Magnetic Resonance Studies of the Active-Site Region of Thermolysin†

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ABSTRACT: The manganese(II) derivative of thermolysin, a thermostable endopeptidase produced by *Bacillus thermoproteolyticus*, and normally containing one zinc ion and four calcium ions per molecule, has been prepared and studied by electron and nuclear magnetic resonance methods. Electron spin resonance measurements have shown that the zinc-free enzyme strongly binds 1 equiv of manganese ion/mol which results in activity equal to 18% of that of the native zinc enzyme. Additional weaker binding may occur at the calcium sites. When both are present in solution, manganese ion and zinc ion are in equilibrium between free and bound environments with the zinc ion having approximately 10–100 times the affinity for the enzyme than the manganese ion. Thermally inactivated enzyme has been shown to have a substantially reduced ability to bind manganese ion. Nuclear magnetic resonance measurements show that the relaxation rate of water protons is enhanced due to enzyme-bound manganese ion. Temperature and frequency dependence studies of the water relaxation show that one exchangeable water molecule is bound in the first coordination sphere of the manganese ion when bound to the enzyme. Addition of inhibitors influences the relaxation in quite different ways suggesting different modes of inhibitor binding to the manganese–enzyme.

The endopeptidase thermolysin, mol wt 34,600, has recently received some attention due to its remarkable heat stability (Ohta *et al.*, 1966; Ohta, 1967; Feder *et al.*, 1971). In addition, the primary sequence (Titani *et al.*, 1972) and X-ray structure (Matthews *et al.*, 1972a,b; Colman *et al.*, 1972) have been determined. It has been shown that thermolysin is a metalloenzyme binding one zinc ion and four calcium ions per molecule (Feder *et al.*, 1971; Latt *et al.*, 1969). While calcium ions have been implicated in contributing to the thermal stability of the enzyme (Feder *et al.*, 1971), the zinc ion, which can be reversibly removed by treatment with 1,10-phenanthroline (Feder and Garrett, 1971), has been shown to be essential for activity (Latt *et al.*, 1969), and is bound at the active site of the enzyme (Matthews *et al.*, 1972a). In this study, information concerning the active site of the molecule has been obtained by replacing the zinc ion in the native enzyme with the paramagnetic manganese ion. As has been demonstrated in other macromolecular systems, bound paramagnetic ions can serve as built-in magnetic probes of the detailed structure of these systems in the vicinity of the bound ion. Electron spin resonance (esr) and nuclear magnetic resonance (nmr) techniques have been used to monitor the interaction of the manganese ion with the enzyme and the enzyme with three inhibitors. These inhibitors include carbobenzoxy-L-phenylalanine, β-phenylpropionyl-L-phenylalanine, and L-phenylalanyl-L-phenylalaninamide. Details of the binding of the manganese ion at the zinc site of the enzyme, the nature of the coordination sphere of the bound manganese ion, and its perturbation by the binding of inhibitors are presented in this communication.

Experimental Section

Materials. Thermolysin, three times crystallized, A grade, and Hepes,[1] A grade, were obtained from Calbiochem. 1,10-Phenanthroline monohydrate was obtained from Mallinckrodt. Sephadex G-25 and G-100 were purchased from Pharmacia Fine Chemicals, Inc. Carbobenzoxy-L-phenylalanine, β-phenylpropionyl-L-phenylalanine, L-phenylalanyl-L-phenylalaninamide, and FAGLA were purchased from Cyclo Chemical Co. All other chemicals were reagent grade of the highest quality generally available. Double distilled water was used throughout and care was taken to avoid contamination by metal ions.

Methods. Enzyme devoid of zinc ion but retaining its four calcium ions was prepared by dissolving 50–100 mg of starting material in 4 ml of buffer containing 0.01 M Hepes (pH 7.2) (Radiometer Model 26), 0.01 M $CaCl_2$, 2×10^{-3} M 1,10-phenanthroline, and 20% glycerol (v/v), placing on a Sephadex G-25 (medium) column (1 × 25 cm) and eluting with the same buffer. The enzyme was detected by the method of Lowry *et al.* (1951) because of the high uv absorption of the solution due to the 1,10-phenanthroline, and by assay using FAGLA as a substrate. Fractions containing significant concentrations of enzyme were pooled and then separated from the 1,10-phenanthroline by passing the material through a second G-25 column (2.5 × 30 cm) containing 0.01 M Hepes (pH 7.2), 0.01 M $CaCl_2$, and 10% glycerol. The enzyme was detected by measuring the optical density of the fractions at 280 mμ with a Cary Model 14 spectrophotometer and calculating the concentration using the known extinction coefficient of the enzyme (Ohta *et al.*, 1966). Metal ion concentrations were determined using a Techtron Model 5 atomic absorption spectrometer with an acetylene–air flame. Zinc, manganese, and calcium measurements using the appropriate hollow-cathode lamp operated at 5 mA current were performed at 213.9, 279.5, and 422 mμ, respectively. Freshly prepared standards, dissolved in the same buffer, were run with each set of samples.

Kinetic measurements were performed by following the hydrolysis of FAGLA at 345 mμ using a Cary Model 14 spectrophotometer as described previously (Feder, 1968). Assay conditions were: 1.0×10^{-3} M FAGLA, $\sim 10^{-5}$ M enzyme, 0.01 M $CaCl_2$, 0.01 M Hepes (pH 7.2), and 3×10^{-5} M $ZnCl_2$ when zinc-free enzyme was used. Solutions of 1.0×10^{-4}–1.0×10^{-2} M of the inhibitors in the assay mixture were added to the

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[1] Abbeviations used are: FAGLA, furylacryloylglycyl-L-leucinamide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

TABLE I: Two Typical Chromatographies of Native Thermolysin on Sephadex G-25.[a]

Fraction	E (M)	Zn (M)	Ca (M)	Zn/E	Ca/E
15	1.3×10^{-5}	1.7×10^{-5}	6.0×10^{-5}	1.31	4.61
16	7.5×10^{-5}	9.1×10^{-5}	3.3×10^{-4}	1.21	4.40
17	2.8×10^{-5}	3.5×10^{-5}	1.3×10^{-4}	1.25	4.64
18	6.0×10^{-6}	7.0×10^{-6}	2.7×10^{-5}	1.17	4.50
15	1.8×10^{-5}	2.2×10^{-5}	7.2×10^{-5}	1.22	4.00
16	6.7×10^{-5}	7.5×10^{-5}	2.4×10^{-4}	1.12	3.58
17	1.7×10^{-5}	2.1×10^{-5}	7.2×10^{-5}	1.24	4.24
18	3.3×10^{-6}	3.5×10^{-6}	1.4×10^{-5}	1.06	4.24

[a] 25 mg of starting material in 2 ml of buffer was placed on the Sephadex G-25 column and eluted with buffer containing 0.01 M Hepes (pH 7.2) and 10% (v/v) glycerol. Enzyme concentration was determined from the known extinction coefficient at 280 mμ. Zinc and calcium ion concentrations were obtained by atomic absorption.

