J. Biochem. **141,** 843–853 (2007)
doi:10.1093/jb/mvm088

Kinetic Analysis of the Activation-and-Inhibition Dual Effects of Cobalt Ion on Thermolysin Activity

Yasuhiko Hashida and Kuniyo Inouye*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Received February 10, 2007; accepted March 28, 2007; published online April 3, 2007

Thermolysin activity as well as its stability is remarkably enhanced by high concentration of neutral salts consisting of Na^+, K^+, Cl^- and Br^- in the synthesis and hydrolysis of *N*-carbobenzoxy-L-aspertyl-L-phenylalanine methyl ester and hydrolysis of *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA) [Inouye, K. (1992) *J. Biochem.* 112, 335–340]. However, effect of divalent salts on thermolysin activity has not been investigated systematically. In this study, effect of Co^{2+} ion on thermolysin activity in the hydrolysis of FAGLA was examined. Thermolysin activity increased 3–4 times with increasing the Co^{2+} concentration to 2 mM, but the enhanced activity was considerably reduced with higher Co^{2+} concentration (2–18 mM). The activation-and-inhibition dual effects of Co^{2+} ion were analysed kinetically. Release of the catalytic Zn^{2+} ion from thermolysin, concomitantly occurred with the Co^{2+}-dependent activation, was measured with a Zn^{2+}-specific fluorescent probe. This indicates that the activation is caused by substituting Co^{2+} ion for the catalytic Zn^{2+} ion. Meanwhile, the Co^{2+}-dependent activation was inhibited competitively by Zn^{2+} ion (0.1–1.0 μM) added, similarly to that it is inhibited by higher concentration of Co^{2+} ion. These lines of evidence provide a strategy for regulating thermolysin activity with Co^{2+} and Zn^{2+} ions.

Key words: cobalt ion, inhibition, metalloproteinase, thermolysin, zinc ion.

Abbreviations: FA-, *N*-[3-(2-furyl)acryloyl]-; FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; ZDFM, *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester; ZnAF-2, 6-[*N*-[*N*',*N*'-bis(2-pyridinylmethyl)-2-aminoethyl]amino-3',6'-dihydroxy-spiro [isobenzofuran-1(3H), 9'-[9H] xanthen]-3-one, tetrahydrochloride.

Thermolysin [EC 3. 4. 24. 27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (*1–4*). It requires one zinc ion for enzyme activity and four calcium ions for structural stability (*5–7*), and catalyses specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (*8*). The amino acid sequence (*9*, *10*) and three-dimensional structure (*11*) are available, and a reaction mechanism has been proposed (*12–14*).

We have reported that high concentrations (1–5 M) of neutral salts cause a remarkable activation of the thermolysin-catalysed hydrolysis and synthesis of *N*-carbobenzoxy-L-asparatyl-L-phenylalanine methyl ester (ZDFM), a precursor of a synthetic sweetener (*15*), and hydrolysis of *N*-[3-(2-furyl)acryloyl] (FA)-dipeptide amides with different amino acids at the scissile bond (*16*). The activation is brought about most effectively by NaCl and NaBr, and the activity increases in an exponential fashion with increasing salt concentration. The degree of activation at x M NaCl is expressed by 1.9^x at pH 7.5 (*16*, *17*). The molecular activity, k_{cat}, and Michaelis constant, K_m, can be evaluated separately in the cases of ZDFM, FA-L-leucyl-L-alanine amide (FALAA), and FA-L-phenylalanyl-L-alanine amide (FAFAA), and the activation has been demonstrated to be induced solely by an increase in k_{cat}, and K_m is not affected at all by the presence of salts (*15–17*). Recently, we have reported that the activation degree is dependent on the electrostatic charges on the substrate, and is higher with the substrate carrying positive charge than that carrying negative charge (*18*). We have observed a characteristic absorption difference spectrum on mixing thermolysin with NaCl and NaBr, suggesting changes in the states of tyrosyl and tryptophyl residues (*15*, *19*). The specific interaction between cations and thermolysin might be involved in the activation, and effectiveness is in the order of $Na^+ > K^+ > Li^+$ (*15–17*). We also demonstrated that a change in the ionization state on the surface of thermolysin affects the NaCl-dependent activation by means of nitration and amination of tyrosyl residues in the enzyme (*20*). Accordingly, the salt-dependent activation might be related to electrostatic interactions of thermolysin with ions in the medium. The solubility of thermolysin increases greatly in the presence of high concentrations of salts (*21*), and the thermal stability is also increased by the addition of NaCl (*22*). Unique interactions of the molecular surface of thermolysin with ions might change the solubility and thermal stability as well as the activity. Unique interactions of the molecular surface of thermolysin with ions might change the solubility and thermal stability as well as the activity. To explore the mechanism of the salt-induced activation, we recently reported the preliminary X-ray crystallopraghic analysis of thermolysin in the presence of 4 M NaCl (*23*). Recently, we have established novel efficient methods for

*To whom correspondence should be addressed. Tel: +81-75-753-6266, Fax: +81-75-753-6265, E-mail: inouye@kais.kyoto-u.ac.jp

purification and expression of thermolysin, which enable us to prepare a wide range of thermolysin variants and to examine the reaction mechanism precisely (*24–27*). We have also reported that the mechanism of autodegradation of thermolysin and provided strategies to enhance its thermostability (*28*, *29*).

In comparison with the systematic study on the effects of monovalent ions on thermolysin activity, the effect of divalent ions has not been studied well. Thermolysin contains intrinsic five divalent ions (one Zn^{2+} ion and four Ca^{2+} ions). The Zn^{2+} ion contributes to the catalytic activity of thermolysin, and the Ca^{2+} ions to its stability. The addition of divalent ions might create new interactions between thermolysin and the added ions in addition to that of the molecular surface of thermolysin and ions. In this study, the effect of cobalt ion on thermolysin activity is described by comparing the effect of zinc ion. At lower concentration of Co^{2+} ion, thermolysin activity is enhanced up to 3–4 times, but the enhanced activity is reduced by the addition of higher concentration of Co^{2+} or Zn^{2+} ions in a competitive manner. It is demonstrated that the catalytic Zn^{2+} ion is replaced by Co^{2+} ion as examined by determining the Zn^{2+} ion released to the solution from the active site of the enzyme, although the Co^{2+}-substituted thermolysin (CoTLN) is inhibited by higher concentrations of Zn^{2+} or Co^{2+} ions.

EXPERIMENTAL PROCEDURES

Materials—A three-times-crystallized and lyophilized preparation of thermolysin (Lot T5CB491; 8,720 proteinase units/mg according to the supplier) was purchased from Daiwa Kasei (Osaka, Japan). This preparation was used without further purification. The solution of thermolysin was filtered through a Millipore membrane filter, Type HA (pore size: 0.45 μm) before use. The concentration of thermolysin was determined spectrophotometrically using an absorption value, *A* (1 mg/ml), at 277 nm of 1.83 (*15*), and a molecular mass of 34.6 kDa (*9*). FAGLA (Lot 57H5800) was purchased from Sigma (St Louis, MO, USA). The concentration of FAGLA was determined spectrophotometrically using the molar absorption coefficient, ε_{345} of 766 $M^{-1}cm^{-1}$ (*15*, *30*). 6-[*N*-[*N*,′ *N*′-bis(2-pyridinylmethyl)-2-aminoethyl] amino-3′, 6′-dihydroxy-spiro [isobenzofuran-1(3H), 9′-[9H] xanthen]-3-one, tetrahydrochloride (ZnAF-2, Lot 001RIZ), a zinc-selective fluorescent reagent (*31*), was purchased from Daiichi Pure Chemicals (Tokyo, Japan). The concentration of ZnAF-2 was evaluated using the data provided by the supplier. All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto, Japan). All spectrophotometric measurements were done with a Shimadzu UV-visible recording spectrophotometer UVmini-1240 (Kyoto, Japan).

Hydrolysis of FAGLA—Hydrolysis of FAGLA by thermolysin was performed in 40 mM HEPES-Na buffer (pH 7.0) (designated as buffer A) at 25°C or in buffer A containing 10 mM $CaCl_2$ (buffer B). The enzyme solution (1.0 ml) which was pre-incubated with $CoCl_2$ and $ZnCl_2$ for 30 min was added to the substrate solution (2.0 ml). The initial concentrations of thermolysin and FAGLA in the reaction mixture at the start of the hydrolysis were 0.10 μM and 0.10–0.32 mM, respectively. The hydrolysis of FAGLA was followed by continuously monitoring the decrease in absorbance at 345 nm. The amount of substrate hydrolysed was estimated using the molar absorption difference on the hydrolysis, $\Delta\varepsilon_{345} = -310\,M^{-1}cm^{-1}$ (*15*). Because of the high K_m (Michaelis constant, approximately 30 mM) and sparing solubility of FAGLA, it was difficult to perform reactions at FAGLA concentrations large enough to separate the kinetic parameters, k_{cat} (catalytic constant) and K_m (*15*). The hydrolysis was performed under the condition of $[FAGLA]_o << K_m$, where pseudo-first-order kinetics is valid, and thus the activity is expressed by the specificity constant, k_{cat}/K_m, throughout this study.

Preparations of Apo-thermolysin—Apo-thermolysin was prepared according to the method previously reported (*32*) with some modification. Ten millilitres of thermolysin (25 μM) in buffer B containing 5 mM 1,10-phenanthroline was applied to a Sephadex G-50 Fine column [size: 3.0 cm (inner diameter) × 10 cm] (Amersham Pharmacia, Uppsala, Sweden) and eluted with buffer B containing 5 mM 1,10-phenanthroline. Fractions containing thermolysin were collected, and then applied to a PD-10 column (Sephadex G-25) equilibrated with buffer A. Apo-thermolysin was eluted from the column with the same buffer.

