J. Biochem. 130, 783–788 (2001)

Effects of Cobalt-Substitution of the Active Zinc Ion in Thermolysin on Its Activity and Active-Site Microenvironment[1]

Keiko Kuzuya and Kuniyo Inouye[2]

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

Received August 8, 2001; accepted September 26, 2001

Thermolysin is remarkably activated in the presence of high concentrations (1–5 M) of neutral salts [Inouye, K. (1992) *J. Biochem.* 112, 335–340]. The activity is enhanced 13–15 times with 4 M NaCl at pH 7.0 and 25°C. Substitution of the active site zinc with other transition metals alters the activity of thermolysin [Holmquist, B. and Vallee, B.L. (1974) *J. Biol. Chem.* 249, 4601–4607]. Cobalt is the most effective among the transition metals and doubles the activity toward *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide. In this study, the effect of NaCl on the activity of cobalt-substituted thermolysin was examined. Cobalt-substituted thermolysin, with 2.8-fold increased activity compared with the native enzyme, is further activated by the addition of NaCl in an exponential fashion, and the activity is enhanced 13–15 times at 4 M NaCl. The effects of cobalt-substitution and the addition of salt are independent of each other. The activity of cobalt-substituted thermolysin, expressed as k_{cat}/K_m, is pH-dependent and controlled by at least two ionizing residues with pK_a values of 6.0 and 7.8, the acidic pK_a being slightly higher compared to 5.6 of the native enzyme. These pK_a values remain constant in the presence of 4 M NaCl, indicating that the electrostatic environment of cobalt-substituted thermolysin is more stable than that of the native enzyme, the acidic pK_a of which shifts remarkably from 5.6 to 6.7 at 4 M NaCl. Zincov, a competitive inhibitor, binds more tightly to the cobalt-substituted than to native thermolysin at pH 4.9–9.0, probably because of its preference for cobalt in the fivefold coordination. The cobalt substitution has been shown to be a favorable tool with which to explore the active-site microenvironment of thermolysin.

Key words: cobalt, halophilicity, metalloproteinase, thermolysin, zinc.

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (*1, 2*). It requires one zinc ion for enzyme activity and four calcium ions for structural stability (*3–5*), and catalyzes specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (*6*). The amino acid sequence (*7, 8*) and three-dimensional structure (*9*) are available, and a reaction mechanism has been proposed (*10, 11*).

We have reported that high concentrations of neutral salts cause a remarkable activation of the thermolysin-catalyzed hydrolysis and synthesis of *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (ZDFM), a precursor of a synthetic sweetener (*12*), and hydrolysis of *N*-[3-(2-furyl)-acryloyl] (FA)–dipeptide amides with different amino acids at the scissile bond (*13*). The activation is brought about most effectively by NaCl and NaBr, and the activity increases in an exponential fashion with increasing salt concentration. 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, and FA-L-phenylalanyl-L-alanine amide, 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 (*12, 13*). 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 (*12, 14*). The specific interaction between cations and thermolysin might be involved in the activation, and effectiveness is in the order of $Na^+ > K^+ > Li^+$ (*12, 13*). The degree of activation shows a bell-shaped pH-dependence with an optimum pH around 7.0, and decreases significantly with rising temperature and increasing alcohol concentration in the reaction medium (*15*). 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 (*16*). 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 (*17*), and the thermal stability is also increased by the addition of NaCl (*18*). Unique interactions of the molecular surface of ther-

[1] This study was supported in part (K.I.) by Grants-in-Aid for Scientific Research (nos. 11167248 and 11460040) from the Ministry of Education, Science, Sports and Culture of Japan, and grants (nos. 9856 and 0049) from the Salt Science Foundation (Tokyo).

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

Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; zincov, 2-(*N*-hydroxycarboxamido)-4-methyl pentanoyl-L-alanyl-glycine amide.

molysin with ions might change the solubility and thermal stability as well as the activity.