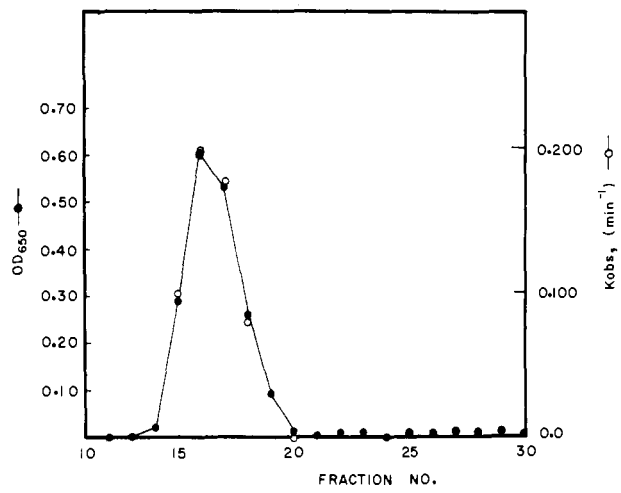

FIGURE 1: Sephadex G-25 chromatography of thermolysin in the presence of 1,10-phenanthroline. The buffer contained 0.01 M Hepes (pH 7.2), 10% glycerol, 0.01 M $CaCl_2$, and 2.0×10^{-3} M 1,10-phenanthroline. Protein concentrations (●) were determined by the Lowry method and enzyme activity (○) was measured using the FAGLA assay as described in the text.

assay mixture together with a constant enzyme concentration for the determination of the inhibition constants of the inhibitors. The cell was thermostated throughout to maintain a temperature of 25 ± 1°.

Esr spectra were obtained with a Varian E-4 spectrometer equipped with a Varian Model V-4350 variable-temperature accessory to maintain the temperature at 25 ± 1°. For quantitative measurements, samples were introduced into the cavity by placing exactly 100 μl of solution in a disposable micropipet which was then placed inside a quartz esr tube repositioned exactly in the cavity for each measurement. Instrument settings were the same (except for receiver gain) for each determination: field setting 3400 G, scan range 1000 G, modulation amplitude 2.0×10^0, microwave power 32 mW, time constant 1.0 sec and scan time 8 min. For experiments determining the binding of manganese and/or zinc ions to the zinc-free enzyme, 1.00 ml of zinc-free enzyme was mixed with 10 μl of the appropriate concentration of standard $ZnCl_2$ and/or $MnCl_2$ solution. Calcium concentration was also varied over the range from 1 to 100 mM to test the effect of Ca(II) concentration on the binding.

The longitudinal or spin–lattice relaxation time, T_1, of the water protons of samples containing enzyme with and without metal ions were measured at 25 MHz using an NMR Specialities PS-60A pulsed nuclear magnetic resonance spectrometer system. Measurements at 100 MHz were performed using a Varian XL100 nuclear magnetic resonance spectrometer operating in the Fourier transform mode together with a 16K 620i Varian computer. With both systems, T_1 was measured using a π, τ, $\pi/2$ RF pulse sequence in which the spin population is first inverted with a π pulse, allowed to relax for a variable time τ, followed by a $\pi/2$ observing pulse. Values of T_1 were calculated using a least-squares fit to a single exponential. Reproducibility of the T_1 values obtained was usually about 1%. Spin–spin relaxation times, T_2, were measured at 25 MHz using the Carr–Purcell (1954) pulse sequence with Meiboom and Gill (1958) modification. The amplitudes of the set of spin-echo signals are seen to decay exponentially with time with a time constant of T_2. T_2 relaxation times at 100 MHz were determined from line-width measurements with $T_2 = 1/(\pi \cdot \nu_{1/2})$ where $\nu_{1/2}$ is the width at half-height of the resonance line.

Results

Preparation of Thermolysin Devoid of Zinc Ion (Zinc-Free Thermolysin). Initial attempts to prepare the zinc-free enzyme invariably resulted in a rather high zinc content (usually about 0.5 mol/mol of enzyme). This is apparently due to the high affinity of the enzyme for zinc ion and the tendency of zinc ion to bind to various chromatography supports where it could be scavenged by the enzyme. A rather elaborate purification scheme had to be devised to prepare zinc-free enzyme with only 1–2 mol % zinc. The purification also removes the peptides produced by autolysis of the enzyme. Chromatography of commercial enzyme preparations on Sephadex G-25 or G-100, using 0.01 M Hepes (pH 7.2) and 10% glycerol buffer, gives a single major protein peak. Analysis of this peak for metal ions shows one zinc ion and four calcium ions per mole of enzyme (Table I), suggesting that these ions are tightly bound. This agrees with earlier solution data and recent X-ray results. Activity of the chromatographed material using the FAGLA hydrolysis assay gave $k = k_{obsd}/[E_0] = 1.0 \pm 0.1 \times 10^6$ M^{-1} min^{-1} also in agreement with previous results (Latt *et al.*, 1969). The zinc-free enzyme was prepared by including 2×10^{-3} M 1,10-phenanthroline in the buffer described. As shown in Figure 1, the elution profile of the column when analyzed both with Lowry's method and for enzymatic activity shows an overlap of the two peaks, indicating that treatment with 1,10-phenanthroline does not affect the activity of the enzyme when zinc ion is added back to the mixture. Furthermore, analysis of the elution profile shows that 1,10-phenanthroline removes zinc ion from the enzyme; the zinc ion is seen to be present in the trailing fractions well separated from the enzyme (Figure 2). Chromatography on a second G-25 column served to separate the zinc-free enzyme from the 1,10-phenanthroline. This second Sephadex column was exhaustively eluted with buffer containing 2×10^{-3} M 1,10-phenanthroline to remove bound zinc from the Sephadex. The column was then washed with two column volumes of buffer alone to remove the phenanthroline and the pooled fractions from the first enzyme chromatography were applied. The enzyme was obtained with >98% zinc-free enzyme present in the main fractions (Table II). This material shows full activity when equimolar amounts of zinc ion were added back. Addition of zinc ion over one-to-one amounts

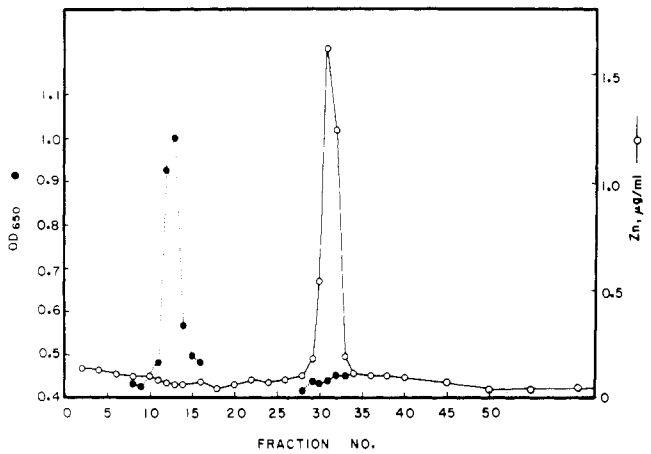

FIGURE 2: Sephadex G-25 chromatography of thermolysin in the presence of 1,10-phenanthroline. The buffer contained 0.01 M Hepes (pH 7.2), 10% glycerol, 0.01 M $CaCl_2$, and 2.0×10^{-3} M 1,10-phenanthroline. Protein concentrations (●) were measured by the Lowry method and zinc ion concentrations (○) determined by atomic absorption spectroscopy as described in the text.