Determination of Zn^{2+} in the Active Site of Thermolysin—Thermolysin (1.0 μM) in buffer B was pre-incubated with 0–1.0 mM $CoCl_2$ for 30 min, and applied to a PD-10 column equilibrated with buffer A to remove free Co^{2+} and Zn^{2+} ions released from the active site of thermolysin. A fraction containing thermolysin was mixed with the same volume of 10% SDS and boiled for 10 min to unfold thermolysin and release Zn^{2+} in the active site of thermolysin. The boiled thermolysin solution was cooled at 25°C for 5 min, followed by the addition of ZnAF-2. The concentrations of thermolysin and ZnAF-2 in the mixture were 0.71 and 1.0 μM, respectively. The fluorescence emission was measured at 514 nm with excitation at 492 nm using a Shimadzu fluorescence spectrophotometer RF-5300PC (Kyoto, Japan).

RESULTS

Inhibitory Effect of Zinc Ion on Thermolysin Activity—Thermolysin activity was measured using FAGLA as substrate in buffer B (pH 7.0) at 25°C in the presence of various concentrations of $ZnCl_2$ (0–500 μM). The enzyme activity (k_{cat}/K_m) in the absence of $ZnCl_2$ was $(27.5 \pm 0.3)\,mM^{-1}s^{-1}$, and decreased with increasing $ZnCl_2$ concentration (Fig. 1). It decreased to one-third at 500 μM $ZnCl_2$. It is known that thermolysin activity increased remarkably with high concentration (1–5 M) of chloride salts such as NaCl, KCl and LiCl, and that the activation is given synergistically by monovalent cations and Cl^- ion (*13*, *17*). Based on this evidence, it is implicated that the inhibition of thermolysin by $ZnCl_2$ is raised by Zn^{2+} but not Cl^- ions. By assuming that one molecule of intact thermolysin (which has a catalytic Zn^{2+} ion at the active site, and is hereinafter designated as Zn-thermolysin or ZnTLN) binds with another Zn^{2+} ion

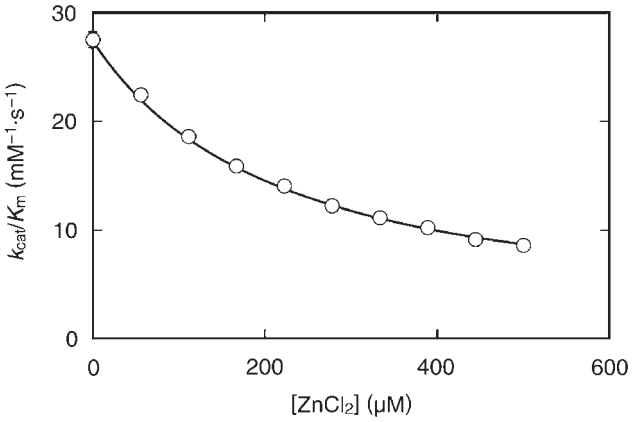
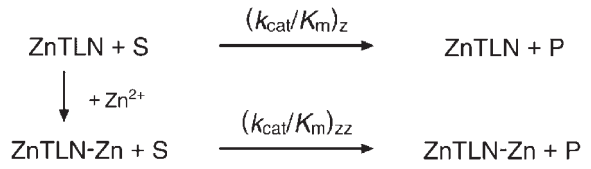


Fig. 1. **Inhibitory effect of Zn^{2+} ion on thermolysin activity.** Thermolysin (0.3 μM) was incubated with $ZnCl_2$ (0–500 μM) for 30 min in 40 mM HEPES-Na buffer (pH 7.0) containing 10 mM $CaCl_2$ (buffer B) at 25°C. Thermolysin-catalysed hydrolysis of FAGLA was performed under the same conditions at the initial concentrations of thermolysin, FAGLA and $ZnCl_2$ of 0.1, 400 and 0–500 μM, respectively. The solid line is a theoretical curve determined by fitting the data to Equation 1 by least-squares-regression method.



Scheme 1. **Change in thermolysin activity by the addition of excessive amount of Zn^{2+} ion.** ZnTLN: intact thermolysin carrying a catalytic Zn^{2+} ion at the active site; and ZnTLN-Zn: ZnTLN bound with Zn^{2+} ion.

to form a thermolysin-Zn^{2+} complex (or ZnTLN-Zn) with no enzyme activity, thermolysin activity was analysed by Equation 1 [Scheme 1 where k_{cat}/K_m for ZnTLN-Zn, $(k_{cat}/K_m)_{zz}$ is set to zero].

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)_o\, K_i}{K_i + [ZnCl_2]} \quad (1)$$

where $(k_{cat}/K_m)_o$, K_i and [$ZnCl_2$] are k_{cat}/K_m in the absence of $ZnCl_2$, the inhibitor constant of Zn^{2+} and the $ZnCl_2$ concentration in the reaction mixture. The data were fitted to Equation 1 with least-squares regression. The K_i value was determined to be (224 ± 2) μM.

Effect of Cobalt Salts on the Thermolysin-catalysed Hydrolysis of FAGLA—Thermolysin activity was measured using FAGLA as substrate in buffer B at various concentrations (0.05–18 mM) of $CoCl_2$ or $CoSO_4$ (Fig. 2). Thermolysin activity (k_{cat}/K_m) increased with increasing the $CoCl_2$ or $CoSO_4$ concentration up to 2 mM, and then decreased gradually with increasing the concentration from 2 to 18 mM (Fig. 2A). The effects of $CoCl_2$ and $CoSO_4$ on the activity were substantially the same, suggesting that the effect of the cobalt salts on thermolysin activity is

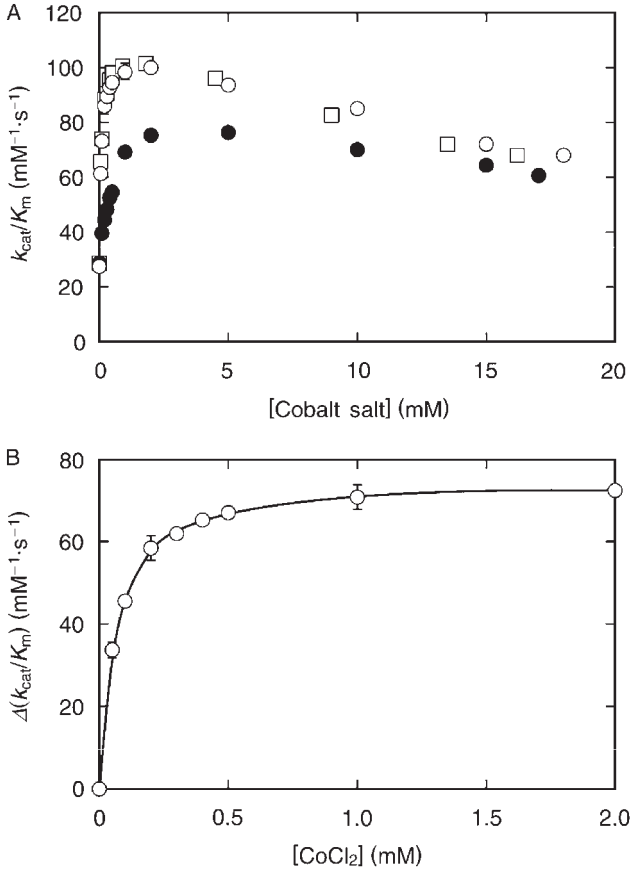
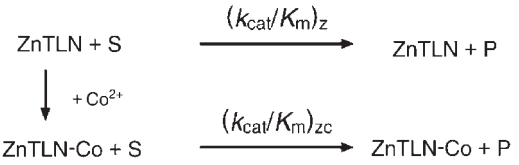
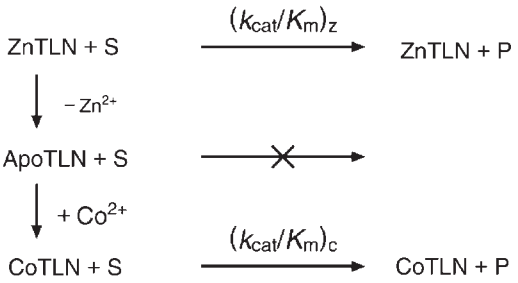


Fig. 2. **Effect of cobalt salts on the thermolysin-catalysed hydrolysis of FAGLA.** The hydrolysis was carried out in buffer B in the presence of 0–18 mM cobalt salt ($CoCl_2$ or $CoSO_4$), at 25°C. The initial concentrations of thermolysin and FAGLA in the reaction mixture ($[TLN]_o$ and $[FAGLA]_o$) were 0.10 μM and 0.10–0.32 mM, respectively. (A) Dependence of thermolysin activity (k_{cat}/K_m) on cobalt salt concentration. Cobalt salts: $CoCl_2$ (open circle); $CoSO_4$, (open square); and $CoCl_2$ with 1 μM $ZnCl_2$, (filled circle). (B) Dependence of the $CoCl_2$-dependent increment [$\Delta(k_{cat}/K_m)$] in thermolysin activity on the $CoCl_2$ concentration. The $\Delta(k_{cat}/K_m)$, which is defined as the difference between the k_{cat}/K_m values under the absence and presence of $CoCl_2$, was calculated from the data shown in panel A. The solid line is a theoretically fitted curve to the experimental data using Equation 3 with least-squares regression.

caused by Co^{2+} ion. The k_{cat}/K_m value was $(27.5 \pm 0.3)\ mM^{-1}\,s^{-1}$ in the absence of Co^{2+} ion, and increased 3.6 times to reach the maximum $[(k_{cat}/K_m)_{max}]$ $(1.00 \pm 0.04) \times 10^2\ mM^{-1}\,s^{-1}$, at 2 mM Co^{2+} ion (in this article, we assume that $CoCl_2$ and $ZnCl_2$ dissociate completely into Co^{2+} and Zn^{2+} and anions in the aqueous solution, and the concentrations of Co^{2+} and Zn^{2+} are for the sake of convenience expressed with the dimension of mol/l or M). The increment of k_{cat}/K_m [$\Delta(k_{cat}/K_m)$] at x M Co^{2+} ion is defined as the difference between k_{cat}/K_m at x M Co^{2+} ion [$(k_{cat}/K_m)_x$] and that in the absence of Co^{2+} ion [$(k_{cat}/K_m)_o$] (Equation 2), which is expressed by Equation 3. The increment increased with increasing the Co^{2+} concentration from 0 to 2 mM in a saturating fashion (Fig. 2B). The saturation curve was analysed by assuming that the binding of Co^{2+}



Scheme 2. **Change in thermolysin activity by the addition of Co^{2+} ion.** ZnTLN-Co: ZnTLN bound with Co^{2+} ion.