Removing the active site zinc of thermolysin yields an inactive apo-enzyme, and replacing the zinc atom with transition metals can restore the activity (*19*). Cobalt has been reported to restore 200% of the activity of the native enzyme toward FA-glycyl-L-leucine amide (FAGLA). The enhanced activity of cobalt-substituted thermolysin may be due to its ability to accept a metal in fivefold coordination, which is considered to stabilize the transition state (*20*).

In this paper, we describe the activation of thermolysin by NaCl as being related to the active-site metal ion by preparing cobalt-substituted thermolysin. The pH dependence of the activity and inhibition by competitive inhibitors of the cobalt-substituted thermolysin are also demonstrated in comparison with that of the native enzyme. The evidence described in this paper provides insights into the molecular expression of the activity of thermolysin by stabilization of the Michaelis complex and transition-state by the addition of salts and substitution of the active-site zinc with cobalt. It is shown that cobalt-substitution might be a favorable tool with which to explore the active-site microenvironment. These results might be also useful for developing industrial applications of thermolysin.

MATERIALS AND METHODS

Materials—A three-times crystallized and lyophilized preparation of thermolysin (Lot T5CB491; 8720 proteinase units/mg according to the supplier) was purchased from Daiwa Kasei (Osaka). 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 was determined spectrophotometrically using an absorptivity value, A (1 mg/ml), at 277 nm of 1.83 (*12*), and a molecular mass of 34.6 kDa (*7*). FAGLA (Lot 57H5800) was purchased from Sigma (St. Louis, MO). The concentration of FAGLA was determined spectrophotometrically using the molar absorptivity, ε_{345} = 766 $M^{-1}\cdot cm^{-1}$ (*12, 21*). 2-(*N*-Hydroxycarboxamido)-4-methylpentanoyl-L-alanyl-glycine amide (zincov) (Lot 293085) was purchased from Calbiochem (La Jolla, CA). The concentration of zincov was estimated from the molecular weight of 302.3. All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto) or Wako Pure Chemicals (Kyoto).

In order to minimize contamination by adventitious metal ions, the buffers used for apo-thermolysin and cobalt-substituted thermolysin were passed through chelating ion exchange resin Chelex 100 (Bio-Rad Laboratories, Hercules, CA). $CaCl_2$ and $CoCl_2$ were then added to the buffers. Cuvettes were soaked in 1 mM EDTA and rinsed thoroughly in Chelex 100–treated distilled water before use.

Preparations of Apo-Thermolysin and Cobalt-Substituted Thermolysin—Thermolysin solution (2 ml) was applied to a Sephadex G-25 Fine column (inner diameter 1.5 cm × 17 cm) equilibrated with 40 mM HEPES–10 mM $CaCl_2$–5 mM 1,10-phenanthroline (pH 7.5) and eluted with the same buffer. Fractions containing thermolysin were collected, applied to a prepacked PD-10 column (Sephadex G-25 M, Amersham Pharmacia), and eluted with 40 mM HEPES–10 mM $CaCl_2$ (pH 7.5) to remove excess 1,10-phenanthroline. Thermolysin, free from the active site metal ion and eluted in the void volume, was collected as apo-thermolysin. Apo-thermolysin was stored frozen at –30°C until use. To prepare cobalt-substituted thermolysin, 100 μM $CoCl_2$ was added to apo-thermolysin. The metal concentration was evaluated by an inductively coupled plasma atomic emission spectrometer (Perkin-Elmer Optima-3000DV) with ultrasonic nebulization (*22*). The respective contents of zinc and cobalt atoms (mol/mol protein) were 1.10 and <0.01 for the native thermolysin; 0.05 and <0.01 for apo-thermolysin; and 0.05 and 0.94 for cobalt-substituted thermolysin. The calcium contents for the three types of thermolysin were 3.7–4.2 mol/mol protein. The zinc atom located in the active site of thermolysin was replaced with a cobalt atom in the cobalt-substitution process keeping 3–4 calcium atoms in the states as have been observed in native thermolysin.