TABLE III: Enzyme–Manganese(II) Activity.[a]

Solution	k_{obsd} (min^{-1})	$k_{obsd}/(E)$ (mol^{-1} min^{-1} l.)	% A^b
Zinc-free enzyme	1.79	4.84×10^4	5
Zinc-free enzyme + Mn(II)	8.79	2.38×10^5	24
Zinc-free enzyme + Mn(II)	7.95	2.15×10^5	22

[a] Assay conditions: 0.500 ml of 7.40×10^{-5} M zinc-free enzyme, 1.0×10^{-2} M $CaCl_2$, 10% glycerol, 0.01 M Hepes (pH 7.2) was mixed with 0.500 ml of Substrate Solution (S) of 2.0×10^{-3} M FAGLA, 1.0×10^{-2} M $CaCl_2$, 10% glycerol, and 0.01 M Hepes (pH 7.2); 10 μl of 0.01 M $MnCl_2$ solution was added to measure the Mn(II) activity. The residual zinc concentration under the assay conditions was 3.7×10^{-6} M as determined by atomic absorption measurements. [b] 100% activity for the native zinc–enzyme equal to 1.0×10^6 M^{-1} min^{-1}.

caused inhibition of enzyme activity as noted previously (Latt *et al.*, 1969). Complete inhibition occurred for Zn(II) concentrations greater than 10 mM. The nature of the inhibition by excess zinc ion is currently being investigated in this laboratory.

Enzyme–Manganese Activity. Using a solution of zinc-free enzyme prepared as described, the activity of the manganese derivative was estimated. The residual zinc present in our most carefully prepared buffers made it necessary to use rather high enzyme concentrations in the assay. At a concentration of 10^{-6} M enzyme only about 8 mol % zinc ion was present. The activity of the enzyme under these conditions was 5% of the native activity, slightly less than expected from the zinc content. Addition of 1 mol of manganese ion/mol of enzyme in the assay mixture gave 23% of the native activity. Thus approximately 18% of the activity is due to the presence of manganese–enzyme complex. It should be pointed out that under the assay conditions, the enzyme-catalyzed hydrolysis of FAGLA is first order in FAGLA concentration. Thus the observed rate includes contributions both from binding and catalysis. Our value of 18% activity refers to this observed rate and it is possible that the binding of FAGLA changes when manganese replaces zinc at the active site. Our studies of inhibitor binding to the manganese enzyme suggest that binding is weakened by the replacement of zinc with manganese ion at the active site. Thus the value of 18% activity is likely to be somewhat low. The results of these experiments are shown in Table III.

TABLE II: Atomic Absorption Data for the Chromatographic Preparation of Zinc-Free Enzyme.[a]

Fraction	E (M)	Zn (M)	% Zn/E
14	1.9×10^{-5}	3.3×10^{-6}	17
15	1.2×10^{-4}	2.5×10^{-6}	2.2
16	1.6×10^{-4}	2.6×10^{-6}	1.6
17	1.5×10^{-4}	2.2×10^{-6}	1.5
18	5.8×10^{-5}	1.0×10^{-6}	1.7
19	1.8×10^{-5}	8.3×10^{-7}	4.6
20	6.0×10^{-6}	4.0×10^{-7}	7.0

[a] Zinc-free enzyme following treatment with 1,10-phenanthroline and Sephadex G-25 chromatography. Enzyme concentration was determined from absorbance at 280 mμ. Zinc ion concentration was measured by atomic absorption.

Determination of K_I for the Inhibitor β-Phenylpropionyl-L-phenylal anine with Thermolysin. For the determination of the dissociation constant of the enzyme–inhibitor complex, a 1.0×10^{-5} M solution of the native purified one zinc, four calcium enzyme in buffer (0.01 M Hepes (pH 7.2), 0.01 M $CaCl_2$, 10% glycerol) was assayed in the presence of inhibitor from 1.0×10^{-4} to 5.0×10^{-3} M inhibitor. From the measured activity, θ, a plot of log $[\theta/(1-\theta)]$ *vs.* log $[I]$ at constant enzyme concentration gave a straight line of slope -1.07 ± 0.05 and $K_I = 1.0 \pm 0.1 \times 10^{-3}$ M. The inhibition constants of the three inhibitors were determined by following the rate of the hydrolysis of FAGLA at different inhibitor concentrations while keeping the substrate and enzyme concentrations constant. The Mn(II) derivative of the enzyme was used since different results were obtained for the Mn(II)–enzyme and the native zinc–enzyme for β-phenylpropionyl-L-phenylalanine. This inhibitor had a value of K_I equal to $1.0 \pm 0.1 \times 10^{-3}$ M with the native zinc–enzyme whereas the manganese–enzyme derivative gave a $K_I \simeq 3 \times 10^{-3}$ M. Inhibition constants for two other inhibitors, carbobenzoxy-L-phenylalanine and L-phenylalanyl-L-phenylalaninamide, also were on the order of $3\text{–}5 \times 10^{-3}$ M. Exact results were difficult to obtain due to the interference of zinc in the assay mixtures. Accordingly for nmr relaxation experiments 0.01 M solutions of the inhibitors were used so that the majority of the enzyme molecules were saturated with inhibitor.

