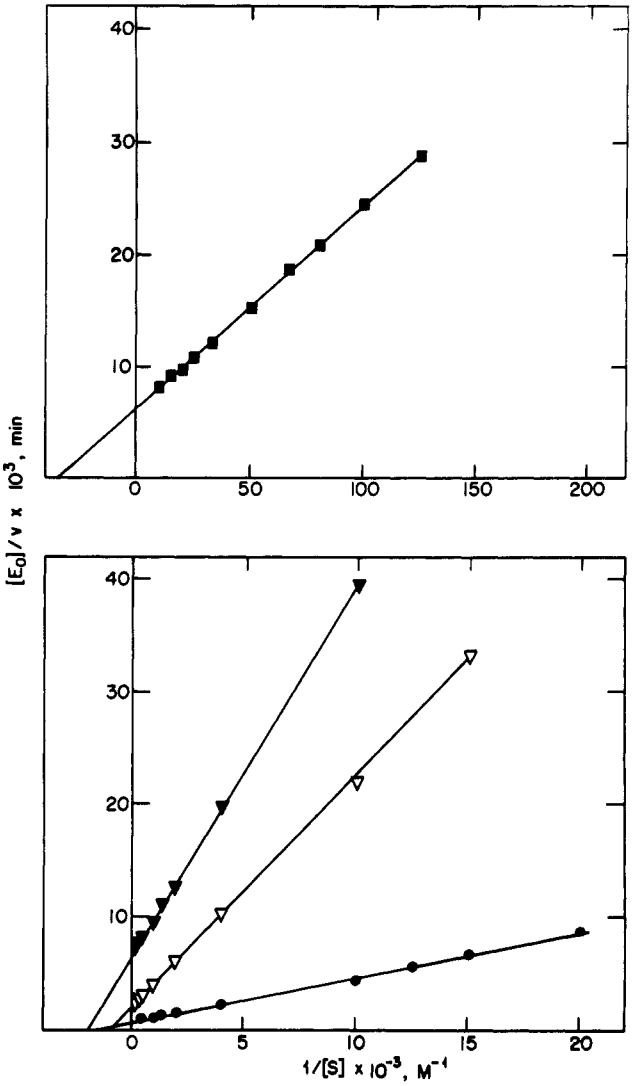

FIGURE 6: Double-reciprocal plots for the hydrolysis of FALGPA by various [(γ-CHC)M(II)], where M(II) = (▽) Zn(II), (●) Co(II), (▼) Ni(II), and (■) Cu(II). Assays were carried out at 25 °C in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at varying concentrations of FALGPA in the presence of excess metal ions as described in the text.

Table III: Kinetic Parameters for the Hydrolysis of FALGPA by Various [(γ- or ζ-CHC)M(II)][a]

	M(II)	[M(II)] in assay (mM)	k_{cat} (min^{-1})	K_M (mM)	$10^{-4}(k_{cat}/K_M)$ (M^{-1} min^{-1})
[(γ-CHC)M(II)]	Zn(II)		530	1.1	50
	Co(II)	3.0	1300	0.51	260
	Cu(II)	0.050	160	0.030	520
	Ni(II)	1.0	150	0.51	30
[(ζ-CHC)M(II)]	Zn(II)		3000	0.32	950
	Co(II)	3.0	3200	0.090	3600
	Ni(II)	1.0	3400	0.33	1000

[a] All assays were carried out in 5 mM Hepes, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5 at 25 °C.

Discussion

The study of active site metal substituted collagenases has the potential to provide insights into the role of the metal ion in catalysis. It is preferable to be able to study such species without first preparing the apoenzymes, since the latter are laborious to prepare and are easily contaminated by adventitious metal ions. This is particularly true when only small quantities of enzyme are available. Holmquist and Vallee have shown that metal replacements can be effected for thermolysin

Table IV: Comparison of the Peptidase Activities of Several Active Site Metal Substituted Metalloproteinases

enzyme	activity,[a] 100 ($v_{M(II)}/v_{Zn(II)}$)						ref
	Zn(II)	Co(II)	Cu(II)	Ni(II)	Cd(II)	Hg(II)	
γ-collagenase	100	500	1000	60	<5	<5	this work
ζ-collagenase	100	380	<5	110	<5	<5	this work
thermolysin	100	200	<5	<5	<5	<5	*b*
carboxypeptidase A	100	160	<5	110	<5	<5	*c*
angiotensin converting enzyme	100	55	<5	<5	<5	<5	*d*
Aeromonas aminopeptidase	100	510	7300	5200		<5	*e*

[a] The activities listed reflect k_{cat}/K_M values for the following peptide substrates: FALGPA for both collagenases; FA-Gly-Leu-NH_2 for thermolysin; Cbz-Gly-Phe for carboxypeptidase A; FA-Phe-Gly-Gly for angiotensin converting enzyme; and Ala-*p*-nitroanilide for *Aeromonas* aminopeptidase. [b] Holmquist and Vallee (1974). [c] Coleman and Vallee (1961). [d] Bunning and Riordan (1985). [e] Prescott et al. (1985). This enzyme has two metal-binding sites. The activities listed are those for species with M(II) at the active site and Zn(II) at the second site.