Hydrolysis of FAGLA—Thermolysin or cobalt-substituted thermolysin solution (0.1 ml) was added to FAGLA solution (2.0 ml) in a cuvette, and the hydrolysis of FAGLA was followed by continuous monitoring of the decrease in absorptivity at 345 nm with a Shimadzu UV-visible recording spectrophotometer UV-240 (*12*). The amount of FAGLA hydrolyzed was estimated using the molar absorptivity difference on hydrolysis, $\Delta\varepsilon_{345}$ = –310 $M^{-1}\cdot cm^{-1}$ (*12*). The standard conditions for FAGLA hydrolysis were in 40 mM HEPES buffer containing 10 mM $CaCl_2$ and 0–4 M NaCl, pH 7.5, at 25°C. When cobalt-substituted thermolysin was examined, 100 μM $CoCl_2$ was added to the buffer.

Because of the high K_m (Michaelis constant) value and poor solubility of FAGLA, it was difficult to perform reactions at FAGLA concentrations large enough to separate the k_{cat} (catalytic constant) and K_m values. All reactions were performed under conditions ($[FAGLA]_0 \ll K_m$) where pseudo-first-order kinetics is valid, and the activity was expressed by the specificity constant, k_{cat}/K_m.

The effects of salt and pH on the thermolysin-catalyzed hydrolysis of FAGLA were examined in 40 mM sodium acetate buffer at pH 4.5–5.6, 40 mM sodium maleate buffer at pH 5.4–7.0, 40 mM HEPES buffer at pH 6.8–8.2, or 40 mM TAPS buffer at pH 8.0–9.0, all containing 10 mM $CaCl_2$ and 0–4 M NaCl, at 25°C. When the cobalt-substituted thermolysin-catalyzed hydrolysis of FAGLA was examined, 100 μM $CoCl_2$ was added to each buffer.

Inhibition of Thermolysin and Cobalt-Substituted Thermolysin by Zincov—Thermolysin or cobalt-substituted thermolysin was mixed with zincov in the various buffers (pH 4.9–9.0) described above for 30 min prior to measuring the enzyme activity. The enzyme-inhibitor solution (0.1 ml) was then mixed with 2.0 ml of FAGLA dissolved in the same buffer and the hydrolysis of FAGLA was measured at 25°C. The initial concentrations of thermolysin, cobalt-substituted thermolysin, FAGLA, and zincov were 100 nM, 30 nM, 130–750 μM, and 0–800 nM, respectively. The inhibitor constant, K_i, was determined from a modified form of Scatchard plot (*23, 24*).

RESULTS AND DISCUSSION

Effect of NaCl on the Hydrolysis of FAGLA by Thermolysin and Cobalt-Substituted Thermolysin—Under the standard conditions, the specificity constant, k_{cat}/K_m, for the thermolysin-catalyzed hydrolysis of FAGLA was (2.5 ± 0.2) × 10^4 $M^{-1}\cdot s^{-1}$. The activity of apo-thermolysin was nearly

undetectable, and completely recovered by adding $ZnCl_2$ at an equimolar concentration of apo-enzyme. The k_{cat}/K_m value for the hydrolysis of FAGLA by cobalt-substituted thermolysin was $(7.1 \pm 0.2) \times 10^4$ $M^{-1}\cdot s^{-1}$, 280% of that of native thermolysin. According to Holmquist and Vallee (*19*), the activity of cobalt-substituted thermolysin is 200% that of the native enzyme. The difference may be attributed to the purity of the enzyme preparation or the buffer used in each case. The k_{cat}/K_m value has been reported to be $(2.2 \pm 0.2) \times 10^4$ $M^{-1}\cdot s^{-1}$ in 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 25°C (*13, 17*). It was noticed that the activity of thermolysin toward FAGLA and the degree of activation by cobalt-substitution were relatively higher in HEPES than in Tris at pH 7.5.