Measurements of the Binding of Mn(II) to Zinc-Free Enzyme. The esr spectrum of manganese(II) ion in aqueous solution consists of six relatively sharp resonances. Usually the esr spectrum of manganese(II) ion when bound to a macromolecule is broadened beyond detection. Comparison of the esr intensity of a given manganese(II) ion concentration to the intensity of the same concentration of ion in the presence of the enzyme provides a relatively simple method for determination of the number of bound manganese ions. Of course, this does not establish where the ions are bound, only that binding occurs somewhere on the macromolecule. A 1.0×10^{-4} M solution of $MnCl_2$ in buffer (0.01 M Hepes (pH 7.2), 0.01 M $CaCl_2$, and

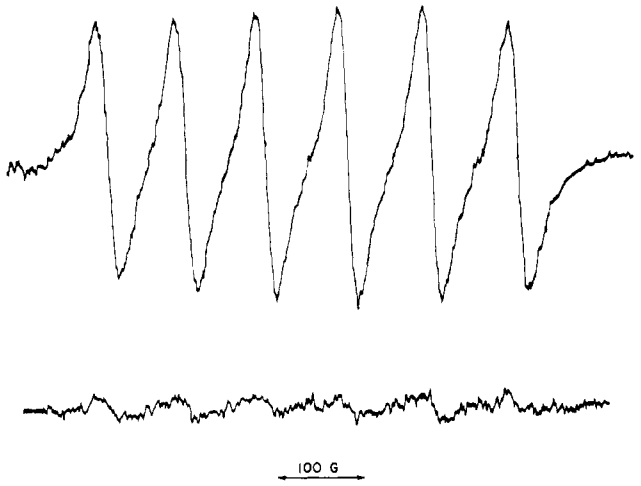

FIGURE 3: Esr spectra of Mn(II) and Mn(II) + zinc-free enzyme. The top spectrum is that of a solution containing 1.0×10^{-4} M Mn(II) in Hepes buffer (pH 7.2), 10% glycerol, and 0.01 M $CaCl_2$. The bottom spectrum is the result of the addition of 9.0×10^{-5} M zinc-free enzyme.

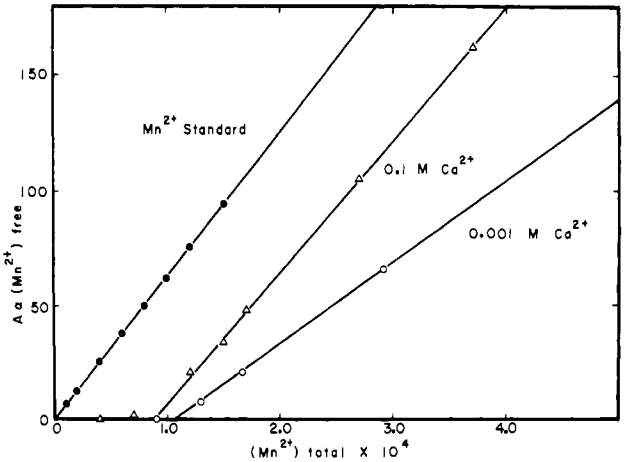

FIGURE 4: Esr titration of zinc-free enzyme with Mn(II). Aliquots of 0.01 M Mn(II) were added to 1.0×10^{-4} M solutions of the zinc-free enzyme in Hepes buffer (pH 7.2), 10% glycerol, and the indicated Ca(II) concentrations, and the esr spectra were recorded. The amplitude, A, of the signal was taken as the average of the intensities of the six lines. The Mn(II) standard line was determined by adding Mn(II) to the buffer only.

10% glycerol) gives the expected six-line spectrum with peak-to-peak widths of about 20 G and overall spectrum width of about 650 G in the X-band esr spectrometer. For quantitative intensity measurements, the average of the amplitudes of the six lines was used. Studies from 10^{-5} to 0.01 M Mn(II) showed a linear dependence of line intensity on concentration over the entire range. As shown in Figure 3, addition of zinc-free enzyme reduces the intensity of the esr signal with no other changes in the spectrum.

When Mn(II) concentrations less than the zinc-free enzyme concentrations were employed, no esr signal could be detected, implying that all the Mn(II) was bound to the enzyme. Upon addition of excess Mn(II) the characteristic six-line spectrum of free Mn(II) returned. The amplitude of the signal was proportional to the excess Mn(II) added. Quantitative measurements of the signal intensity revealed that the intensity of the Mn(II) spectrum in the presence of constant concentrations of zinc-free enzyme was also sensitive to the Ca(II) concentration. At higher concentrations of Ca(II) an increase in intensity of the Mn(II) signal was observed.

Figure 4 shows plots of the esr intensity of Mn(II) as a function of added Mn(II) to a constant concentration of zinc-free enzyme solutions containing 1, 10, and 100 mM Ca(II), respectively. In each case the esr intensity was zero until 1 equiv of Mn(II)/mol of enzyme had been added. However, the slopes of the plots were different after 1 equiv of Mn(II) ion had been added. The slope was steepest at the highest Ca(II) concentration and, as can be seen in Figure 4, the slope in the presence of 100 mM $CaCl_2$ is the same for the addition of Mn(II) to a solution lacking the enzyme. These results show that Mn(II) ions beyond 1 mol/mol of enzyme can be bound rather weakly at site(s) competitive with Ca(II). At high Ca(II) concentrations, however, the Mn(II) appears to be directed to a single site. Thus care must be taken to use sufficiently high Ca(II) concentrations and low Mn(II) concentrations to assure that all the Mn(II) is at a single site if quantitative, interpretable relaxation rate studies are to be performed on the enzyme–Mn(II) complex.

Enzyme–Manganese–Zinc Equilibrium. Experiments in which zinc-free enzyme was mixed with both Mn(II) and Zn(II) were also attempted. From earlier qualitative experiments it was clear that addition of Zn(II) to solutions containing equimolar amounts of zinc-free enzyme and Mn(II) and which gave no esr signal resulted in the appearance of the characteristic Mn(II) spectrum, suggesting that Zn(II) and Mn(II) ions were competitive. To understand the nature of the relationship between the binding of the ions to the zinc-free enzyme, quantitative esr experiments were performed. In a typical experiment a constant amount of zinc-free enzyme, usually 1.0×10^{-4} M, was mixed with varying amounts of Mn(II) and Zn(II). In a separate experiment, the same concentrations were employed with the order of addition of the ions reversed. Thus in one series of experiments, various amounts of Zn(II) (10^{-5}–10^{-2} M) were added to an equimolar mixture of zinc-free enzyme and Mn(II). An alternative series consisted of adding 1 equiv of Zn(II) to the zinc-free enzyme followed by varying amounts of Mn(II) and then observing the esr spectra. Figure 5 shows the result of such a study. The intensities of the esr spectra containing the same concentrations of ions were identical, independent of the order of addition of the metal ions. Thus Mn(II) and Zn(II) competition for the enzyme is

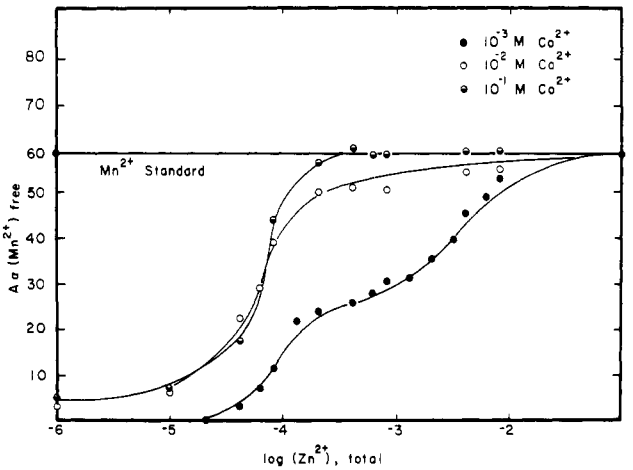

FIGURE 5: Titration of the Mn(II)-enzyme with Zn(II). Zn(II) was added to solutions containing 1.0×10^{-4} M Mn(II)-enzyme in Hepes buffer (pH 7.2), 10% glycerol, and the indicated Ca(II) concentrations. The Mn(II) standard contained 1.0×10^{-4} M Mn(II) in the same buffer.

