Metal Substitutions and Inhibition of Thermolysin: Spectra of the Cobalt Enzyme*

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SUMMARY

Removal of zinc from thermolysin results in an inactive, metal-free apoenzyme. Zn\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\), when added in stoichiometric amounts, restore 100, 200, and 10\% of the activity of the native enzyme. Fe\(^{2+}\) in high molar excess restores about 60\% of the native activity. Zinc is bound much more firmly than is cobalt, as shown by mutual displacement and the resultant enzymatic activities. Zinc in excess of that required to induce activity inhibits the enzyme, seemingly by binding to a specific, inhibitory site. The zinc atom does not seem to contribute to the stability of the protein toward heat denaturation. Chelating agents, e.g., 1,10-phenanthroline, inhibit native thermolysin by competing with the enzyme for its zinc atom and removing it. The inhibition is fully reversible on dilution or on addition of Zn\(^{2+}\) ions and is a function of concentrations of enzyme and chelating agents.

The enzymatically active cobalt thermolysin exhibits absorption and circular dichroic spectra indicative of an unusual environment of the cobalt atom. There is a shoulder near 500 nm and a maximum at 555 nm (ε ~90). These data together with spectral perturbations of the CD, magnetic circular dichroic, and absorption spectra lead to the conclusion that the cobalt atom is coordinated in a distorted tetrahedral geometry. This deduction is consistent both with those other cobalt-substituted zinc enzymes which exhibit similarly unusual properties as compared with Co(III) complex ions and with interpretations of x-ray structure analysis of native thermolysin.

Thermolysin is an endopeptidase first isolated from the thermolabile organism Bacillus thermoproteolyticus (Rokko) by Endo (1). Its neutral pH optimum, specificity for substrates containing hydrophobic residues, inhibition by chelating agents, and insensitivity to the inhibitors of sulfhydryl and seryl proteases are characteristic of a large number of bacterial endopeptidases (2). We have previously demonstrated that crystalline thermolysin contains 1 g atom of zinc and 3 to 4 g atoms of calcium per mole of enzyme. Zinc can be removed by means of chelating agents with concomitant loss of enzymatic activity which returns on restoration of the metal (3).

Thermolysin is remarkably stable to heat (1, 4, 5), it lacks disulfide bonds (5), but calcium atoms (3) stabilize its structure (4–6). The relationship of its composition and structure to its function and to those of other bacterial and mammalian metalloproteases has given particular impetus to the study of this enzyme.

A catalytically essential zinc atom is one feature which thermolysin shares with all of these enzymes (2, 7). It is likely involved either in substrate binding, catalysis, or both. In the course of our efforts to delineate details of the catalytic action of thermolysin we have directed our attention first to the inhibition of thermolysin by chelating agents and next to the functional and spectral consequence of substituting other metals for the native zinc atom.

MATERIALS AND METHODS

Thermolysin was obtained from Diawa Kasei (Osaka, Japan) or from Calbiochem (Los Angeles) as a lyophilized, 3 times recrystallized material containing 30\% calcium acetate. Strains of this organism needed to prepare the enzyme are not accessible. Although the enzyme can be recrystallized by dissolution at high pH, followed by crystallization at neutrality (8), it was found more convenient to recrystallize by dissolving the enzyme (50 mg per ml) in 5 M NaBr, 0.05 M Tris, 0.01 M Ca\(^{2+}\), pH 7.5, freshly extracted with dithizone followed by dialysis to low ionic strength with the same buffer but in the absence of NaBr. Needle-shaped crystals with hexagonal cross section and of high specific activity were obtained in this manner. Thermolysin as obtained from the supplier is a mixture of the enzyme and its autodigestion products which can be separated by gel filtration on Sephadex G-75 to isolate enzyme of high purity and maximal specific activity (9).