The addition of NaCl markedly enhanced the activity of both thermolysin and cobalt-substituted thermolysin for the hydrolysis of FAGLA (Fig. 1). At every NaCl concentration examined, the activity of cobalt-substituted thermolysin was 260–290% that of the native enzyme. In the presence of 4 M NaCl, the k_{cat}/K_m values of thermolysin and cobalt-substituted thermolysin were $(3.6 \pm 0.4) \times 10^5$ and $(9.6 \pm 2.0) \times 10^5$ $M^{-1}\cdot s^{-1}$, respectively. Both were activated 13–15 times in the presence of 4 M NaCl compared with the absence of NaCl. The activity increased in an exponential fashion with increasing NaCl concentration. As we demonstrated previously (*13, 17*), the activity (k_{cat}/K_m) at x M salt is expressed as

$$\log(k_{cat}/K_m)_x = \log(k_{cat}/K_m)_0 + a\cdot x \quad (1)$$

where x and 0 refer to the salt concentrations of x and 0 M, respectively, and a is the slope of the straight line in the plot of $\log(k_{cat}/K_m)$ against the salt concentration. Equation 1 can be converted to

$$(k_{cat}/K_m)_x/\log(k_{cat}/K_m)_0 = 10^{\alpha x} = \alpha^x \quad (2)$$

where 10^a is expressed as α. Equation 2 indicates that the degree of activation at x M salt, $(k_{cat}/K_m)_x/\log(k_{cat}/K_m)_0$, is equal to $10^{\alpha x}$. In the case of the activation of native thermolysin by NaCl, the value is 1.90^x (*13, 17*). According to the data in Fig. 1, the degrees of activation at x M NaCl were calculated to be 1.96^x and 1.89^x, for the native and cobalt-substituted thermolysins, respectively. Because these values are regarded to be constant, the activating effect of cobalt substitution and the addition of NaCl are considered to be independent of each other. That is, together, the active site metal substitution and the addition of 4 M NaCl result in the activity of thermolysin being enhanced as much as 40-fold.

The reason that high concentrations of neutral salts enhance the activity of thermolysin is not clearly understood at present. The enhanced activity is solely due to the increase in k_{cat} (*12, 13*), and the possibility of slight conformational changes in the presence of salts is indicated (*14*). The change in the electrostatic environment is considered to be an important factor, and there may be specific interactions between the ions and the enzyme (Inouye and Mizuno, unpublished results).

According to X-ray crystallographic studies, the active site zinc is tetrahedrally coordinated by three protein ligands, His-142, His-146, and Glu-166, and a water molecule (*9*). Cobalt is quite similar to zinc in size and its ability to adopt a penta-coordinate geometry (*20, 25*). Cobalt in the active site of thermolysin is proposed to have a five-coordinate geometry, even in the absence of substrate/inhibitor, with two water molecule ligands, while zinc ligation is pre-

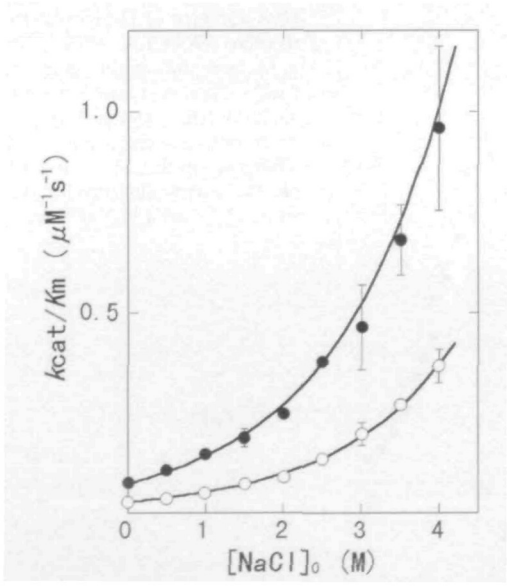

Fig. 1. **Effects of NaCl on the hydrolytic activity of thermolysin (○) and cobalt-substituted thermolysin (●) on FAGLA.** The initial concentrations of thermolysin, cobalt-substituted thermolysin, and FAGLA were 10–100 nM, 10–100 nM, and 0–170 μM, respectively, in 40 mM HEPES buffer (pH 7.5) containing 10 mM $CaCl_2$ (and 100 μM $CoCl_2$ in the case of cobalt-substituted thermolysin) at 25°C. The solid lines were calculated as $(k_{cat}/K_m)_x = 10^{ax}$ $(k_{cat}/K_m)_0$ where x and 0 refer to the NaCl concentrations x and 0 M, and a is the slope of the straight line in the plot $\log(k_{cat}/K_m)$ *vs.* NaCl concentration.