Scheme 3. **Change in thermolysin activity by the addition of Co^{2+} ion to apo-thermolysin.** ApoTLN: apo-thermolysin.

ion activates Zn-thermolysin (or ZnTLN) (Scheme 2). In this scheme, k_{cat}/K_m should correlate linearly with the concentration of the Co^{2+}-bound thermolysin, [ZnTLN-Co].

$$\Delta\left(\frac{k_{cat}}{K_m}\right) = \left(\frac{k_{cat}}{K_m}\right)_x - \left(\frac{k_{cat}}{K_m}\right)_o \quad (2)$$

$$\Delta(k_{cat}/K_m) = \Delta(k_{cat}/K_m)_{max}/(K_{d\ (ZnTLN-Co)} + [Co^{2+}]) \quad (3)$$

where $\Delta(k_{cat}/K_m)_{max}$ and $K_{d\ (ZnTLN-Co)}$ are the maximum value of $\Delta(k_{cat}/K_m)$ and the dissociation constant for the interaction between Co^{2+} and ZnTLN, respectively. From Fig. 2B, the $\Delta(k_{cat}/K_m)_{max}$ and $K_{d\ (ZnTLN-Co)}$ values were determined to be $(75.8 \pm 0.7)\,mM^{-1}\,s^{-1}$ and $(62.8 \pm 2.2)\,\mu M$, respectively. The $(k_{cat}/K_m)_{max}$ $[= (k_{cat}/K_m)_o + \Delta(k_{cat}/K_m)_{max}]$ of $(1.00 \pm 0.04) \times 10^2\,mM^{-1}\,s^{-1}$ is in good agreement with the k_{cat}/K_m $(1.16 \times 10^2\,mM^{-1}\,s^{-1})$ determined with the Co^{2+}-substituted thermolysin (CoTLN) (*32*), which was prepared by adding Co^{2+} ion to apo-thermolysin (ApoTLN) but not to ZnTLN and the catalytic Zn^{2+} ion in the active site was replaced with a Co^{2+} ion. Here, $(k_{cat}/K_m)_{max}$ is the k_{cat}/K_m of ZnTLN-Co corresponding to $(k_{cat}/K_m)_{zc}$ in Scheme 2 and the k_{cat}/K_m of CoTLN is shown by $(k_{cat}/K_m)_c$ in Scheme 3. This suggests two possibilities. In one possibility, a thermolysin species (ZnTLN-Co) which is formed by the addition of Co^{2+} to thermolysin (ZnTLN) is the same as cobalt-substituted thermolysin (CoTLN), in which the catalytic Zn^{2+} ion of ZnTLN is replaced with Co^{2+} ion. In the other possibility, ZnTLN-Co has the same k_{cat}/K_m value with CoTLN $[(k_{cat}/K_m)_{zc} = (k_{cat}/K_m)_c]$.

Competitive Effect of Zn^{2+} ion on the Co^{2+}-induced Activation of Thermolysin—Activity of thermolysin (ZnTLN) enhanced by the addition of $CoCl_2$ (0–18 mM) was suppressed considerably in the presence of 1.0 μM $ZnCl_2$ (Fig. 2A). The $(k_{cat}/K_m)_{max}$ value in the presence of 1.0 μM $ZnCl_2$ was observed at 5.0 mM Co^{2+}, and was $(76.2 \pm 0.5)\,mM^{-1}\,s^{-1}$. This value was 82% of that $[(92.0 \pm 0.5)\,mM^{-1}\,s^{-1}]$ obtained in the absence of $ZnCl_2$ at 5.0 mM Co^{2+}. The activity (k_{cat}/K_m) in the presence of 1.0 μM $ZnCl_2$ decreased with increasing $[Co^{2+}]$ from 2 to 17 mM, and the k_{cat}/K_m was $60\,mM^{-1}\,s^{-1}$ at 17 mM Co^{2+} ion. This value is almost the same as that observed at 17 mM Co^{2+} in the absence of Zn^{2+} (Fig. 2A). On the other hand, thermolysin activity in the presence of 1 mM Co^{2+} decreased with increasing $[Zn^{2+}]$ from 0 to 10 μM, and it shows a reversed saturation curve (Fig. 3A). The $-\Delta(k_{cat}/K_m)$ increment

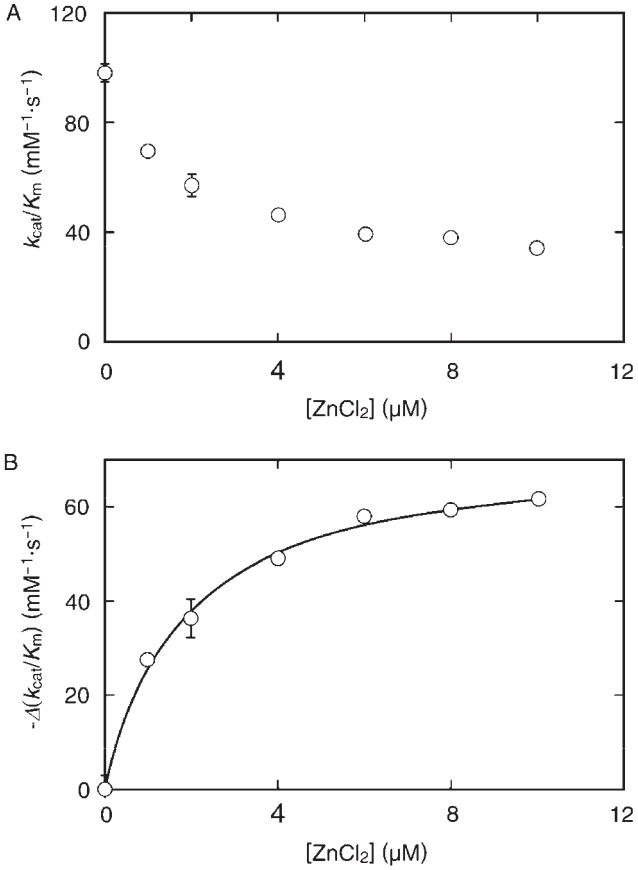
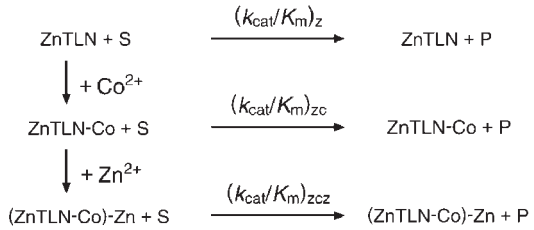


Fig. 3. **Suppression of the Co^{2+}-dependent activation of thermolysin by $ZnCl_2$.** The thermolysin-catalysed hydrolysis of FAGLA was carried out in buffer B containing 1 mM $CoCl_2$ in the presence of 0–10 μM $ZnCl_2$, at 25°C. $[TLN]_o = 0.10$ μM and $[FAGLA]_o = 0.10$–0.32 mM. (A) Dependence of thermolysin activity (k_{cat}/K_m) activated by $CoCl_2$ on the $ZnCl_2$ concentration. (B) Dependence of the $ZnCl_2$-dependent decrement $[-\Delta(k_{cat}/K_m)]$ in thermolysin activity activated by $CoCl_2$ on the $ZnCl_2$ concentration. The $\Delta(k_{cat}/K_m)$, which is defined as the difference between the k_{cat}/K_m under the absence and presence of $ZnCl_2$, was calculated from the data shown in panel A. The solid line is a theoretically fitted curve to the experimental data using Equation 5 with least-squares regression.



Scheme 4. **Change in thermolysin activity by the addition of Co^{2+} and Zn^{2+} ions successively.** (ZnTLN-Co)-Zn: ZnTLN bound with Co^{2+} ion at the secondary site, and with Zn^{2+} at the tertiary site.

was defined as follows (Equation 4) for analysing this curve.

$$-\Delta\left(\frac{k_{cat}}{K_m}\right) = \left(\frac{k_{cat}}{K_m}\right)_o - \left(\frac{k_{cat}}{K_m}\right)_x \quad (4)$$

where $(k_{cat}/K_m)_o$ and $(k_{cat}/K_m)_x$ are the k_{cat}/K_m values at 0 M and x M Zn^{2+}, respectively. The plot of $-\Delta(k_{cat}/K_m)$ *versus* $[ZnCl_2]$ was fitted well to Equation 5 (Fig. 3B). The equation was formulated by assuming that Zn^{2+} ion binds to a thermolysin species (ZnTLN-Co), which is activated with 1 mM Co^{2+}, to form another species (ZnTLN-Co)-Zn with a decreased k_{cat}/K_m value.

$$-\Delta(k_{cat}/K_m) = -\Delta(k_{cat}/K_m)_{max}/(K_{d\,[(ZnTLN-Co)-Zn]} + [ZnCl_2]) \quad (5)$$

$$K_{d[(ZnTLN-Co)-Zn]} = [ZnTLN - Co][Zn]/[(ZnTLN - Co) - Zn] \quad (6)$$

where $K_{d\ [(ZnTLN-Co)-Zn]}$ is the dissociation constant for the interaction of Zn^{2+} with a thermolysin species ZnTLN-Co which is activated with 1 mM $CoCl_2$ (Scheme 4).