Re crystallization either from NaOH or NaBr effectively removes these contaminants, resulting in a product which exhibits a single peak of absorption at 280 nm on Sephadex G-75 chromatography. Importantly, the specific activity of the recrystallized enzyme is constant and equal to that of the enzyme purified by chromatography. Prior to studies of activities the enzyme was recrystallized at least once. Protein concentration was determined by measuring absorbance at 280 nm with the use of the absorbity E\(_{280}^{1%}\) = 17.05 reported by Ohta (5).

Solutions of metal ions were prepared from the spectroscopically pure sulfate salts (Johnson-Matthey “Specpure” grade). Ferric oxide (Johnson-Matthey) was dissolved in 6 M hydrochloric acid (Aristar, BDH chemicals). Furylacryloylglycyl-\(L\)-leucyl amide (Cyclo Chemical Co.), thioglycolic acid (Eastman Chemicals),
constant for the corresponding 1:1 metal-inhibitor complexes; p = 0.1 M with NaCl. At this substrate concentration, reactions were first order for at least 88% of total hydrolysis, unless otherwise noted. Values of $k_{cat}$, the observed first order rate constant, were calculated from plots of $\log (A_0 - A_t)/(A_0 - A_i)$ versus time, where $A_0$ is the initial absorbance, $A_t$ is the absorbance after complete hydrolysis and $A_i$ is the absorbance at time $t$. Enzyme activity is expressed as $k_{cat}/[E]$ in units of M$^{-1}$ min$^{-1}$, where $[E]$ is the enzyme concentration determined from absorption measurements.

Inhibition studies with metal binding agents were performed by adding concentrated solutions of these agents to the substrate, followed by enzyme to initiate the reaction. In certain studies a reverse order of addition was employed in order to preincubate enzyme with inhibitor. In such instances substrate was added to inhibit the reaction.

Values of $K_I$, the concentration of inhibitor needed to achieve 50% inhibition, and the average number of moles of ligand complexed per mole of enzyme, $n$, were obtained by use of the equation $k_0/k_i = 1 + K_I/[L]$ (12, 13) where $k_0$ is the control activity of the native enzyme and $k_i$ is the activity in the presence of free inhibitor, $[I_{free}]$ (Table I). Values of $K_I$ and $n$ were obtained from linear plots of $\log (k_0/k_i) - 1$ versus $\log [I_{free}]$.

The absorption spectra of the cobalt enzyme were recorded by means of a Cary 14 spectrophotometer with a 0 to 0.1 A slide-wire using 1-cm pathlength quartz cuvettes. A blank consisting of apoenzyme at a concentration identical with that of the cobalt enzyme was stored routinely at 4°C and was usually used within a week. For the preparation could be reactivated completely with zinc after storage at 4°C for at least 2 years. In studies of the apoenzyme special preventative measures were necessary to ensure freedom from contamination with adventitious metal ions, especially during spectrophotometric assays of the residual activity. In addition to the precautions outlined by Thiers (10), it was found necessary, prior to measurements, to wash the cuvettes used in the assay with a dilute solution of apoenzyme or with a solution of EDTA, 1 mM, followed by extensive washing with metal-free distilled water.

**Results**

Inhibition Studies—The metal-binding agents, 2-mercaptoethylyamine, thioglycolic acid, 1,10-phenanthroline, cyanide, $\alpha$, $\alpha'$-bipyridyl, and imidazole, known to form complexes with zinc, inhibit thermolysin. The inhibition is the same, either when measured after the initial 30 s of reaction, or after 2 hours of preincubation of the enzyme in the presence of inhibitor but in the absence of substrate. Fig. 1 shows the dependence of the degree of inhibition on inhibitor concentration, and Table I gives the corresponding values of $K_I$ and $n$, the number of inhibitor molecules bound to the metal.

When the assay mixture contains any of these agents and thermolysin is added to initiate hydrolysis, there is a delay, confined to the very initial phase of the reaction, before inhibition becomes apparent. Fig. 2 shows this effect with the use of 1,10-phenanthroline. In its absence the rate is linear for over 3 half-lives.