TABLE IV: Temperature Dependence of T_1 Relaxation at 25 MHz.[a]

Temp (°C)	$(T_1)_E$ (sec)	$(T_1)_{E-Mn}$ (sec)	$(T_1)_p$ (sec)
22	1.62	0.188	0.213
32	1.78	0.212	0.241
44	2.06	0.236	0.266
58	2.44	0.285	0.322

[a] Zinc-free enzyme concentration was 1.0×10^{-4} M. 1 equiv of Mn(II) was added to obtain $(T_1)_{E-Mn}$. $(T_1)_p$ was calculated using $1/(T_1)_p = 1/(T_1)_{E-Mn} - 1/(T_1)_E$.

sufficiently rapid to have reached equilibrium in the 5–10 min required to make the esr measurement. Again, the presence of relatively high concentrations of Ca(II) is necessary to assure that Mn(II) is only going to the Zn(II) site.

From the intensity data shown in Figure 5, one can estimate that the binding of Mn(II) is approximately 10–100 times weaker than the binding of Zn(II) to the enzyme. Feder *et al.* (1971) have shown that the dissociation constant of Zn(II) from the enzyme is about 10^{-13} M. This suggests a value of 10^{-10}–10^{-11} M for the enzyme–Mn(II) dissociation constant.

Finally the interaction of Mn(II) with zinc-free enzyme was tested by taking a sample which contained a 1.0×10^{-4} equimolar mixture of Mn(II) and zinc-free enzyme. This solution gave no esr signal. Heating of this solution to 95° for 10 min resulted in complete loss of enzymic activity. Reexamination of the heated sample in the esr spectrometer showed a spectrum of intensity nearly equal to that of 1.0×10^{-4} manganese standard.

Nmr Relaxation. Zinc-free enzyme solutions (1.0–1.3×10^{-4} M) were prepared in 0.01 M Hepes buffer (pH 7.2) and 10% glycerol, containing 0.01 M $CaCl_2$. An equivalent amount of Mn(II) was added to form the Mn(II)–enzyme complex. Experiments in the presence of inhibitors were done with inhibitor concentrations of 0.01 M, well in excess of the K_I's which were previously determined.

For this system, T_{ip}, the weighted average between the relaxation rates in the first coordination sphere of the Mn(II) and the bulk solvent was determined by measuring the relaxation time of the zinc-free enzyme, T_{io}, then adding slightly less than 1 equiv amount of Mn(II) and measuring the relaxation time again, $T_{i,obsd}$. T_{ip} was then calculated from (Swift and Connick, 1962)

$$\frac{1}{T_{ip}} = \frac{1}{T_{i,obsd}} - \frac{1}{T_{io}} \qquad i = 1, 2 \qquad (1)$$

As seen from Table V the relaxation rate of water due to the enzyme-bound Mn(II) is considerably enhanced compared to the value for Mn(II) free in solution. This suggests that the Mn(II) is tightly bound to the enzyme, and that it is accessible to solvent. The reduction of the relaxation rate in the presence of a saturating concentration of the inhibitors β-phenylpropionyl-L-phenylalanine and L-phenylalanyl-L-phenylalaninamide also suggests that these inhibitors restrict this accessibility to some degree.

In order to understand the details of the interaction between the enzyme-bound Mn(II) and the water protons, the paramagnetic contribution of Mn(II) to the relaxation, T_{1M}, was carefully examined. The general relation between T_{1p} and T_{1M}, the paramagnetic contribution to the relaxation time, neglecting outer sphere relaxation, is given by (Swift and Connick, 1962; Luz and Meiboom, 1964)

$$1/T_{1p} = np/(T_{1M} + \tau_m) \qquad (2)$$

A similar expression can be written for T_2 relaxation, where T_{1p} is the ratio of the concentration of the paramagnetic ion to the concentration of the ligand, n is the number of ligands in the first coordination sphere of the paramagnetic ion, and τ_m is the lifetime of the ligand in the first coordination sphere. To determine the relative magnitudes of T_{1M} and τ_m, the temperature dependence of T_{1p} was studied. As seen from Table IV, T_{1p} is seen to increase slightly with increasing temperature over the range of 20–60°. Thus $1/T_{1p}$ decreases with increasing temperature. This suggests that at these temperatures τ_m is small compared to T_{1M} since τ_m will always decrease with increasing temperature and should be strongly temperature dependent.

Since the paramagnetic contribution to the relaxation time determines the observed relaxation, the theoretical treatment of the relaxation as derived by Soloman and Bloembergen (Soloman, 1955; Bloembergen, 1957) applies. One has for "spin only" paramagnetic ions

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma^2 g^2 \beta^2}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right] + \frac{2}{3}\frac{S(S+1)A^2}{\hbar^2}\frac{7\tau_e}{1+\omega_s^2\tau_e^2} \qquad (3)$$

and

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma^2 g^2 \beta^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\tau_e \qquad (4)$$

where S is the electron spin quantum number (5/2 for Mn(II)); γ is the nuclear magnetogyric radio; g, the electronic "g" factor; β, the Bohr magneton; ω_I and ω_s, the Larmor angular precession frequency for the nuclear and electron spins, respectively; A, the hyperfine coupling constant; r, the ion–proton internuclear distance; and τ_c and τ_e, the correlation times for dipolar and spin exchange interactions, respectively. τ_c and τ_e are themselves composed of several factors. In general

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_m} \qquad (5)$$

where τ_r is the correlation time of the rotational motion of the internuclear ion–nucleus radius vector, τ_s is the electron spin relaxation time, and τ_m is the residence time of the nuclear species in the first coordination sphere of the paramagnetic ion. The correlation time, τ_c, will be determined by the fastest process, *i.e.*, whichever time is shortest, τ_r, τ_s, or τ_m.