by direct metal exchange (Holmquist & Vallee, 1974). For example, the addition of increasing amounts of Co(II) to native thermolysin results in an instantaneous increase in activity due to the production of Co(II)–thermolysin. The addition of a slight excess of Zn(II) is sufficient to reverse this process and restore both the activity and Zn(II) content to that of the native enzyme. The present study demonstrates that several [(γ- or ζ-CHC)M(II)] can also be prepared by direct metal exchange. The addition of Co(II), Cu(II), Ni(II), Cd(II), and Hg(II) to [(γ- or ζ-CHC)Zn(II)] results in changes in activity that parallel those observed for the reconstitution of the respective apoenzymes with these metal ions (Angleton & Van Wart, 1988).

The rates of the metal-exchange reactions for these collagenases depend upon the identity of the metal ion. For both collagenases, the exchange of Co(II) or Cu(II) for Zn(II) is complete within 1 min (Figures 1 and 4). However, the exchange of Ni(II), Cd(II), and Hg(II) for Zn(II) in [(γ-CHC)Zn(II)] and Cd(II) and Hg(II) for Zn(II) in [(ζ-CHC)Zn(II)] is measurably slower. It is not possible to conclude anything about the time dependence of the exchange of Ni(II) for Zn(II) in [(ζ-CHC)Zn(II)], since both species have almost the same kinetic parameters. The finding that the exchange rates in these experiments for the same collagenase depend on the identity of the metal ion means that the rates are not determined solely by the off-rate of Zn(II). In fact, this suggests that the mechanism by which the exchange occurs depends upon the properties of the individual metal ions and their interactions with the enzymes.

It should be noted that excess Zn(II) instantaneously inhibits the native collagenases. As with thermolysin and other zinc metalloproteases, this inhibition is probably due to the binding of a second atom of Zn(II) to amino acid residues other than those located at the normal metal binding pocket. The *C. histolyticum* collagenases are known to contain essential Tyr and carboxyl residues in the active site (Bond & Van Wart, 1984b). It is possible that the inhibition by Zn(II) is due to binding to one of these residues. In thermolysin, the inhibition by Zn(II) is believed to arise from binding to an essential His residue (Holmquist & Vallee, 1974).

The effects of metal substitution on activity are different for the two collagenases. The most significant difference is observed on substitution of Cu(II) for Zn(II). For [(γ-CHC)Zn(II)], this results in a 10-fold increase in k_{cat}/K_M, while for [(ζ-CHC)Zn(II)], there is a total loss of activity. Together with the other more subtle differences in the kinetic parameters for the active γ- and ζ-metallocollagenases listed in Table III, this exemplifies further the differences in the active-site structures of these two classes of *C. histolyticum* collagenases. Thus, the differences in their metal-binding characteristics can be added to the list of criteria used to distinguish the two classes.

It is of interest to compare the results presented here with those of earlier studies of the effect of divalent metal ions on *C. histolyticum* collagenases. Such comparisons can only be speculative, since earlier studies were not performed on individual, chromatographically purified enzymes. Yagisawa and co-workers (1965) have reported that the peptidase activity (as measured by the hydrolysis of 10 mM benzyloxycarbonyl-Gly-Pro-Leu-Gly-Pro) of collagenase is increased 2.5-fold when assayed in the presence of 2 mM Co(II). Similarly, dialysis of collagenase against Co(II) or the inclusion of Co(II) in the *C. histolyticum* growth medium has been reported to yield an enzyme with 1.8-times the activity of the native enzyme (Seifter & Harper, 1970, 1971). These results are qualitatively consistent with the present finding that the activities of both [(γ-CHC)Co(II)] and [(ζ-CHC)Co(II)] are higher than that for the respective Zn(II) enzymes.

Most recently, Evans and Mason (1986) have studied the effects of Co(II) on the rate of hydrolysis of 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg by an unfractionated, commercial preparation of clostridial collagenase. These workers were unable to prepare the apocollagenase, but reported that the V_{max}/K_M value of the native preparation is increased about 1.7-fold on addition of 1 mM Co(II). They found that this was due to an 11-fold increase in V_{max} and a 7-fold increase in K_M for this substrate. The authors postulated the existence of a second transition metal binding site distinct from that of the active-site Zn(II) and attributed the effects of Co(II) on the activity of the enzyme to binding at this site.

While the increase in V_{max}/K_M brought about by Co(II) is in general agreement with the results reported here, all of the other results and conclusions are incompatible with our findings. First, the results presented here and in the previous paper (Angleton & Van Wart, 1988) clearly establish for both classes of collagenase that the changes in activity observed on the addition of Co(II) to both the native and apoenzymes are due solely to the binding of this metal ion at the active site. The inability of these authors to prepare the apoenzyme and to stoichiometrically reconstitute it with Co(II) made it impossible for them to differentiate between exchange of Co(II) for Zn(II) at the active site and binding of Co(II) to a second site. Second, the *large increase* in V_{max} and *increase* in K_M are substantially different trends from those found here (Table III).