The degree of inhibition by metal-binding agents depends upon the concentration of both the enzyme and the inhibitor. The inhibitory effects of 1,10-phenanthroline were examined when the concentration of enzyme was varied (Table II). In the presence of 1,10-phenanthroline, $10^{-4} M$, and over a 10-fold range of enzyme concentration, the percentage of inhibition decreases from 65.5 to 24.7%, while over the same enzyme concentration range and 1,10-phenanthroline, $2 \times 10^{-4} M$, the percentage of inhibition decreases from 31.8 to 4.1%.

Addition of Zn$^{2+}$ reverses the inhibition by metal-chelating agents. $\alpha$, $\alpha'$-Bipyridyl, $10^{-4} M$, and 1,10-phenanthroline, $10^{-4} M$, inhibit the activity of native enzyme to 18 and 30% of the control, respectively (Fig. 3), but addition of increasing concent-

| Metal-binding agent | Zinc thermolysis |
|---------------------|------------------|
|                     | $pK_{a}$ | $n$ | $pK_{L}$ |
| 1,10-Phenanthroline | 4.4     | 1.8 | 6.4     |
| $\alpha$, $\alpha'$-Bipyridyl | 3.4     | 1.8 | 4.9     |
| Thioglycolic acid   | 5.1     | 1.8 | 7.9     |
| Imidazole           | 1.3     | 1.2 | 2.5     |
| Cyanide             | 3.9     | 2.7 | 4.8$^a$ |
| 2-Mercaptoethylyamine | 6.1     | 1.4 | 8.1     |

- Reactions performed under standard assay conditions initiated by the addition of enzyme to an equilibrated solution of substrate and inhibitor.
- The values are those of the negative log of the concentration of free inhibitor, calculated from their respective acid dissociation constants, required for 50% inhibition of the native enzyme.
- The values are those of the negative log of the dissociation constant for the corresponding 1:1 metal-inhibitor complexes (Ref. 14).
- From Ref. 15.

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3.2 X 10^-6 M, was added to the standard assay mixture, 2.5 ml, near 10^-6 M, it is equal to that of the native enzyme. Higher concentrations of Zn^2+ inhibit.

2.4 X 10^-7 M; imidazole (ZM), 2.4 X 10^-7 M. Conditions: pH 7.5, 0.05 M Tris, 0.01 M Ca^2+, 0.1 M NaCl, 25°C, 1 mM furylacryloylglycyl-L-leucyl amide.

Fig. 4, samples of enzyme, -1 mg per ml, were dialyzed against Tris, pH 6 and 10 does not remove it. In the experiment shown in Fig. 4, samples of enzyme, -1 mg per ml, were dialyzed against the agent: 2-mercaptoethylamine (MEA), 3.71 X 10^-7 M; thioglycolic acid (TGA), 3.71 X 10^-7 M; l,lO-phenanthroline (OP), 0.05 X 10^-7 M, an^2-bipyridine (a,b), 2.0 X 10^-7 M; cyanide, 2.4 X 10^-7 M; imidazole (IM), 2.4 X 10^-7 M. Conditions: pH 7.5, 0.05 M Tris, 0.01 M Ca^2+, 0.1 M NaCl, 25°C, 1 mM furylacryloylglycyl-L-leucyl amide.

Fig. 2. First order rates for the time course of furylacryloylglycyl-L-leucyl amide hydrolysis in the presence of varying concentrations of 1,10-phenanthroline. Fifty microliters of enzyme, 1.4 X 10^-6 M, was added to the standard assay mixture, 2.5 ml, containing the following concentrations of 1,10-phenanthroline: O, none; □, 1.5 X 10^-4 M; △, 2 X 10^-4 M; ●, 2.3 X 10^-4 M.