Similarly, the spin exchange rate $1/\tau_e$ is given by

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_m} \qquad (6)$$

Since $\omega_s = 650\omega_I$, $\omega_s^2\tau_c^2$ and $\omega_s^2\tau_e^2$ are expected to be much greater than one so the above equations can be reduced to

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)\gamma^2 g^2 \beta^2}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] \qquad (7)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma^2 g^2 \beta^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\tau_e \qquad (8)$$

Using these equations one can now, in theory, calculate the distance between the Mn(II) and the water protons in the first hydration sphere if the number of water ligands, n, is known. Alternatively having or guessing a value of r, one can determine the number of water molecules bound to Mn(II). In order to carry out these calculations one needs a value of τ_c. Presumably, a value of τ_c can be obtained from the frequency dependence of the relaxation time (Peacocke *et al.*, 1969). This treatment assumes that τ_c itself is independent of frequency. This need not be the case if τ_c contains a large contribution from τ_s, the electronic spin relaxation time. Accordingly the method of Navon (1970) was used to calculate the correlation time and hydration number of the Mn(II). Combining eq 2, 7, and 8 gives the following equation from which the correlation time may be calculated

$$\frac{T_{1p}}{T_{2p}} = \frac{1}{2} + \left[\left(\frac{2}{3} + 0.022\,\frac{\tau_e}{\tau_c}\right)(1 + \omega_I^2\tau_c^2)\right] \quad (9)$$

Taking $\tau_e \sim \tau_c$ one can substitute this expression for τ_c into eq (7) and combining with eq 2 obtain the following expression for n

$$n = \left(\frac{15}{6}\,\frac{\gamma^6}{S(S+1)\gamma^2g^2\beta^2}\right) \times \frac{(T_{1p}/T_{2p} - 0.500)}{(T_{1p}/T_{2p} - 1.189)^{1/2}}\,\frac{\omega_I}{T_{1p}}\,\frac{55.6}{N} \quad (10)$$

where N is the concentration of bound Mn(II). Substituting values of S, γ, g, β, and r for the $Mn(II)(H_2O)_6$ complex and accounting for the 10% v/v of glycerol in the solvent gives

$$n = (2.93 \times 10^{-14})\,\frac{(T_{1p}/T_{2p} - 0.500)}{(T_{1p}/T_{2p} - 1.189)^{1/2}}\,\frac{\omega_I}{NT_{1p}} \quad (11)$$

Initial results of experiments performed at 25 MHz confirmed the validity of the assumptions used in arriving at eq 9 and 10. For correlation times in the range expected for this system of between 10^{-8} to 10^{-10} sec and taking $\tau_e/\tau_c \sim 1$, one has from eq 9: $T_{1p}/T_{2p} = 1.189$ for $\omega_I^2\tau_c^2 \ll 1$. The observed value is $T_{1p}/T_{2p} = 1.206$. Thus taking $\tau_e/\tau_c \sim 1$ is justified and substitution of the observed value of T_{1p}/T_{2p} into eq 9 gives $\tau_c = 1.1 \times 10^{-9}$ sec at 25 MHz. The data taken at 25 MHz could not be used with confidence to calculate the hydration number of the Mn(II) using eq 11 because the term involving the difference between the T_{1p}/T_{2p} ratio observed and 1.189 was too small to allow for an accurate answer. In order to use this equation experiments at 100 MHz were performed where the T_{1p}/T_{2p} ratio was expected to be significantly larger than 1.189. As shown in Table V, the value of T_{1p}/T_{2p} at 100 MHz was found to be 2.95. A value of τ_c at 100 MHz of 2.5×10^{-9} sec is obtained using eq 9. The value for n, the number of freely exchanging water sites on the bound Mn(II) ion, is calculated to be 1.1, using eq 11.

TABLE V: Relaxation Measurements and Calculated Correlation Times and Hydration Numbers at 25 and 100 MHz.[a]

Sample	T_1 (sec)	T_2 (sec)	T_{1p}/T_{2p}	τ_c (sec)	n
25 MHz					
E	2.10	0.915			
E–Mn	0.187	0.143	1.21	$\leq 1.1 \times 10^{-9}$	~1.09
100 MHz					
E	2.44	0.1415			
E–Mn	0.273	0.0601	2.95	2.5×10^{-9}	1.11

[a] E is zinc-free enzyme, concentration 1.2×10^{-4} M, and E–Mn is the Mn(II) derivative, concentration 1.0×10^{-4} M. Temperature 25 ± 1°.

Discussion

Treatment of native thermolysin, which normally contains one bound zinc ion and four bound calcium ions per molecule (Table I), with 2×10^{-3} M 1,10-phenanthroline effectively removes the zinc ion resulting in inactive zinc-free enzyme (Figures 1 and 2 and Tables II and III). Separation of the zinc-free enzyme from the 1,10-phenanthroline and subsequent addition of equimolar amounts of Zn(II) or Mn(II) results in reactivation of the enzyme. Full restoration of activity was obtained with the addition of Zn(II) and about 18% activity was obtained for the addition of Mn(II) (Table III).

Electron spin resonance studies of the Mn(II) in the presence of zinc-free enzyme and other ions, notably Zn(II) and Ca(II), revealed that Mn(II) does indeed bind to the enzyme and that the binding is influenced by both of these other ions. Addition of up to equimolar amounts of Mn(II) to zinc-free enzyme results in complete loss of observable esr signal from the manganese. The complete absence of an observable esr signal suggests that the electron spin relaxation time is shortened when Mn(II) is bound to the enzyme. Changes in the shape and/or intensity of Mn(II) bound to macromolecules have been observed in a number of other systems (Malmstrom *et al.*, 1958; Mildvan and Cohn, 1963, 1965; O'Sullivan and Cohn, 1966; Miller *et al.*, 1968; Nicolau *et al.*, 1969; Reed and Cohn, 1970). Alterations in the shape and/or intensity of the manganese spectrum when bound result from the restriction of the rotational motion and/or the lowering of the electron spin relaxation time of the manganese.

Since the esr signal is a measure of the Mn(II) free in solution, esr was used to quantitatively determine the binding of Mn(II) to the zinc-free enzyme. Titration of the zinc-free enzyme with Mn(II) is shown in Figure 4. Extrapolation of the sloped portion of the line gave the binding of 1.0 ± 0.1 mol of Mn(II)/mol of zinc-free enzyme. Evidence for additional interaction of Mn(II) with the enzyme is inferred from the dependence of the slope of the line with Ca(II) concentration. If only one binding site per enzyme molecule were available to the Mn(II), then the slope of the graph after 1 equiv of Mn(II) has been added should be equal to that of Mn(II) free in solution. The data presented in Figure 4 show that only as the concentration of Ca(II) approaches 100 mM do the slopes become equal. At lower Ca(II) concentrations the slope is dependent on the total enzyme concentration suggesting that the Ca(II) binding sites serve as additional weak binding sites for Mn(II) or that Ca(II) and Mn(II) compete for weak nonspecific metal binding sites on the enzyme. Further evidence for the strong and specific binding of Mn(II) to the zinc-free enzyme was obtained in experiments in which an equimolar mixture of Mn(II) and zinc-free enzyme giving no esr signal was heated to 95° for 10 min, which irreversibly inactivates the enzyme. The intensity of esr signal is about 80% that of the standard, indicating that inactivation involves the loss of the ability of the molecule to bind Mn(II). The Ca(II) in the experiment was 1 mM, low enough to allow some of the Mn(II) to still be bound to the active enzyme. Since only about 80% of the intensity is observed after heating, it is possible that the denatured enzyme still possesses some nonspecific metal ion binding ability.