The values of $K_{d[(ZnTLN-Co)-Zn]}$ and $-\Delta(k_{cat}/K_m)_{max}$ were estimated to be (1.88 ± 0.17) μM and $(75.3 \pm 2.0)\,mM^{-1}\,s^{-1}$, respectively. The value of $-\Delta(k_{cat}/K_m)_{max}$ was in good agreement with the $\Delta(k_{cat}/K_m)_{max}$ value $(75.8\,mM^{-1}\,s^{-1})$ observed in the activation of ZnTLN by Co^{2+} ion (Fig. 2B). This result indicates that Zn^{2+} ion suppresses the Co^{2+}-induced activation of thermolysin (ZnTLN). In order to examine this suppression mechanism, the effect of $ZnCl_2$ on the Co^{2+}-induced activation of thermolysin activity was measured at the $CoCl_2$ concentration lower than 0.5 mM (Fig. 4). The $\Delta(k_{cat}/K_m)$ values are plotted against $[CoCl_2]$ in the presence of various concentrations of $ZnCl_2$ (0–1.0 μM). Saturation curves were observed (Fig. 4A) as similar to that observed in Fig. 2B. The curves at respective $ZnCl_2$ concentrations were analysed according to Equation 3. The activation of thermolysin induced by $CoCl_2$ was suppressed with increasing $[ZnCl_2]$ added to the reaction mixture. In the $1/\Delta(k_{cat}/K_m)$ *versus* $1/[CoCl_2]$ plot, linear lines obtained at different $ZnCl_2$ concentrations were crossed on the vertical axis, indicating that Zn^{2+} ion suppresses the activation induced by binding of Co^{2+} to ZnTLN in a competitive manner (Fig. 4B). This suggests that Zn^{2+} ion might bind to the same site of thermolysin as Co^{2+} binds (Scheme 5). ZnTLN is

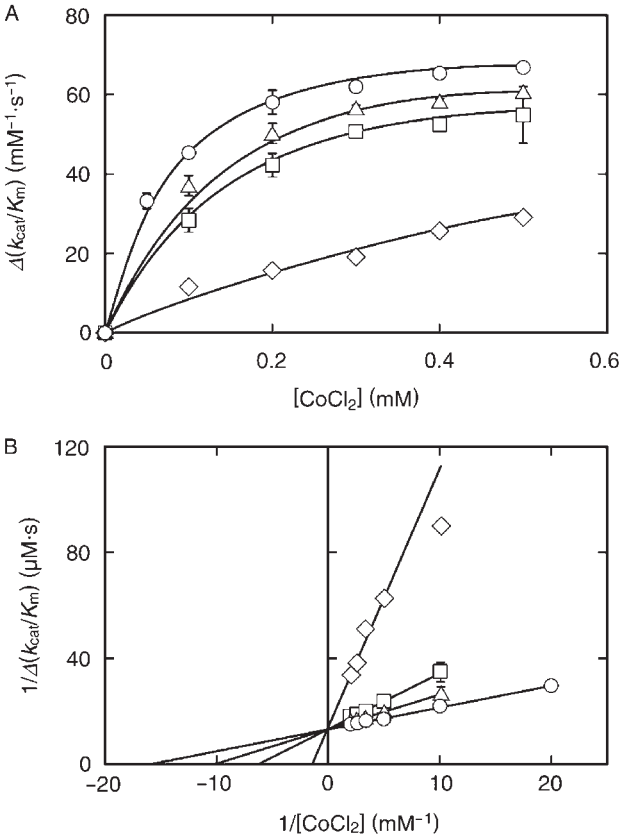
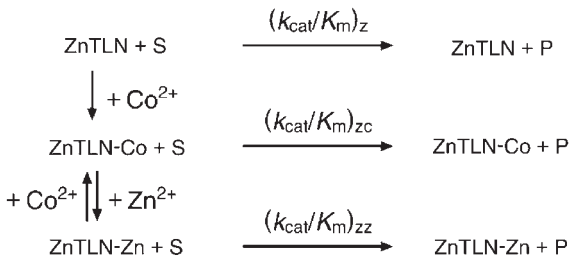


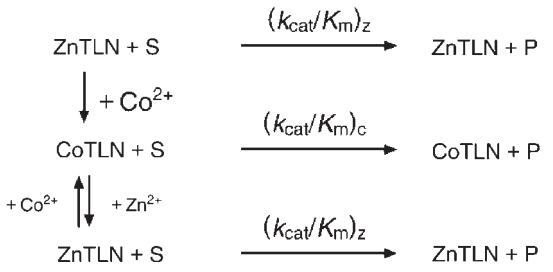
Fig. 4. **Competitive inhibition of Zn^{2+} ion against the Co^{2+}-dependent activation of thermolysin.** (A) Effect of various concentrations of Zn^{2+} ion on the increment of the k_{cat}/K_m $[\Delta(k_{cat}/K_m)]$ induced by the addition of Co^{2+} ion. The reaction was carried out in buffer B in the presence of 0–20 mM $CoCl_2$, and 0–1 μM $ZnCl_2$, at 25°C. $[TLN]_o = 0.1$ μM. The $ZnCl_2$ concentrations are 0 M (open circle); 0.1 μM (open triangle); 0.2 μM (open square) and 1.0 μM (open diamond). Solid lines are the theoretical curves drawn by fitting the data to Equation 6. (B) The double reciprocal plot of the $\Delta(k_{cat}/K_m)$ values induced by Co^{2+} ion at various concentrations of Zn^{2+} ion against the concentration of Co^{2+} ion. The $ZnCl_2$ concentrations are 0 M (open circle); 0.1 μM (open triangle); 0.2 μM (open square) and 1.0 μM (open diamond).

converted to ZnTLN-Co in the presence of Co^{2+} ion,



Scheme 5. **Replacement of Co^{2+} ion with Zn^{2+} at the secondary site.**

and ZnTLN-Co is converted possibly by the binding with Zn^{2+} ion to either (ZnTLN-Co)-Zn or ZnTLN-Zn. Considering the competitive inhibition by Zn^{2+}



Scheme 6. **Replacement of Co^{2+} ion with Zn^{2+} at the catalytic site.**

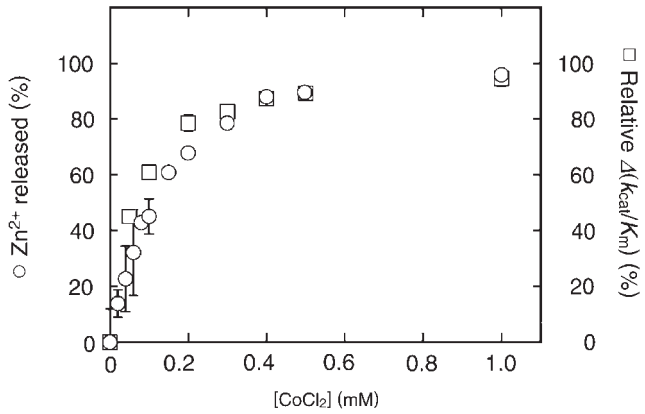


Fig. 5. **Release of Zn^{2+} ion from the active site of thermolysin on the addition of Co^{2+} ion**. Thermolysin (ZnTLN, 1.0 μM) and $CoCl_2$ (0–1.0 mM) were incubated in buffer B at 25°C. Zn^{2+} ion released from the active site of thermolysin was estimated by determining Zn^{2+} ion in the reaction mixture using a fluorescent probe. Thermolysin activity was measured for the mixture of thermolysin and $CoCl_2$ at their concentrations of 0.1 μM and 0–1.0 mM, respectively, in buffer B. The amounts of Zn^{2+} ion released (open circle) and the increment of k_{cat}/K_m (open square) induced by the addition of Co^{2+} ion were plotted against [$CoCl_2$].

against the Co^{2+}-dependent activation, formation of (ZnTLN-Co)-Zn might be declined and ZnTLN-Co could be converted to ZnTLN-Zn (Scheme 5). Presently, we have no evidence if the thermolysin species designated as ZnTLN-Co is the same as the species CoTLN, but if it was so as suggested in Fig. 2, Zn^{2+} ion at the concentration of 0–1.0 μM could replace the Co^{2+} ion bound at the active site of thermolysin (Scheme 6).

Replacement of the Catalytic Zn^{2+} ion in the Thermolysin Active Site by Co^{2+} ion—The $(k_{cat}/K_m)_{max}$ value [$(1.00 \pm 0.04) \times 10^2\,mM^{-1}\,s^{-1}$] obtained by adding 2.0 mM $CoCl_2$ to thermolysin (ZnTLN) (Fig. 2) was essentially the same as that ($116\,mM^{-1}\,s^{-1}$) of CoTLN (*22*), indicating that $(k_{cat}/K_m)_c$ is the same as $(k_{cat}/K_m)_{zc}$ in Schemes 2 and 4. Zn^{2+} ion inhibits competitively the binding of Co^{2+} ion to thermolysin (Fig. 4). These results suggest two possibilities. One is that the catalytic Zn^{2+} ion of thermolysin (ZnTLN) can be replaced by Co^{2+} ion on the addition of 2.0 mM $CoCl_2$ and CoTLN is formed (Scheme 5). The other is that the catalytic Zn^{2+} ion of ZnTLN was not replaced from the active site on the Co^{2+} addition, and ZnTLN-Co is formed (Scheme 4). In this case, ZnTLN-Co and CoTLN should have the same (k_{cat}/K_m) values $[(k_{cat}/K_m)_{zc} = (k_{cat}/K_m)_c]$, and also ZnTLN and ZnTLN-Zn should have the same (k_{cat}/K_m) values $[(k_{cat}/K_m)_z = (k_{cat}/K_m)_{zz}]$. We examined whether the catalytic Zn^{2+} ion of ZnTLN can be replaced with Co^{2+} ion on the addition of Co^{2+} ion and released into the solution. Zn^{2+} ion, if any was released from the active site, can be detected by a Zn^{2+}-specific fluorophore, ZnAF-2, and the Zn^{2+} concentration remaining in the active site is estimated as the difference between the total thermolysin concentration [ZnTLN] and the increment of Zn^{2+} concentration on the addition of Co^{2+} ion determined by ZnAF-2. The ratio of the Zn^{2+} concentration released to the total thermolysin concentration increased with increasing the Co^{2+} ion concentration added to the reaction mixture (Fig. 5). The behaviour of the increase in the Zn^{2+} ion concentration ([ΔZn^{2+}]) observed with increasing [Co^{2+}] was in good agreement with that of the increase in thermolysin activity [$\Delta k_{cat}/K_m$)] induced by the addition of Co^{2+} ion (Fig. 4). This result suggests strongly that the catalytic Zn^{2+} ion is replaced directly when Co^{2+} ion is added to ZnTLN, and that CoTLN but not ZnTLN-Co is formed. In other words, ZnTLN-Co and CoTLN must be the same (Schemes 5 and 6).