**Table II**

*Effect of enzyme concentration on 1,10-phenanthroline inhibition of zinc thermolysin*

| [Thermolysin] [1,10-Phenanthroline] | \( k_f/k_c \times 10^b \) |
|-------------------------|-----------------|
| \( \nu \times 10^5 \)  | \( \nu \times 10^6 \) |  |
| 14                      | 1               | 65.5 |
| 7                       | 1               | 22.7 |
| 1.4                     | 1               | 24.7 |
| 14                      | 2               | 31.8 |
| 7                       | 2               | 18.2 |
| 1.4                     | 2               | 4.1  |

Enzyme was added to 1,10-phenanthroline in 0.1 M NaCl, 0.06 M Tris, pH 7.5, 0.01 M Ca^2+, furylacryloylglycyl-L-leucyl amide, 1 mM, 25°C.

\( k_f \) = rate of inhibited enzyme; \( k_c \) = rate of native control.

100 ml of buffer for 96 hours at the pH values shown. The dialysis saccs were then opened and analyzed for total protein, zinc, and activity. The enzyme retained both 1 g atom of zinc and full activity, and there was no loss of protein over this pH interval. Below pH 6, however, total protein, zinc, and activity were all lost, such that at pH 4 less than 5% active enzyme remained. However, the specific activity and the ratio of zinc to protein remain constant for each sample, irrespective of the pH of dialysis.

**Preparation and Characterization of Apothermolysin**—In the presence of Ca^2+, zinc can be removed from thermolysin by dialysis of the native enzyme against 1,10-phenanthroline, followed by dialysis with buffer to remove excess reagent. Preparations of the apoenzyme containing less than 1% of the 1 g atom of zinc present in the native enzyme and having less than 1% peptidase activity were obtained reproducibly.

Activity regenerated by stoichiometric additions of zinc to apothermolysin is proportional to the amount of zinc added until 1 g atom of zinc per mole is incorporated and full native activity is restored (Fig. 5). Zn^2+ in excess of 1 g atom per mole of enzyme does not increase activity further, and, in fact, inhibits markedly (Fig. 5, inset). Thus, 50% of the activity is lost at a concentration of Zn^2+, 1.8 X 10^-4 M. Zn^2+ inhibits the native enzyme in identical manner. The inhibition, moreover, is completely reversible by dilution and independent of enzyme concentration when [Zn] >> [E]. Zn^2+, 6 X 10^-4 M, added to enzyme, 1.4 X 10^-7 M, results in 28% activity of the control. Ten-fold dilution results in activity precisely that observed when Zn^2+, 0 X 10^-4 M, is added, i.e. 85% activity. Further Zn^2+, 2.0 X 10^-4 M, always inhibits the native enzyme to the same degree, 56%, of the control, while enzyme concentration is varied 10-fold, *i.e. from 1.4 X 10^-7 to 1.4 X 10^-6 M.*

While active site zinc is indispensable for the catalytic activity of thermolysin it might also maintain conformation or stabilize the enzyme against heat inactivation. To examine these possibilities the thermal stability, circular dichroic, and absorption spectra of native and apothermolysin were compared. When incubated at 80°C, the activity of native thermolysin, containing 1 g atom of zinc per mole, decreases exponentially, with a half-life of ~50 min (1, 4). The thermal denaturation rates of native and apothermolysin (Fig. 6) do not differ significantly, indicating that the functionally active zinc atom alone does not noticeably contribute to the unusual thermal stability of this enzyme. The circular dichroic spectrum of native thermolysin exhibits a negative Cotton effect centered at 275 nm with \( [\theta]_{275}^{\text{deg}} \approx 8 \times 10^4 \text{ deg cm}^2 \text{ decimole}^{-1} \). The position and intensity of the 275 and 225
MOLES Zn²⁺ / MOLE APOENZYME

FIG. 5 (left). Restoration of peptidase activity to apothermolysin, 5.0 × 10⁻⁵ M, by addition of Zn²⁺. Inset, effect of excess Zn²⁺ on peptidase activity. Activity measurements as described in the text.