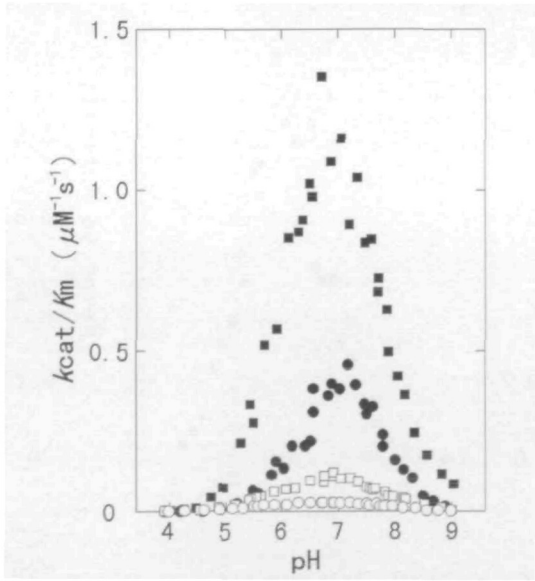

Fig. 2. **Effects of pH on the activation of thermolysin and cobalt-substituted thermolysin by NaCl in the hydrolysis of FAGLA.** The reaction was performed in 40 mM sodium acetate buffer at pH 4.5–5.6, 40 mM sodium maleate buffer at pH 5.4–7.0, 40 mM HEPES buffer at pH 6.8–8.2, and 40 mM TAPS buffer at pH 8.0–9.0, all containing 10 mM $CaCl_2$ (and 100 μM $CoCl_2$ in the case of cobalt-substituted thermolysin) at 25°C. Symbols are; thermolysin in 0 M (○) and 4 M NaCl (●), cobalt-substituted thermolysin in 0 M (□) and 4 M NaCl (■).

sumed to change from a four-coordinate to a five-coordinate geometry during catalysis (*20*). This feature of cobalt may contribute to a more stable transition state and enhanced activity toward some substrates. The addition of high concentrations of salts seems to have no effect on the coordination of the active site metal ion.

Effect of NaCl on the pH-Dependence of FAGLA Hydrolysis—Figure 2 shows the pH-dependence of the k_{cat}/K_m value on the hydrolysis of FAGLA by thermolysin and cobalt-substituted thermolysin in the absence and presence of NaCl at 25°C. A bell-shaped pH-dependence with an optimal pH around 7 was observed in each case. The relative activity (the maximum value of k_{cat}/K_m at the optimum pH set as 1) was plotted against pH for thermolysin (Fig. 3A) and cobalt-substituted thermolysin (Fig. 3B). Plots of $\log(k_{cat}/K_m)$ *vs.* pH (Dixon Plots) were fitted with three straight lines with slopes of +1, 0, and −1, and two pK_a values were determined in each case (Table I), indicating that the reaction is controlled by at least two ionizable residues.

In the absence of NaCl, substituting zinc with cobalt at the active site of thermolysin causes the acidic pK_a (pK_{a1}) value to shift from 5.6 to 6.0, while the alkaline pK_a (pK_{a2}) value seems stable (7.9–7.8). Increasing the NaCl concentration from 0 to 4 M shifts the pK_{a1} value of thermolysin from 5.6 to 6.7. This result is consistent with our previous data (*15*), although the buffers used were different in part. This shift in the pK_{a1} induced by NaCl seems to correspond with the degree of activation by NaCl (*15*). In the case of cobalt-substituted thermolysin, neither pK_{a1} (6.0–6.1) nor pK_{a2} (7.8–7.7) shifted when NaCl was added. The ionizable residue responsible for pK_{a1} seems to be sensitive to environmental changes both in the local region (substitution of the metal ion in the active site) and in the medium (salt concentration), and may be closely related to the increase in the activity. However, the fact that the pK_{a1} of cobalt-substituted thermolysin does not change even at 4 M NaCl might indicate that the shift in pK_{a1} is not directly related to the enhanced activity at high salt concentrations, and that the higher pK_{a1} might not be necessary for the higher activity.