Estimation of K_{dCo} Using Apo-thermolysin—The dependence of thermolysin (ZnTLN) activity on [$CoCl_2$] shows a saturation curve (Fig. 2), and the $K_{d(ZnTLN\text{-}Co)}$ of Co^{2+} ion for its interaction with ZnTLN was determined to be (62.8 ± 2.2) μM. However, it should be noted that the value was determined under the condition where Zn^{2+} ion (at the concentration equal to that of ZnTLN) was contained in the reaction mixture. When ZnTLN-Co is assumed to be formed in the interaction between ZnTLN and Co^{2+} ion, the Zn^{2+} ion must stay as a bound-form in the active site of the enzyme, and when CoTLN is assumed to be formed, it must be released from the active site into the reaction mixture. Thus, the $K_{d(ZnTLN\text{-}Co)}$ should not be the same as the K_d for the interaction of Co^{2+} with apo-thermolysin where no Zn^{2+} ion exists in the reaction mixture, even if the species ZnTLN-Co formed in the interaction between ZnTLN and Co^{2+} ion is CoTLN. In order to estimate the K_d for the interaction between Co^{2+} and apo-thermolysin, the dependence of k_{cat}/K_m for apo-thermolysin on [Co^{2+}] was examined in the absence of Zn^{2+} ion (Fig. 6). Apo-thermolysin was shown to have a small activity due to the slightly remained Zn^{2+} ion in the active site of the enzyme, and the k_{cat}/K_m value was $(0.181 \pm 0.026)\,mM^{-1}\,s^{-1}$, which is 6.6% of that ($27.0\,mM^{-1}\,s^{-1}$) of the native thermolysin (ZnTLN). The activity of apo-thermolysin increased with increasing [$CoCl_2$], and the maximum activity [$(k_{cat}/K_m)_c$] observed was $(1.05 \pm 0.07) \times 10^2\,mM^{-1}\,s^{-1}$, which is essentially the same as that

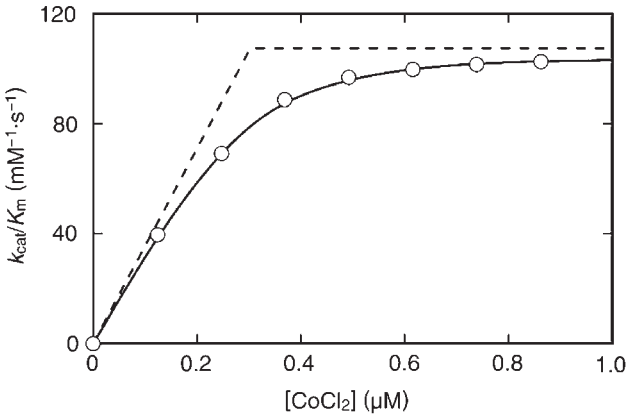


Fig. 6. **Recovery of thermolysin activity of apo-thermolysin by the addition of Co^{2+} ion.** Thermolysin activity of apo-thermolysin was determined in the presence of 0–0.86 μM $CoCl_2$ in buffer B at 25°C. The thermolysin concentration in the reaction mixture was 0.3 μM. The solid line is a theoretical curve drawn by fitting the data to Equation 6 using the values of K_{dCo} and $(k_{cat}/K_m)_c$ of (27.3 ± 6.1) nM and (1.08 ± 0.01) × 10^2 $mM^{-1}s^{-1}$, respectively, by assuming that one mole of Co^{2+} ion binds to one mole of thermolysin. The dashed lines are asymptotes for the theoretical curve.

$[(k_{cat}/K_m)_{zc} = (1.00 \pm 0.04) \times 10^2\,mM^{-1}\,s^{-1}]$ observed when $CoCl_2$ was added to ZnTLN (Fig. 2A).

The binding of Co^{2+} ion to apo-thermolysin was analysed by Scheme 3, where the activity (k_{cat}/K_m) is described as follows:

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)_c[\text{CoTLN}]}{[\text{ApoTLN}]_o} \tag{7}$$

where [CoTLN], $[\text{ApoTLN}]_o$ and $(k_{cat}/K_m)_c$ are the concentrations of the Co^{2+}-substituted thermolysin (CoTLN), the initial concentration of apo-thermolysin and the maximum k_{cat}/K_m which is the k_{cat}/K_m obtained when all apo-thermolysin molecules carry the catalytically active Co^{2+} ion at the active site, respectively. The dissociation constant (K_{dCo}) for the interaction of Co^{2+} ion with thermolysin is defined as follows:

$$K_{dCo} = \frac{[\text{ApoTLN}][Co^{2+}]}{[\text{CoTLN}]} \tag{8}$$

where [ApoTLN], $[Co^{2+}]$ and [CoTLN] are the concentrations of apo-thermolysin (ApoTLN), free Co^{2+} and CoTLN, respectively. The initial concentrations of thermolysin and Co^{2+} ion ($[\text{ApoTLN}]_o$ and $[Co^{2+}]_o$) are given as follows.

$$[\text{ApoTLN}]_o = [\text{ApoTLN}] + [\text{CoTLN}] \tag{9}$$

$$[Co^{2+}]_o = [Co^{2+}] + [\text{CoTLN}] \tag{10}$$

Substituting for [ApoTLN] and $[Co^{2+}]$ in Equation 8 by those in Equations 9 and 10 gives

$$K_{dCo} = \frac{([\text{ApoTLN}]_o - [\text{CoTLN}])([Co^{2+}]_o - [\text{CoTLN}])}{[\text{CoTLN}]}$$

This is converted to the following equations:

$$\begin{aligned} K_{dCo}[\text{CoTLN}] &= [\text{CoTLN}]^2 - ([E]_o + [Co^{2+}]_o)[\text{CoTLN}] \\ &\quad + [\text{ApoTLN}]_o[Co^{2+}]_o \\ 0 &= [\text{CoTLN}]^2 - (K_{dCo} + [\text{ApoTLN}]_o \\ &\quad + [Co^{2+}]_o)[\text{CoTLN}] + [\text{ApoTLN}]_o[Co^{2+}]_o \end{aligned}$$

when $[Co^{2+}]_o = 0$, [CoTLN] must be zero, and thus, the concentration of CoTLN is expressed as Equation 11.

$$[\text{CoTLN}] = \left(\alpha - \sqrt{\alpha^2 - 4\beta}\right)/2 \tag{11}$$

where α and β are given by

$$\alpha = K_{dCo} + [\text{ApoTLN}]_o + [Co^{2+}]_o$$
$$\beta = [\text{ApoTLN}]_o[Co^{2+}]_o$$

The right side of Equation 11 was substituted for [CoTLN] in Equation 7. In Equation 11, $[\text{ApoTLN}]_o$ was set in the experimental conditions, and $[Co^{2+}]_o$ was determined from the α and β values. Thus, k_{cat}/K_m can be expressed with unknown parameters, K_{dCo} and $(k_{cat}/K_m)_{max}$ [namely $(k_{cat}/K_m)_c$], in Equation 7. From the best fitting as shown by the solid line in Fig. 6, the values of K_{dCo} and $(k_{cat}/K_m)_{max}$ were estimated to be $(27.3 \pm 6.1)\,nM$ and $(1.08 \pm 0.01) \times 10^2\,mM^{-1}\,s^{-1}$, respectively, by assuming that one mole of Co^{2+} ion binds to one mole of thermolysin.