Only Yagisawa and co-workers (1965) have reported the effects of any of the other divalent metal ions studied here on the activity of a clostridial collagenase. These authors found that Zn(II) and Ni(II) inhibited peptidase activity by greater than 90% at concentrations over 10 mM. This result for Zn(II) is in reasonable agreement with the results obtained here for both collagenases. However, the results obtained here with Ni(II) are somewhat different. Specifically, while the present study shows that Ni(II) partially inhibits [(γ-

CHC)Zn(II)], the inhibition obtained by Yagisawa and associates for Ni(II) is almost complete and the inhibition curve lacks the plateau region shown in Figure 2.

A comparison of the effects of active site metal substitutions in these collagenases with those found for other zinc metalloproteinases (Holmquist & Vallee, 1974; Coleman & Vallee, 1961; Bunning & Riordan, 1985; Prescott et al., 1985) is given in Table IV. The relative peptidase activities at pH 7.5 are listed, as measured by k_{cat}/K_M, with the activity of the Zn(II) enzymes assigned a value of 100. Substitution of Co(II) for Zn(II) at the active site of all six proteinases is accompanied by retention of activity. In contrast, only *Aeromonas* aminopeptidase (Prescott et al., 1985) and γ-collagenase are active when Cu(II) resides at the active site. Generally speaking, the *Clostridium* collagenases accept metal substitutions with retention of activity better than most of the other proteinases.

The active-site metal ion in metalloproteinases can function in both the binding and catalytic steps. Thus, substitution of the active-site metal can result in changes in both K_M and k_{cat}. The changes in these parameters on metal substitution can therefore provide information on the role of the metal ion in catalysis. Carboxypeptidase A, for example, has both peptidase and esterase activities (Davies et al., 1968). Metal substitutions in this enzyme produce opposite sets of changes in k_{cat} and K_M for these two activities (Auld & Holmquist, 1974). For peptidase activity, active site metal substitutions produce marked changes in the values of k_{cat}, while K_M remains almost constant. For esterase activity, on the other hand, these substitutions produce significant changes in the values of K_M, while k_{cat} remains almost invariant. This suggests that the metal ion participates primarily in the catalytic step for the hydrolysis of peptides, but primarily in the binding step for the hydrolysis of esters.

The effects of active site metal substitution in the clostridial collagenases are not as clear-cut as for carboxypeptidase A. For γ-collagenase, there is a variation in both k_{cat} and K_M upon metal substitution (Table III). These variations suggest that the metal ion is required for both the binding and catalytic steps in the hydrolysis of FALGPA. For ζ-collagenase, however, the primary change is in K_M. This suggests that these two enzymes have somewhat different mechanisms, at least with regard to the rate-determining step for this reaction. Spectral studies of these chromophoric metallocollagenases will provide an opportunity to study in detail the environment and role of the active-site metal in these enzymes.

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REFERENCES

Angleton, E. L., & Van Wart, H. E. (1988) *Biochemistry* (preceding paper in this issue).

Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355–4360.

Bond, M. D., & Van Wart, H. E. (1984a) *Biochemistry 23*, 3077–3085.

Bond, M. D., & Van Wart, H. E. (1984b) *Biochemistry 23*, 3092–3099.

Bunning, P., & Riordan, J. F. (1985) *J. Inorg. Biochem. 24*, 183–198.

Coleman, J. E., & Vallee, B. L. (1961) *J. Biol. Chem. 236*, 2244–2249.

Davies, R. C., Riordan, J. F., Auld, D. S., & Vallee, B. L. (1968) *Biochemistry 7*, 1090–1099.

Evans, C. H., & Mason, G. C. (1986) *Int. J. Biochem. 18*, 89–92.

Good, N. E., & Izawa, S. (1972) *Methods Enzymol. 24*, 53–68.

Holmquist, B., & Vallee, B. L. (1974) *J. Biol. Chem. 249*, 4601–4607.

Prescott, J. M., Wagner, F. W., Holmquist, B., & Vallee, B. L. (1985) *Biochemistry 24*, 5350–5356.

Seifter, S., & Harper, E. (1970) *Methods Enzymol. 19*, 613–635.

Seifter, S., & Harper, E. (1971) *Enzymes (3rd Ed.) 3*, 649–697.

Thiers, R. T. (1957) *Methods Biochem. Anal. 5*, 273–335.

Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283–430.

Van Wart, H. E., & Steinbrink, D. R. (1981) *Anal. Biochem. 113*, 356–365.

Yagisawa, S., Morita, F., Nagai, Y., Noda, H., & Ogura, Y. (1965) *J. Biochem. (Tokyo) 58*, 407–416.