FIG. 6 (right). The effect of temperature on the activity of native zinc thermolysin and of apothermolysin. Native (●) and apothermolysin (X), both at 1.3 × 10⁻⁵ M, were each incubated in sealed tubes at pH 7.5, 0.05 M Tris, 0.01 M Ca²⁺, in an oil bath at 80°C. At the times indicated, aliquots were removed, cooled in water to 25°C, and assayed under standard assay conditions. After ascertaining that the apoenzyme was still inactive, it was assayed (see text) after addition of a 1.5-fold excess Zn²⁺.

MOLES Me²⁺ / MOLE APOENZYME

FIG. 7 (left). Restoration of peptidase activity on addition of the sulfate salts of Co²⁺ (●, left axis) and Mn²⁺ (○, right axis), to apothermolysin, 6.3 × 10⁻⁵ M. Activities were measured immediately after metal (Me) addition under standard assay conditions, as described in the text.

FIG. 8 (right). Enhancement of native thermolysin activity by Co²⁺ (m) and inhibition by Zn²⁺ (●). Assays were performed by adding native enzyme, 3.4 × 10⁻⁷ M, containing 1 g atom of Zn²⁺ to a cuvette containing buffer and substrate, plus either Co²⁺ or Zn²⁺ of the concentrations indicated and monitoring hydrolysis immediately. Addition of 10 eq of Zn²⁺ to the enzyme in the presence of 1 mM Co²⁺ (vertical arrow) restores full native activity (▲). The assay mixture contained Ca²⁺, 0.01 M, 0.05 M Tris, 0.1 M NaCl, 1 mM furylacryloylglycyl-L-leucyl amide, pH 7.5, 23°C.

nm bands are identical both in the apoenzyme and when 1 g atom of zinc per mole is added.

In the region from 250 to 300 nm the absorption spectra of zinc and apothermolysin differ somewhat. Upon addition of zinc to apothermolysin absorbance at 288 nm decreases slightly (Δε = 230) indicating perturbation of aromatic chromophores.

Addition of Metals to Apothermolysin—Co²⁺ or Mn²⁺, 1 g atom per mole of apothermolysin, instantaneously and maximally restore activity to approximately 200% and 10% of that of the native enzyme, respectively (Fig. 7). In concentrations up to 1 mM, these metals fail to inhibit catalytic activity.

Addition of excess Co²⁺ to native zinc thermolysin generates the activity characteristic of the cobalt-substituted enzyme. Activity increases until at a cobalt concentration near 1 mM, activity becomes twice that of the native enzyme (Fig. 8). Results were identical whether experiments were performed by addition of Co²⁺ in the presence of substrate or by preincubation of Co²⁺ with the zinc enzyme for up to 1 hour. In turn, addition of Zn²⁺ restores the activity to that characteristic of the native enzyme (Fig. 8).

Iron thermolysin exhibits 60% of the activity of the native enzyme. However, for this to be observed a 30-fold molar excess of Fe²⁺ over apoenzyme is required, and Fe³⁺ is completely ineffective. Consistent with this, Zn²⁺ restores native activity when added either to Fe²⁺ or Fe³⁺ thermolysin.

Mg²⁺, Cr³⁺, Ni²⁺, Cu²⁺, Mo⁶⁺, Pb²⁺, Hg²⁺, Cd²⁺, Nd²⁺, Pr³⁺, or Dy³⁺ all fail to restore activity which was <2% that of the native enzyme. For this purpose 5- and 50-fold molar excess of each metal was added to a reaction cuvette containing apoenzyme and substrate, followed immediately by measurement of activity. In addition, the metal and apoenzyme were preincubated for 1 and 12 hours, and metal concentrations were varied up to 200-
molysin is a metalloenzyme containing one zinc atom per mole which is required for catalytic activity.