Estimation of the Affinity of Zn^{2+} ion in Thermolysin by Analysing the Competitive Binding of Zn^{2+} and Co^{2+} ions to Apo-thermolysin—It is considerably difficult to estimate the K_d (K_{dZn}) for the interaction of Zn^{2+} ion with apo-thermolysin by measuring the change in thermolysin activity with changing $[Zn^{2+}]$ added to apo-thermolysin because of too small K_d values. Therefore, thermolysin activity (k_{cat}/K_m) was measured by changing $[Zn^{2+}]$ with the coexistence of Co^{2+} ion, and the K_{dZn} value was evaluated by analysing competitive binding of Co^{2+} and Zn^{2+} ions to apo-thermolysin. When Zn^{2+} and Co^{2+} ions are added to the reaction mixture, the activity (k_{cat}/K_m) can be described using the concentrations of ZnTLN, CoTLN and the total thermolysin species (ZnTLN, CoTLN and ApoTLN), which are designated [ZnTLN], [CoTLN] and $[\text{TLN}]_o$, respectively, as follows:

$$\begin{aligned} k_{cat}/K_m &= (k_{cat}/K_m)_z[\text{ZnTLN}]/[\text{TLN}]_o \\ &\quad + (k_{cat}/K_m)_c[\text{CoTLN}]/[\text{TLN}]_o \end{aligned} \tag{12}$$

where $(k_{cat}/K_m)_z$ and $(k_{cat}/K_m)_c$ are k_{cat}/K_m values of the thermolysin species (ZnTLN and CoTLN) accommodating Zn^{2+} and Co^{2+} ions at the active site, respectively. The dissociation constants of the interactions of thermolysin with Zn^{2+} and Co^{2+} are defined, respectively, as follows:

$$K_{dZn} = [\text{ApoTLN}][Zn^{2+}]/[\text{ZnTLN}] \tag{13}$$

$$K_{dCo} = [\text{ApoTLN}][Co^{2+}]/[\text{CoTLN}] \tag{14}$$

The initial concentrations of thermolysin, Zn^{2+} ion and Co^{2+} ion ($[TLN]_o$, $[Zn^{2+}]_o$ and $[Co^{2+}]_o$) are given as follows.

$$[TLN]_o = [ApoTLN] + [ZnTLN] + [CoTLN] \quad (15)$$

$$[Zn^{2+}]_o = [Zn^{2+}] + [ZnTLN] \quad (16)$$

$$[Co^{2+}]_o = [Co^{2+}] + [CoTLN] \quad (17)$$

The initial concentration of Co^{2+} ion ($[Co^{2+}]_o$) is much higher than that of thermolysin ($[TLN]_o$), and thus

$$[Co^{2+}]_o \approx [Co^{2+}] \quad (18)$$

Furthermore, the initial concentrations of Zn^{2+} and Co^{2+} ($[Zn^{2+}]_o$ and $[Co^{2+}]_o$) are much higher than the values of K_d for each ions, namely, $[Zn^{2+}]_o >> K_{dZn}$ and $[Co^{2+}]_o >> K_{dCo}$; and thus Equation 15 is transformed to

$$[TLN]_o \approx [ZnTLN] + [CoTLN] \quad (19)$$

The quotient of K_{dZn} and K_{dCo} are given from Equations 13 and 14 as follows:

$$K_{dZn}/K_{dCo} = ([Zn^{2+}][CoTLN])/([Co^{2+}][ZnTLN]) \quad (20)$$

Substituting $[Zn^{2+}]$, $[Co^{2+}]$ and [CoTLN] in Equation 19 by those in Equations 16, 18 and 19 yields the following equation.

$$K_{dZn}/K_{dCo} = ([Zn^{2+}]_o - [ZnTLN])([TLN]_o - [ZnTLN])/[Co^{2+}]_o[ZnTLN]$$

This is converted to

$$0 = [ZnTLN]^2 - ([TLN]_o + [Zn^{2+}]_o + K_{dZn}/K_{dCo}[Co^{2+}]_o) \times [ZnTLN] + [TLN]_o[Zn^{2+}]_o$$

Thus, the concentration of ZnTLN is expressed as follows:

$$[ZnTLN] = \left(\gamma \pm \sqrt{\gamma^2 - 4\delta}\right)/2 \quad (21)$$

where γ and δ are given by

$$\gamma = [TLN]_o + [Zn^{2+}]_o + K_{dZn}/K_{dCo}[Co^{2+}]_o$$
$$\delta = [TLN]_o[Zn^{2+}]_o$$

As the solution of [ZnTLN] must be limited to the condition of $[ZnTLN] \leq [TLN]_o$, [ZnTLN] is expressed as follows:

$$[ZnTLN] = \left(\gamma + \sqrt{(\gamma^2 - 4\delta}\right)/2 \quad (22)$$

The right side of Equation 22 was substituted for [ZnTLN] in Equation 12. It was also substituted for [ZnTLN] in Equation 19, and the resulting [CoTLN] was substituted into Equation 12. Thus, Equation 12 was expressed with $(k_{cat}/K_m)_z$, $(k_{cat}/K_m)_c$, $[Zn^{2+}]_o$, $[Co^{2+}]_o$, K_{dZn} and K_{dCo}. The experimentally obtained values of 27.4 and 108 $mM^{-1}s^{-1}$ were substituted for $(k_{cat}/K_m)_z$ and $(k_{cat}/K_m)_c$, respectively, and the values of $[TLN]_o$ (0.10 μM), $[Zn^{2+}]$ and $[Co^{2+}]_o$ were set in the experimental conditions in Equation 12. Subsequently, the unknown parameter K_{dZn} was tried to be determined by best-fitting the data in Fig. 7 to Equation 12 by the least-squares regression, although the fitting was declined. This suggests that the estimation of the initial concentration of Zn^{2+} ion ($[Zn^{2+}]_o$) was inappropriate. It should be improved so that the sum of the Zn^{2+} concentration ($[Zn^{2+}]_{TLN}$) accommodated to the active site of the native thermolysin (ZnTLN) and the Zn^{2+} ion concentration ($[Zn^{2+}]_{Added}$) was added to the reaction mixture in the form of $ZnCl_2$. Accordingly, $[Zn^{2+}]_o$ is not expressed with $[Zn^{2+}]_{TLN}$ but should be expressed as follows:

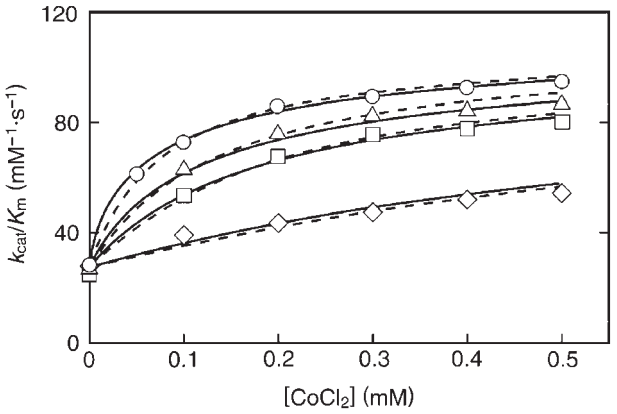


Fig. 7. **Estimation of the affinity constant ($K_{d\ Zn}$) of Zn^{2+} ion in thermolysin (ZnTLN) from the competitive binding of Zn^{2+} and Co^{2+} ions to thermolysin.** The reaction was carried out in buffer B in the presence of 0–20 mM $CoCl_2$, and 0–1 μM $ZnCl_2$, at 25°C. The thermolysin concentration for activity measurement was 0.1 μM, and the $ZnCl_2$ concentrations were 0 M (open circle); 0.1 μM (open triangle); 0.2 μM (open square) and 1.0 μM (open diamond). The experimental data are the same as those shown in Fig. 4A. The solid lines are the theoretical curves drawn by fitting the data to Equation 12. The dashed lines, which are essentially the same drawn by the dotted lines in Fig. 4A, are the theoretical curves drawn by fitting the data to Equation 5. The K_{dZn} value was estimated to be (36.7 ± 0.1) pM.

$$[Zn^{2+}]_o = [Zn^{2+}]_{TLN} + [Zn^{2+}]_{Added} \quad (23)$$

The $[Zn^{2+}]_o$ value determined by Equation 23 was substituted into Equation 12, and the best-fitting was tried to the data of Fig. 7. Theoretically fitted curves drawn by solid lines in Fig. 7 are well fitted to the data, and the values of K_{dZn} and $[Zn^{2+}]_{TLN}$ were estimated to be (36.7 ± 0.1) pM and (0.136 ± 0.002) μM, respectively. The $[Zn^{2+}]_{TLN}$ value is in good agreement with the initial concentration of thermolysin (0.10 μM), suggesting verification of the improved treatment used. Therefore, the K_{dZn} value obtained is the correct value by considering the Zn^{2+}concentration bound to thermolysin ($[Zn^{2+}]_{TLN}$) in addition to the Zn^{2+}concentration added. It should be noted that this procedure enables to determine the K_{dZn} value in the competitive binding of Zn^{2+} and Co^{2+} ions to thermolysin.

Table 1. **Comparison of dissociation constants of Zn^{2+} and Co^{2+} ions for various metallopeptidases.**

	$K_{d\ Zn}$ (M)	$K_{d\ Co}$ (M)	$K_{d\ Zn}/K_{d\ Co}$
Thermolysin[a]	3.7×10^{-11}	2.7×10^{-8}	1.4×10^{-3}
Thermolysin[b]	2.4×10^{-13}	3×10^{-10}	0.8×10^{-3}
Carboxypeptidase A[c]	4.7×10^{-9}	1.5×10^{-6}	3.1×10^{-3}
Angiotensin converting enzyme[d]	2.4×10^{-9}	4.5×10^{-7}	5.3×10^{-3}
Dipeptidyl peptidase III[e]	1.9×10^{-13}	8.2×10^{-13}	0.23

[a]This study; [b]Feder *et al.* (*33*); [c] Colemen and Vallee (*36*); [d] Bünning and Riordan (*38*); [e] Hirose *et al.* (*47*).

DISCUSSION

Difference in the Fashion of the Salt-dependent Activation of Thermolysin between $CoCl_2$ and NaCl—It was shown that thermolysin is activated by the addition of $CoCl_2$ at the concentration of up to 2.0 mM (Fig. 2), and this activating fashion is clearly different from that of the remarkable activation observed with high concentrations (1–5 M) of neutral monovalent salts such as NaCl, KCl, NaBr, KBr, *etc.* (*15*, *16*). The enzyme activity (k_{cat}/K_m) increases with increasing [$CoCl_2$] in a saturating fashion, and this suggests that the Co^{2+}-dependent activation might be due to the binding of Co^{2+} ion to thermolysin. On the other hand, the activity increases with increasing [NaCl] uniquely in an exponential fashion, and not only Na^+ ion but also Cl^- ion is known to contribute to the activation, although the mechanism of this activation has not yet been clearly revealed (*15–22*). The degree of activation between $CoCl_2$ and NaCl is also very different. The activity increases 3–4 times at 1–2 mM $CoCl_2$, but 2 times and 12–15 times at the NaCl concentrations of 1 and 4 M, respectively. This difference in thermolysin activation between $CoCl_2$ and NaCl suggests the difference in their activation mechanism. Because the solubility of $CoCl_2$ is very low compared with that of NaCl, the effect of high concentration of $CoCl_2$ on thermolysin activity could not be examined, but it can be described that $CoCl_2$ is a low-concentration activator, whereas NaCl and other monovalent neutral salts are high-concentration activators.