The change in pK_{a1} induced by adding NaCl or by substituting the active-site zinc ion with a cobalt ion can be attributed to a change in the electrostatic environment around the pK_{a1} group, which destabilizes the ionized form of the group. The distance of the group from negative charge(s) may be reduced and/or the distance from positive charge(s) increased (*26*). The pK_{a1} has been suggested to be due to Glu-143 (*10, 27*) or to the water molecule (Lewis acid) coordinated to the active site zinc ion (*11, 28*). Our observation that the pK_{a1} of thermolysin is 6.7 at 4 M NaCl suggests that the group bearing pK_{a1} might be a water molecule bound to the Zn ion rather than Glu-143 (*15*). The present data suggest that the state of the cobalt ion is more stable than that of the zinc ion and is unaffected by the change in the salt concentration of the medium. This may be related to the observation that the cobalt ion is always in the penta-coordinate geometry, while the zinc is usually tetra-coordinate. If we consider that the functional group(s) responsible for pK_{a1} is a water molecule(s) liganding to the metal ion (*11, 28*), it may be considered that a water molecule liganding the zinc is sensitive, but two water molecules liganding the cobalt are insensitive to the change in salt

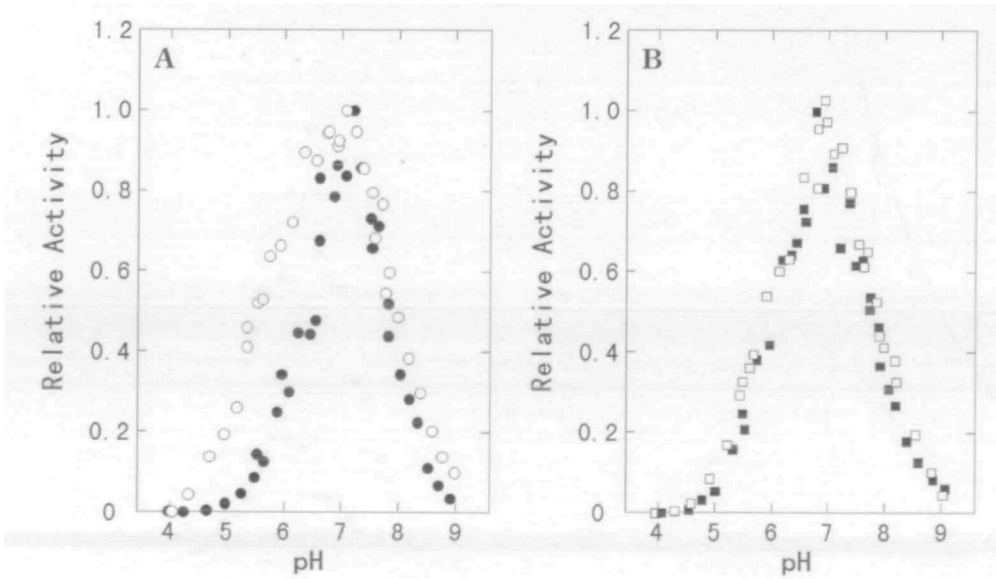

Fig. 3. **Effects of pH on the relative activity of thermolysin to hydrolize FAGLA.** The maximum value of k_{cat}/K_m at the optimum pH at the NaCl concentration examined was set as 1. The reaction was performed as described in the legend to Fig. 2. A: Thermolysin in 0 M (○) and 4 M NaCl (●). B: Cobalt-substituted thermolysin in 0 M (□) and 4 M NaCl (■).