Both zinc content and activity remain constant in the course of six recrystallizations (3). Neither extensive washing of the crystals with water nor gel filtration of its solutions at pH 6.3 remove zinc. Dialysis at low pH frequently removes metals from metalloproteins, presumably by competition of H+ with the metal for the protein ligands (7). This mode of removing zinc has not proven suitable for thermolysin which is only stable to extensive dialysis above pH 6. Below this pH, zinc, activity, and total protein are all lost simultaneously suggesting extensive autocatalytic digestion, as verified by concomitant appearance of membrane-permeable peptides.

Indeed, in the absence of calcium, thermolysin is inactivated rapidly even above pH 6, a result of autocatalytic digestion, a process which calcium minimizes when in excess, >10^{-4}M (4, 6). However, at low pH values, the protective effect of excess calcium is lost, as would be expected since it is known to be bound to carboxyl groups of aspartyl and glutamyl residues (20).

Chelating agents, however, rapidly remove zinc under conditions which do not result in autolysis. Dialysis against 1,10-phenanthroline removes zinc until <0.01 g atom of zinc per mole of enzyme remains. Enzymatic assay of such preparations generally reveal 1 to 3% of the activity of the native zinc enzyme. However, doubling or quadrupling the amount of apoenzyme does not increase the absolute rate of substrate hydrolysis proportionately, demonstrating the apoenzyme to be virtually metal free.

Chelating agents may inhibit metalloenzymes by way of two predominant mechanisms. According to Equation 1

\[ [E \cdot Me] + \bar{n}I \Leftrightarrow E + [Me(I)\bar{n}] \]  

\( n \) moles of inhibitor, \( I \), remove metal, \( Me \), from the metalloenzyme, \( E \cdot Me \), to form a metal-inhibitor complex, \([Me(I)\bar{n}]\), and apoenzyme, \( E \). This mechanism of inhibition has been observed for other zinc metalloenzymes, such as carboxypeptidase A (21), leucine aminopeptidase (22), and alkaline phosphatase of Escherichia coli (23), among others. Alternatively, chelating agents may inhibit metalloenzymes by forming a ternary, mixed complex between enzyme, metal, and inhibitor as in Equation 2.

\[ [E \cdot Me] + I \Leftrightarrow E \cdot Me \cdot I \]  

as has been observed for horse liver alcohol dehydrogenase (19, 24, 25).

The rapid inactivation of thermolysin by chelating agents, its reversal by Zn^{2+}, and the complete restoration of activity to the apoenzyme by addition of Zn^{2+} suggest that such agents inhibit by removing zinc from the active site (Equation 1) rather than by forming an inactive, mixed complex (Equation 2). The magnitudes of the values of \( K_I \), which parallel the values of \( K_C \) (Table 1) are consistent with such a mechanism, as are the values of \( \bar{n} \), the number of inhibitor molecules bound to the metal, which exceed the value of 1 expected if a mixed complex were to form. The short delay in reaching equilibrium (Fig. 2) indicates that the dissociation of zinc is rate limiting in the early phase of the reaction. This phenomenon is also inconsistent with the formation of a mixed complex. Such a complex would be expected to be inactive, resulting in a slower rather than a more rapid rate during the initial phase. Indeed, attempts to detect an E-zinc-1,10-phenanthroline intermediate by spectrophotometry were also unsuccessful (19, 26).