The interaction of the zinc-free enzyme with Mn(II) and Zn(II) was examined. From preliminary work it was known that a mixture of the Zn(II) and Mn(II) enzymes was obtained from a solution of the native enzyme in a solution of $MnCl_2$

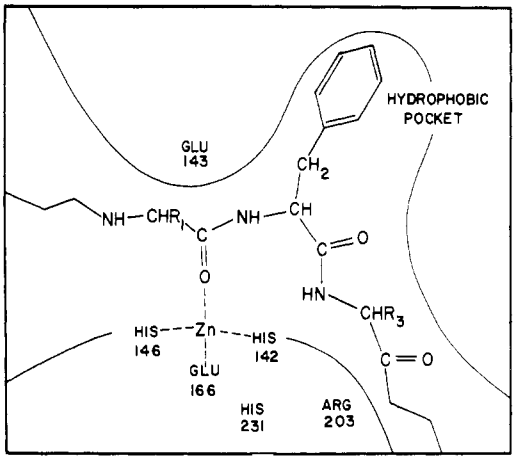

FIGURE 6: Schematic representation of the active site of thermolysin with a polypeptide substrate bound.

when chromatographed on Sephadex G-25. The sum of the concentrations of Zn(II) and Mn(II) in the enzyme-containing fractions was approximately equal to the total enzyme concentration, suggesting that Mn(II) and Zn(II) are competitive for the same site on the enzyme. To test for a condition of competitive equilibrium between the Mn(II) and Zn(II) for the enzyme, various concentrations of Mn(II) and Zn(II) were added to a solution of zinc-free enzyme. As in previous experiments, up to 1 equiv of the sum of Mn(II) plus Zn(II)/mol of zinc-free enzyme were bound. The addition of excess Zn(II) resulted in appearance of an esr Mn(II) signal caused by the displacement of some of the bound Mn(II) by Zn(II). Titration of the Mn(II)-enzyme with Zn(II) or conversely the Zn(II)-enzyme with Mn(II) showed a facile equilibrium exists between the enzyme and the two ions. Thus, for example, solutions of 1.0×10^{-4} M zinc-free enzyme and 1.0×10^{-4} M Mn(II) and Zn(II) have identical esr spectra irrespective of the order of addition of the two ions. Also, as seen in Figure 5, the concentration of Ca(II) influenced the titration results. At low Ca(II) concentration (1.0 mM), a complicated titration curve was obtained with the amplitude of the esr signal approaching the standard only as the Zn(II) concentration was increased to 10 mM. At higher Ca(II) concentrations (10 mM), a more straightforward result was obtained with complete release of bound Mn(II) for Zn(II) concentrations of approximately 1 mM. From the midpoints of the titration curves at higher Ca(II) concentrations, the relative affinities of the two ions for the enzyme can be estimated. A Zn(II) concentration of 6×10^{-5} M results in displacement of one-half of the bound Mn(II) in a 1.0×10^{-4} M equimolar mixture of Mn(II) and zinc-free enzyme, suggesting that Zn(II) has about five times the affinity for the zinc-free enzyme than Mn(II). Allowing for additional nonspecific binding of Mn(II) one might expect the affinity to be 10-100-fold higher for zinc.

Nmr relaxation experiments indicate that one molecule of exchangeable water is bound to the Mn(II) in the Mn-enzyme complex. Results at 25 and 100 MHz show that the dominant correlation time of the interaction between the bound Mn(II) and the water molecules is most probably the electron relaxation time τ_e. This correlation time is field dependent with values of 1.1×10^{-9} sec at a field corresponding to 25 MHz and 2.5×10^{-9} sec at a field corresponding to 100 MHz. An estimate of the relative contributions of rotation, electron relaxation, and exchange to the overall correlation time for the relaxation of the water protons can now be made. Since τ_c has been shown to be field dependent and from eq 5 it can be seen

TABLE VI: Effect of Inhibitors on the T_1 Relaxation.[a]

Sample	I	[I], M	T_1 (sec)
E			2.40
E-Mn			0.275
E-Mn	I_1	0.01	0.43
E-Mn	I_2	0.01	0.28
E-Mn	I_3	0.009	1.4-1.9

[a] Temperature 25 ± 1°; I_1, β-phenylpropionyl-L-phenylalanine; I_2, carbobenzoxy-L-phenylalanine; I_3, L-phenylalanyl-L-phenylalaninamide; [E], 1.2×10^{-4} M; [E-Mn], 1.0×10^{-4} M.

that τ_c will be determined by whichever term is the fastest, τ_c must be largely if not completely determined by electron spin relaxation since the rotational and exchange times should be independent of frequency. An upper value for τ_m, the exchange time, can be calculated using eq 2 and the values of T_1 given in Table V. One obtains $T_{1M} = 4.1 \times 10^{-7}$ and 6.2×10^{-7} sec at 25 and 100 MHz, respectively. From the temperature dependence of the relaxation (Table IV), τ_m must be small compared to T_{1M}, so τ_m must be less than $\sim 10^{-7}$ but greater than $\sim 10^{-9}$ sec.

Experiments done with the complex in the presence of saturating amounts of three inhibitors produced results which can be understood in light of recent X-ray diffraction data taken of the enzyme-inhibitor complexes. A schematic representation of the binding and catalytic site region of thermolysin is shown in Figure 6. Unlike most endo proteases, the specificity of cleavage by thermolysin is determined by the amino acid side chain on the amino group side of the peptide bond. The crystallographic studies (W. Kester and B. W. Matthews, unpublished results) of inhibitor-thermolysin interactions suggest a general hydrophobic pocket toward the interior of the protein which appears to determine cleavage specificity. The figure shows the probable binding orientation of a phenylalanine containing peptide with the phenylalanine residue bound in the specificity pocket (W. Kester and B. W. Matthews, unpublished results) and the peptide carbonyl oxygen atom occupying a ligand position of the zinc atom. The other zinc ligands are histidine residues 142 and 146 and glutamic acid residue 166 (Matthews *et al.*, 1972a).

A similar structure for the manganese-enzyme is suggested from the magnetic resonance studies presented here. Specifically, one water molecule acts as an exchangeable manganese ligand in the free enzyme. In the presence of the inhibitor L-phenylalanyl-L-phenylalani namide, this water molecule is apparently displaced by the inhibitor (Table VI).