Direct Substitution of Co^{2+} ion for the Catalytic Zn^{2+} ion in the Active Site of Thermolysin—The effect of metal substitution has been investigated with many metalloproteases; thermolysin (*32–35*), carboxypeptidase A (*36*, *37*), angiotensin converting enzyme (*38*), aminopeptidases (*39–41*), deuterolysin (*42*), astacin (*43*), serralysin (*44*), collagenases (*45*, *46*) and dipeptidyl peptidase III (*47*). These metalloproteases are activated by the substitution of Co^{2+} ion for the catalytic Zn^{2+} ion except for the case of angiotensin converting enzyme and deuterolysin, but the degree of activation is different by depending on enzyme species. In the present study, it has been shown that the catalytic Zn^{2+} ion of thermolysin (ZnTLN) is replaced directly with Co^{2+} ion added to the enzyme solution. For this replacement, it is not necessary to prepare apo-thermolysin by removing the catalytic Zn^{2+} ion from the active site. Possibility of this type of direct metal replacement has been only suggested with thermolysin (*34*), carboxypeptidase A (*36*) and collagenases (*46*) based on the activation observed when cobalt salt was added to the enzymes. Only in the case of carboxypeptidase A, the concentrations of Zn^{2+} and Co^{2+} ions accommodated by the enzyme were determined using radioactive Zn^{2+} and Co^{2+} ions, and the substitution of a Co^{2+} ion for the catalytic Zn^{2+} ion was shown explicitly (*36*, *48*, *49*). In the present study, we indicated directly the substitution of Co^{2+} for the catalytic Zn^{2+} in thermolysin using a Zn^{2+}-specific fluorescent probe, and this method might be more convenient and useful in comparison with the radioactive method applied to carboxypeptidase A (*33*).

Binding Affinity of Zn^{2+} and Co^{2+} ions to the Active Site of Metallopeptidases—The K_d values in the interactions of thermolysin with Zn^{2+} and Co^{2+} ions (K_{dZn} and K_{dCo} respectively) were estimated to be (36.7 ± 0.1) pM and (27.3 ± 6.1) nM, respectively, at pH 7.0 in the present study. They were also reported to be 0.24 pM and 0.3 nM, respectively, at pH 7.2 based on the inhibition studies (*33*). These values are about 100-fold smaller than those obtained in this study. The cause of this difference is not currently clear, but probably due to the difference in the reaction conditions. On the other hand, the ratio (K_{dZn}/K_{dCo}) estimated in this study is similar to that reported previously (*33*). The K_d values with other metalloproteases were also reported (Table 1). Carboxypeptidase A (*28*) and angiotensin converting enzyme (*38*) have similar K_{dZn} values and also K_{dCo} values as well, and these values are 100 times larger than those of thermolysin. The (K_{dZn}/K_{dCo}) values for carboxypeptidase A and angiotensin converting enzyme are similar to that of thermolysin. The K_{dZn} and K_{dCo} values of dipeptidyl peptidase III, which has a unique zinc binding motif HEXXXH (in reality, HELLGH) but not a common HEXXH, are 0.19 pM and 0.82 pM, respectively (*47*). It should be noted that the (K_{dZn}/K_{dCo}) value of 0.23 observed with dipeptidyl peptidase III is 1000 times larger than the values $[(5.3 - 0.8) \times 10^{-3}]$ observed with thermolysin, carboxypeptidase A and angiotensin converting enzyme. The difference in the affinity of metalloenzymes for Zn^{2+} and Co^{2+} ions might be due to the difference in the size of the binding site to accommodate the metal ions. The metal-binding sites of thermolysin and dipeptidyl peptidase III bind Zn^{2+} ion most tightly, among the enzymes examined, indicating that the metal-binding sites of these enzymes have the most suitable configuration for binding Zn^{2+} ion. Thermolysin recognizes well the difference between Zn^{2+} and Co^{2+} ions, and dipeptidyl peptidase III binds Co^{2+} ion strongly, as well. The ionic radius of Zn^{2+} ion (72.0 pm) is slightly larger than that of Co^{2+} ion (62.5 pm), but a significant difference between them might be that Zn^{2+} has a stable four-coordination in the ground state, and Co^{2+} ion has a five-coordination,

which is believed to be similar to that of Zn^{2+} ion in the transition state (*32*). A thermolysin variant, in which the Zn^{2+}-binding HEXXXH motif ($H_{142}ELTH_{146}$) of the native thermolysin was converted to $H_{142}ELTGH$ by mimicking that ($H_{450}ELLGH_{455}$) of dipeptidyl peptidase III, has no proteolytic activity (Hashida and Inouye, unpublished results), suggesting that the structural geometry of the active-site zinc-binding motif is crucial for thermolysin activity. It should be reminded that the Co^{2+}-substituted dipeptidyl peptidase III has 3–4 times higher activity than the Zn^{2+}-type, native enzyme (*47*), as has been observed also with carboxypeptidase A, thermolysin and so on (*32*). This suggests that the unique Zn^{2+}-binding motif of dipeptidyl peptidase III can bind Co^{2+} ion as well as the common motif does. It is also interesting to note that the difference in the affinity of various peptidases for Zn^{2+} and Co^{2+} ions might be ascribed to the difference in the undefined amino acid designated as XX or XXX residues in the Zn^{2+}-binding motif. The factors determining the binding affinity and specificity of the catalytic metal ion to the active site of the metalloenzymes have not yet been studied well. Recently, we have found that higher concentration (mM-level) of Co^{2+} ion inhibits thermolysin activity in dual manner (*50*). Namely, the Co^{2+}-dependent inhibition is composed of Ca^{2+}-insensitive and Ca^{2+}-sensitive parts, and Co^{2+} ion plays as a competitive inhibitor in the former part, and induces autolysis in the latter one. In the present study, we proposed a convenient method to evaluate the binding affinity of the catalytic metal ion to the active site of the enzymes, which enables us to examine more precisely the mechanism of the metal-ion recognition and control of the enzyme activity of the metallopeptidases.

In the present paper, we have demonstrated the activation-and-inhibition dual effect of Co^{2+} ion on thermolysin activity. The catalytic Zn^{2+} ion in the active site of thermolysin (ZnTLN) is replaced with Co^{2+} ion when 2 mM cobalt salt is added to thermolysin with accompanying the activation of the enzyme. The competitive binding of Zn^{2+} ion at this site is 1000 times as strong as that of Co^{2+}, although the Zn^{2+}-thermolysin (ZnTLN) is less active than Co^{2+}-enzyme (CoTLN). When higher concentration of Zn^{2+} ion (>0.1 μM), ZnTLN activity decreases severely. Similarly, the activity of CoTLN decreases with higher concentration (>5 mM) of Co^{2+} ion. This suggests that there is the second binding site(s) for Zn^{2+} and Co^{2+} ions on the surface of thermolysin, and their binding at the site(s) reduces the activity. These lines of evidence provide a strategy for regulating thermolysin activity with Co^{2+} and Zn^{2+} ions.

This study was supported in part (K.I.) by Grants-in-Aid for Scientific Research (Nos. 14658203 and 17380065) from the Japan Society for the Promotion of Sciences, and grants (Nos. 0150 and 0345) from the Salt Science Foundation (Tokyo).