The ready mutual exchange of cobalt for enzyme-bound zinc and of zinc for enzyme-bound cobalt (Fig. 9) implies a rapid equi-
librium between enzyme-bound and free metal and also lends support to the mechanism of inhibition based on dissociation of the metal atom (Equation 1). Such competition experiments (Fig. 9) demonstrate that the ratio of the equilibrium constants for cobalt and zinc thermolysin is ~600. This value is consistent with the consequences of adding Zn$^{2+}$ to cobalt thermolysin. Thus on addition of 1.4 g atoms of Zn$^{2+}$ to cobalt thermolysin, 2.2 × 10^{-7} \text{ M}, there is complete exchange resulting in activity characteristic of the native enzyme. On further addition of zinc, activity remains constant until inhibition sets in at [Zn$^{2+}$] ~ 10^{-8} \text{ M} (Fig. 9). A difference of stability constants for zinc and cobalt thermolysin of 2.8 (log 600) is quite analogous to those of zinc and cobalt carboxypeptidases (27) whose metal ligands are identical with those of thermolysin.

Addition of Zn$^{2+}$, Co$^{2+}$, or Mn$^{2+}$, 1 g atom per mole, to apothermolysin restores activity (Figs. 5 and 8). However, the cobalt enzyme is twice and the manganese enzyme is one-tenth as active as the native zinc enzyme. The sharp break in the titration curve at zinc concentrations near 10^{-5} \text{ M} set lower limits for the dissociation constants of these metals, 10^{-9} \text{ M}. Indeed, based on inhibition studies, the stability constant for zinc and cobalt thermolysin have been estimated to be 12.6 and 9.5, respectively (28).

While essential to the activity of thermolysin zinc can also inhibit the enzyme. The fact that this inhibition is reversible and is independent of enzyme concentration when [Zn$^{2+}$] > [E] suggests the existence of an additional site to which Zn$^{2+}$ binds and causes inhibition. This inhibition is linear when log ([Zn$^{2+}$]/[E]) is plotted versus log Zn$^{2+}$ with a slope of 1.0, the kinetic order with respect to inhibitor, demonstrating that the binding of 1 g atom of Zn$^{2+}$ mole of enzyme is responsible. It is known that Glu 145 and His 231 are close to the active site zinc atom of thermolysin (20). Moreover, we have shown (29) that histidyl residue is essential to the activity of the enzyme representing a potential site for Zn$^{2+}$ interaction and subsequent inhibition, a subject requiring further investigation. However, K_i = 1.8 × 10^{-9} \text{ M}, the lowest of any reversible inhibition for thermolysin found thus far, must be considered in all measurements of its activity. Particular caution is indicated when studying the enzyme in the presence of added Zn$^{2+}$ ions.

The Fe$^{2+}$ activity-titration curve differs markedly from that of the other metals, since maximal activity, 100% of that of the native enzyme, is observed only at very high Fe$^{2+}$ to apoenzyme ratios. The restoration of activity characteristic of the native enzyme upon addition of Zn$^{2+}$ to the Fe$^{2+}$ enzyme is consistent with the presence of Fe$^{2+}$ at the active site though oxidation to Fe$^{3+}$ cannot be ruled out at present.

Cobalt (II) is an optical probe of the ligand geometry of the active site of thermolysin, much as it has served to explore those of alkaline phosphatase (30), carboxypeptidase (31), procarboxypeptidase A (32), carbonic anhydrase (27), yeast aldolase (33), and liver alcohol dehydrogenase (34, 35), among others. All of these cobalt-substituted zinc metalloenzymes are enzymatically active, and their spectral features are unlike those of most Co$^{2+}$ complex ions soluble in aqueous media. They are generally of low symmetry, and the spectral response to environmental factors such as pH, inhibitors, or substrates correlates with functional properties so that electronic or magnetic changes (or both) in the environment of the metal would appear to reflect in function (26).

The absorption spectrum of cobalt thermolysin (Fig. 9) suggests an irregular tetrahedral geometry of the metal ion. The interpretation of this spectrum is consistent with those of carboxypeptidase A where both Zn$^{2+}$ and Co$^{2+}$ are bound in an irregular tetrahedron (31, 37), for cobalt alkaline phosphatase (30), the low pH form of cobalt carbonic anhydrase (38) and with x-ray structure analysis of thermolysin (20).