The binding of the other two inhibitors is seemingly different from the normal binding mode suggested above. Carbobenzoxy-L-phenylalanine has no effect on the water relaxation rate enhancement due to the presence of the manganese-enzyme. This suggests that this inhibitor does not interact with the metal ion and presumably inhibits by binding only in the hydrophobic pocket. This binding mode can be considered to be nonproductive. The inhibitor β-phenylpropionyl-L-phenylalanine has an intermediate effect on the water relaxation rate. Physically, there must be an integral number of water molecules accessible to the metal ion; thus the simplest interpretation of the observed halving of the water relaxation rate is that this inhibitor binds in more than one orientation in the binding

site. Thus about half the inhibitor molecules appear to be bound in the productive mode and the remaining half appear to be bound non-productively. The preliminary X-ray crystallographic studies of the interaction of thermolysin and β-phenylpropionyl-L-phenylal anine are not inconsistent with the notion of more than one binding orientation for this inhibitor (W. Kester and B. W. Matthews, unpublished results).

There are a number of other residues in the thermolysin binding site region which may affect the bound orientation(s) of the inhibitors. The interaction of arginine residue 203 with the terminal carboxyl groups of the two anomalous inhibitors could be important. There are also possible electrostatic interactions of glutamic acid residue 143 with the free amino group of L-phenylalanyl-L-phenylalaninamide which are not possible with the two anomalous inhibitors. This interaction may explain the stability toward hydrolysis by thermolysin of peptides containing free α-amino groups. These questions of the effects of ionizable groups on the bound orientation of ligands are currently under investigation in this laboratory.

We have interpreted the observed shielding of the manganese ion from water in the presence of L-phenylalanyl-L-phenylalani namide as the replacement of water by the amide carbonyl oxygen atom as the fourth manganese ligand. An alternative explanation is that a water molecule still acts as the fourth metal ligand, but that the accessibility of that water molecule to the bulk solvent has been enormously diminished by inhibitor binding. Examination of the structure of the thermolysin active site suggests no reason to propose a substantially different accessability in the presence of inhibitor unless the water is actually replaced by an inhibitor moiety as the fourth metal ligand. The studies of W. Kester and B. W. Matthews (unpublished results) suggest a direct interaction of the zinc atom and carbonyl group of the inhibitor, although the interpretation of the Fourier difference map in that region is difficult.

If the amide carbonyl oxygen acts as a metal ligand in the productive binding mode, it would appear that this interaction is not the driving force for ligand binding and probably contributes little to the overall binding energy. This view is suggested from the near equality of the binding constants of these three structurally similar inhibitors despite their very different metal ion interactions. This can be rationalized if the difference in interaction energy of water with the metal and the carbonyl group with the metal is nearly zero. The stability of zinc or manganese ion complexes with neutral amides in aqueous solution supports this notion.

The results given here show additional details in the similarities which exist between the Mn(II) derivatives of thermolysin and carboxypeptidase A. Both are native Zn(II) containing enzymes at the active site and in both the metal is required for activity. Both furnish two histidine and one glutamate residues as ligands to the metal ion. Both Mn(II) derivatives are seen to bind one exchangeable water molecule in the first coordination sphere of the Mn(II) and the correlation time in both systems is determined or at least dominated by the electron relaxation time, τ_e. The correlation time in both systems is field dependent and has nearly identical values at 25 and 100 MHz; 1.2×10^{-9} sec for Mn(II)-carboxypeptidase and 1.1×10^{-9} sec for Mn(II)-thermolysin at 25 MHz and 2.3×10^{-9} and 2.5×10^{-9} sec, respectively, at 100 MHz (Navon, 1970). The effect of the bound paramagnetic ion on water relaxation can be eliminated by the addition of saturating amounts of inhibitors β-phenylpropionate for Mn(II)-carboxypeptidase (Shulman *et al.*, 1966) and L-phenylalanyl-L-phenylalaninamide for Mn(II)-thermolysin. Further, both enzymes share the unusual cleavage specificity for the amino acid residue in the amino group side of the peptide bond.

Interestingly, the overall primary structures of the two enzymes are not at all similar and the three-dimensional structures of the regions away from the active site are also completely different. These two enzymes appear to represent an extremely interesting case of convergent evolution in that the active metal site regions are really identical, both in terms of atomic structure as determined by crystallography and electron environment as determined by τ_e, the electron spin relaxation time.

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References

Bloembergen, N. (1957), *J. Chem. Phys. 27,* 572.
Carr, H. Y., and Purcell, E. M. (1954), *Phys. Rev. 94,* 630.
Colman, P. M., Jansonius, J. N., and Matthews, B. W. (1972), *J. Mol. Biol. 70,* 701.
Feder, J. (1968), *Biochem. Biophys. Res. Commun. 32,* 326.
Feder, J., and Garrett, L. R. (1971), *Biochem. Biophys. Res. Commun. 43,* 943.
Feder, J., Garrett, L. R., and Wild, B. S. (1971), *Biochemistry 10,* 4552.
Latt, S. A., Holmquist, B., and Vallee, B. L. (1969), *Biochem. Biophys. Res. Commun. 37,* 333.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40,* 2686.
Malmstrom, B. G., Vanngard, T., and Larsson, M. (1958), *Biochim. Biophys. Acta 30,* 1.
Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., and Neurath, H. (1972b), *Nature (London), New Biol. 238,* 41.
Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., and Dupourque, D. (1972a), *Nature (London), New Biol. 238,* 37.
Meiboom, S., and Gill, D. (1958), *Rev. Sci. Instrum. 29,* 688.
Mildvan, A. S., and Cohn, M. (1963), *Biochemistry 2,* 910.
Mildvan, A. S., and Cohn, M. (1965), *J. Biol. Chem. 240,* 238.
Miller, R. S., Mildvan, A. S., Chang, H.-C., Easterday, R. L., Maruyama, H., and Lane, D. (1968), *J. Biol. Chem. 243,* 6030.
Navon, G. (1970), *Chem. Phys. Lett. 7,* 390.
Nicolau, C. L., Kalb, A. J., and Yariv, J. (1969), *Biochim. Biophys. Acta 194,* 71.
Ohta, Y. (1967), *J. Biol. Chem. 242,* 509.
Ohta, Y., Ogura, Y., and Wada, A. (1966), *J. Biol. Chem. 241,* 5919.
O'Sullivan, W. J., and Cohn, M. (1966), *J. Biol. Chem. 241,* 3104.
Peacocke, A. R., Richards, R. E., and Sheard, B. (1969), *Mol. Phys. 16,* 177.
Reed, G. H., and Cohn, M. (1970), *J. Biol. Chem. 245,* 662.
Shulman, R. G., Navon, G., Wyluda, B. J., Douglass, D. C., and Yamane, T. (1966), *Proc. Nat. Acad. Sci. U. S. 56,* 39.
Soloman, I. (1955), *Phys. Rev. 99,* 559.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37,* 307.
Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1972), *Nature, (London), New Biol. 238,* 35.