REFERENCES

1. Endo, S. (1962) Studies on protease produced by thermophilic bacteria. *J. Ferment. Technol.* **40**, 346–353
2. Matsubara, H. and Feder, J. (1971) Other bacterial, mold, and yeast proteases in *The Enzymes* (Boyer, P.D., ed.) Vol. 3, 3rd edn, pp. 721–795, Academic Press, New York
3. Van der Burg, B. and Eijsink, V. (2004) Thermolysin in *Handbook of Proteolytic Enzymes* (Barrett, J.A., Rawlings, N.D., and Woessner, J. F., eds) Vol. 1, 2nd edn, pp. 374–387, Elsevier, Amsterdam, The Netherlands
4. Inouye, K. (2003) Thermolysin in *Handbook of Food Enzymes* (Whitaker, J.R., Voragen, A.G.J., and Wong, D.W.S., eds) pp. 1019–1028, Marcel Dekker, New York, New York
5. Latt, S.A., Holmquist, B., and Vallee, B.L. (1969) Thermolysin: a zinc metalloenzyme. *Biochem. Biophys. Res. Commun.* **37**, 333–339
6. Feder, J., Garrett, L.R., and Wildi, B.S. (1971) Studies on the role of calcium in thermolysin. *Biochemistry* **10**, 4552–4555
7. Tajima, M., Urabe, I., Yutani, K., and Okada, H. (1976) Role of calcium ions in the thermostability of thermolysin and *Bacillus subtilis* var. *amylosaccharitiicus* neutral protease. *Eur. J. Biochem.* **64**, 243–247
8. Morihara, K. and Tsuzuki, H. (1970) Thermolysin: kinetic study with oligopeptides. *Eur. J. Biochem.* **15**, 374–380
9. Titani, K., Hermodson, M.A., Ericsson, L.H., Walsh, K.A., and Neurath, H. (1972) Amino-acid sequence of thermolysin. *Nature* **238**, 35–37
10. O'Donohue, M.J., Roques, B.P., and Beaumont, A. (1994) Cloning and expression in Bacillus subtilis of the npr gene from *Bacillus thermoproteolyticus* Rokko coding for the thermostable metalloprotease thermolysin. *Biochem. J.* **300**, 599–603
11. Holmes, M.A. and Matthews, B.W. (1982) Structure of thermolysin refined at 1.6 Å resolution. *J. Mol. Biol.* **160**, 623–639
12. Hangauer, D.G., Monzingo, A.F., and Matthews, B.W. (1984) An interactive computer graphics study of thermolysin-catalyzed peptide cleavage and inhibition by *N*-carboxymethyl dipeptides. *Biochemistry* **23**, 5730–5741
13. Mock, W.L. and Aksamawati, M. (1994) Binding to thermolysin of phenolate-containing inhibitors necessitates a revised mechanism of catalysis. *Biochem. J.* **302**, 57–68
14. Mock, W.L. and Stanford, D.J. (1996) Arazoformyl dipeptide substrate for thermolysin. Confirmation of reverse protonation catalytic mechanism. *Biochemistry* **35**, 7369–7377
15. Inouye, K. (1992) Effects of salts on thermolysin: activation of hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, and a unique change in the absorption spectrum of thermolysin. *J. Biochem.* **112**, 335–340
16. Inouye, K., Lee, S.-B., and Tonomura, B. (1996) Effect of amino acid residues at the cleavable site of substrates on the remarkable activation of thermolysin by salts. *Biochem. J.* **315**, 133–138
17. Inouye, K., Lee, S.-B., Nambu, K., and Tonomura, B. (1997) Effects of pH, temperature, and alcohols on the remarkable activation of thermolysin by salts. *J. Biochem.* **122**, 358–364
18. Oneda, H., Muta, Y., and Inouye, K. (2004) Substrate-dependent activation of thermolysin by salt. *Biosci. Biotechnol. Biochem.* **68**, 1811–1813
19. Inouye, K., Kuzuya, K., and Tonomura, B. (1994) A spectrophotometric study on the interaction of thermolysin with chloride and bromide ions, and the state of tryptophyl residue 115. *J. Biochem.* **116**, 530–535
20. Inouye, K., Lee, S.-B., and Tonomura, B. (1998) Effects of nitration and amination of tyrosyl residues in thermolysin on its hydrolytic activity and its remarkable activation by salts. *J. Biochem.* **124**, 72–78
21. Inouye, K., Kuzuya, K., and Tonomura, B. (1998) Effect of salts on the solubility of thermolysin: a remarkable increase in the solubility as well as the activity by the addition of

salts without aggregation or dispersion of thermolysin. *J. Biochem.* **123**, 847–852
22. Inouye, K., Kuzuya, K., and Tonomura, B. (1998) Sodium chloride enhances markedly the thermal stability of thermolysin as well as its catalytic activity. *Biochim. Biophys. Acta* **1388**, 209–214
23. Kamo, M., Inouye, K., Nagata, K., and Tanokura, M. (2005) Preliminary X-ray crystallographic analysis of thermolysin in the presence of 4 M NaCl. *Acta Crystallogr.* **D61**, 710–712
24. Yasukawa, K., Kusano, M., Nakamura, K., and Inouye, K. (2006) Characterization of Gly-D-Phe, Gly-L-Leu, and D-Phe as affinity ligands to thermolysin. *Protein Expr. Purif.* **46**, 332–336
25. Inouye, K., Minoda, M., Takita, T., Sakurama, H., Hashida, Y., Kusano, M., and Yasukawa, K. (2006) Extracellular production of recombinant thermolysin expressed in *Escherichia coli*, and its purification and enzymatic characterization. *Protein Expr. Purif.* **46**, 248–255
26. Kusano, M., Yasukawa, K., Hashida, Y., and Inouye, K. (2006) Engineering of the pH dependence of thermolysin activity as examined by site-directed mutagenesis of Asn 112 located at the active site of thermolysin. *J. Biochem.* **139**, 1017–1023
27. Tatsumi, C., Hashida, Y., Yasukawa, K., and Inouye, K. (2007) Effects of site-directed mutagenesis of the surface residues Gln128 and Gln225 of thermolysin on its catalytic activity. *J. Biochem.* **141** (in press); doi:10.1093/jb/mvm087
28. Matsumiya, Y., Nishikawa, K., Aoshima, H., Inouye, K., and Kubo, M. (2004) Analysis of autodegradation sites of thermolysin and enhancement of its thermostability by modifying Leu155 at an autodegradation site. *J. Biochem.* **135**, 547–553
29. Matsumiya, Y., Nishikawa, K., Inouye, K., and Kubo, M. (2005) Mutational effect for stability in a conserved region of thermolysin. *Lett. Appl. Microbiol.* **40**, 329–334
30. Feder, J. (1968) A spectrophotometric assay for neutral protease. *Biochem. Biophys. Res. Commun.* **32**, 326–332
31. Hirano, T., Kikuchi, K., Urano, Y., and Nagano, T. (2002) Improvement and biological applications of fluorescent probes for zinc, ZnAFs. *J. Am. Chem. Soc.* **124**, 6555–6562
32. Kuzuya, K. and Inouye, K. (2001) Effect of cobalt-substitution of active zinc ion in thermolysin on its activity and active-site microenvironment. *J. Biochem.* **130**, 783–788
33. Feder, J., Garrett, L.R., and Kochavi, D. (1971) Studies on the inhibition of neutral proteases by 1,10-phenanthroline. *Biochim. Biophys. Acta* **235**, 370–377
34. Holmquist, B. and Vallee, B.L. (1974) Metal substitutions and inhibition of thermolysin: Spectra of the cobalt enzyme. *J. Biol. Chem.* **249**, 4601–4607
35. Holland, D.R., Hausrath, A.C., Juers, D., and Matthews, B.W. (1995) Structural analysis of zinc substitutions in the active site of thermolysin. *Protein Sci.* **4**, 1955–1965
36. Coleman, J.E. and Vallee, B.L. (1960) Metallocarboxypeptidases. *J. Biol. Chem.* **235**, 390–395
37. Coleman, J.E. and Vallee, B.L. (1961) Metallocarboxypeptidases: stability constants and enzymatic characteristics. *J. Biol. Chem.* **236**, 2244–2249
38. Bünning, P. and Riordan, J.F. (1985) The functional role of zinc in angiotensin converting enzyme: implications for the enzyme mechanism. *J. Inorg. Biochem.* **24**, 183–198
39. Allen, M.P., Yamada, A.H., and Carpenter, F.H. (1983) Kinetic parameters of metal-substituted leucine aminopeptidase from bovine lens. *Biochemistry* **22**, 3778–3783
40. Prescott, J.M., Wagner, F.W., Holmquist, B., and Vallee, B.L. (1985) Spectral and kinetic studies of metal-substituted *Aeromonas* aminopeptidase: nonidentical, interacting metal-binding sites. *Biochemistry* **24**, 5350–5356
41. Li, J.Y., Chen, L.L., Cui, Y.M., Luo, Q.L., Li, J., Nan, F.J., and Ye, Q.Z. (2003) Specificity for inhibitors of metal-substituted methionine aminopeptidase. *Biochem. Biophys. Res. Commun.* **307**, 172–179
42. Doi, Y., Lee, B.R., Ikeguchi, M., Ohoba, Y., Ikoma, T., Tero-Kubota, S., Yamauchi, S., Takahashi, K., and Ichishima, E. (2003) Substrate specificities of deuterolysin from *Aspergillus oryzae* and electron paramagnetic resonance measurement of cobalt-substituted deuterolysin. *Biosci. Biotechnol. Biochem.* **67**, 264–270
43. Gomis-Ruth, F.X., Grams, F., Yiallouros, I., Nar, H., Kusthardt, U., Zwilling, R., Bode, W., and Stocker, W. (1994) Crystal structures, spectroscopic features, and catalytic properties of cobalt(II), copper(II), nickel(II), and mercury(II) derivatives of the zinc endopeptidase astacin. A correlation of structure and proteolytic activity. *J. Biol. Chem.* **269**, 17111–17117
44. Park, H.I. and Ming, L.J. (2002) Mechanistic studies of the astacin-like *Serratia* metalloendopeptidase serralysin: highly active (>2000%) Co(II) and Cu(II) derivatives for further corroboration of a "metallotriad" mechanism. *J. Biol. Inorg. Chem.* **7**, 600–610
45. Angleton, E.L. and Van Wart, H.E. (1988) Preparation and reconstitution with divalent metal ions of class I and class II *Clostridium histolyticum* apocollagenases. *Biochemistry* **27**, 7406–7412
46. Angleton, E.L. and Van Wart, H.E. (1988) Preparation by direct metal exchange and kinetic study of active site metal substituted class I and class II Clostridium histolyticum collagenases. *Biochemistry* **27**, 7413–7418
47. Hirose, J., Iwamoto, H., Nagao, I., Enmyo, K., Sugao, H., Kanemitu, N., Ikeda, K., Takeda, M., Inoue, M., Ikeda, T., Matsuura, F., Fukasawa, K.M., and Fukasawa, K. (2001) Characterization of the metal-substituted dipeptidyl peptidase III (rat liver). *Biochemistry* **40**, 11860–11865
48. Hirose, J., Ando, S., and Kidani, Y. (1987) Excess zinc ions are a competitive inhibitor for carboxypeptidase A. *Biochemistry* **26**, 6561–6565
49. Larsen, K.S. and Auld, D.S. (1989) Carboxypeptidase A: mechanism of zinc inhibition. *Biochemistry* **28**, 9620–9625
50. Hashida, Y. and Inouye, K. (2007) Molecular mechanism of the inhibitory effect of cobalt ion on thermolysin activity and the suppressive effect of calcium ion on the cobalt ion-dependent inactivation of thermolysin. *J. Biochem.* **141** (in press); doi:10.1093/jb/mvm089