We have emphasized the predictive value of MCD in the assignment of the over-all geometry of cobalt (II) complex ions and that of cobalt-substituted metalloenzymes (39). The MCD spectrum of thermolysin indicates the geometry of Co$^{2+}$ at the active site to be tetrahedral, in agreement with conclusions from absorption spectra (Fig. 9).

The competitive inhibitor, $\beta$-phenylpropionyl-$\beta$-phenylalanine, interacts with the active center as is evident from the marked perturbation of the absorption and MCD spectra of cobalt thermolysin, manifesting both as an increase in resolution and over-all intensity. This could be due either to direct interaction with the cobalt atom, to indirect effect on the ligands or to binding-induced conformational changes of protein structure. Whatever the mechanism, the MCD data indicate that the over-all coordination geometry of the active site cobalt remains unchanged in the presence of the inhibitor (39). Such alterations in the spectra must signal a change in the microscopic rather than in the over-all geometry of the metal upon interaction of the inhibitor with the active site. The natural circular dichroism both for the cobalt enzyme and its inhibitor complex is consistent with such deductions, though these spectra are of much lower magnitude (Fig. 9, inset).

The x-ray structure analysis of thermolysin (20) shows the protein to consist of two halves which are linked through residues 153 to 158. Zinc is bound to Glu 166 located in one of the halves and to His 142 and His 146, positioned in a region of a helix interstitial to the two halves. The metal complex thus appears to bridge the two halves in a manner which could be thought to contribute both to structural stabilization and the formation and integrity of the active site cleft (20), though experiments thus far give no support to such a view.

The apoenzyme is remarkably stable and activity, identical with that of the native enzyme, is restored by readdition of zinc, even after extended periods (up to 2 years). Further, the rates of heat denaturation both of the apo- and native enzymes at high temperatures are similar. Different results would be expected if the coordination of zinc to the active site ligands would contribute to the conformation stability of this enzyme. The absence of significant alterations of either the CD or absorption spectra upon addition of Zn$^{2+}$ to apothermolysin indicates that conformational changes are minimal.

Sequence and x-ray structure analysis (9, 20) do not reveal sequence homology between thermolysin and carboxypeptidase A, and their tertiary structures differ markedly. Yet, there are some functional and structural analogies in the region of the active centers. In both enzymes zinc is bound to one glutamyl and two histidyl residues in a tetrahedron of low symmetry. The metal is functional but does not serve a structural role. Zinc can be replaced with Co$^{2+}$ resulting in similar spectral features. Further, Co$^{2+}$, Mn$^{2+}$, and Fe$^{2+}$ can replace zinc and restore peptide activity to both apoenzymes. Functional amino acid residues of thermolysin have recently also been described. Thus, modification of histidyl (29), carboxyl (40), and perhaps tyrosyl (41) residues markedly affect activity. Both carboxyl and tyrosyl residues are also known to be critical to the function of carboxypeptidase A (42), suggesting considerable mechanistic similarities (7).

The present studies have been limited to an examination of the active site metal of thermolysin and a delineation of its possible
roles in catalysis. Such knowledge is, of course, vital to the ultimate elucidation of the over-all mechanism. Additionally, knowledge of the active site metal and of residues involved in catalysis is mandatory for the interpretation of other features exhibited by this enzyme, notably its calcium requirement and its relationship to thermostabilization.

Further explorations of the nature of thermolysin catalysis are thus indicated. The substrate used here is convenient for accurate activity measurements. However, it is lacking in several important features. Its low solubility and high $K_m$ value (11) limit its usefulness to the determination of the ratio $k_{cat}/K_m$. However, $k_{cat}$ and $K_m$ cannot be determined definitively under such conditions, as would be required e.g. for the delineation of the consequences of metal substitution and the mode of inactivation by various inhibitors. Such considerations have led us to synthesize substrates more suitable for this purpose, and details of their kinetics will be reported.

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