TABLE I. **Estimation of the pK_a values of thermolysin and cobalt-substituted thermolysin in the hydrolysis of FAGLA and in the inhibition by zincov in the absence and presence of NaCl at 25°C.** The pK_{a1} and pK_{a2} values were evaluated from the Dixon plot {$\log(k_{cat}/K_m)$ *vs.* pH} using the data shown in Figs. 2 and 3.

Thermolysin	NaCl (M)	Hydrolysis of FAGLA		Inhibition by zincov	
		pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
Native thermolysin	0	5.6 ± 0.1	7.9 ± 0.1	5.6 ± 0.2	7.6 ± 0.2
	4	6.7 ± 0.1	7.5 ± 0.1	—	—
Cobalt-substituted thermolysin	0	6.0 ± 0.1	7.8 ± 0.1	6.2 ± 0.2	7.6 ± 0.2
	4	6.1 ± 0.1	7.7 ± 0.1	—	—

concentration. Upon the addition of NaCl, the microenvironment of the active site of the native thermolysin might change so that the pK_{a1} value is increased. In contrast, the microenvironment of cobalt-substituted thermolysin seems to be changed little by the addition of salt. The active site of cobalt-substituted thermolysin accommodates two water molecules, and the microenvironment of the active site might be too crowded to be influenced by the salt addition.

Inhibition by Zincov—The inhibitor constant K_i values of zincov for thermolysin and cobalt-substituted thermolysin in the absence of NaCl at pH 7.5 are 230 and 12 nM, respectively. The K_i values are much smaller with cobalt-substituted thermolysin than with native thermolysin over the pH range (4.9–9.0) examined (Fig. 4), indicating tighter binding of zincov to the cobalt-substituted thermolysin than to the native enzyme. The inhibition shows a bell-shaped pH-dependence, and the pK_a values were estimated (Table I). The values were almost the same as the pK_a values obtained for the hydrolysis of FAGLA by both the native and cobalt-substituted thermolysins. The hydrolysis of FAGLA and the inhibition by zincov are likely to be controlled by the same ionizable residues in the enzyme.

The smaller K_i values observed for the cobalt-substituted enzyme than the native enzyme suggest the possibility that the activation by cobalt-substitution is induced by tighter binding of the substrate to the enzyme (*i.e.*, a decrease in the K_m value). However, the degree of activation (2.8-fold) is not in relation to the difference in the K_i values (2 to 18-fold). Zincov is proposed to bind thermolysin bidentately, with the carbonyl oxygen and the hydroxyl oxygen in its hydroxamate moiety each approximately 2.0 Å apart from the metal ion, resulting in the zinc being penta-coordinate (*29*). It is reasonable that zincov may be accommodated more favorably at the active site of thermolysin containing cobalt instead of zinc, as long as the cobalt is penta-coordinate from the beginning of the reaction cycle. Phosphoramidon, *N*-(α-L-rhamnopyranosyl-oxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, is another competitive inhibitor of thermolysin, and it is a presumed to be a transition state analog similar to the tetrahedral intermediate formed during catalysis (*30, 31*). In the case of phosphoramidon, the K_i values for thermolysin and cobalt-substituted thermolysin at pH 7.5 are almost the same at 30 nM (detailed data not shown). The K_i values reflect the structure of each inhibitor and how it is accommodated in the active site, but do not directly predict what happens during the catalysis of a specific substrate such as FAGLA. By comparing the K_i values of zincov and phosphoramidon against the native and cobalt-substituted thermolysins, the possibility that the substitution of zinc by cobalt stabilizes the Michaelis complex but not the transition state is suggested. Perturbation of the microenvironment of the active site of thermolysin by replacing the zinc ion essential for activity with a cobalt ion, as well as by the addition of high concentrations of NaCl, might be useful for shedding light on the reaction mechanism of thermolysin. The results obtained in this study provide valuable information for the use of thermolysin in industrial applications such as the thermolysin-catalyzed synthesis of the artificial sweetener, aspartame (L-aspartyl-L-phenylalanine ethyl ester).

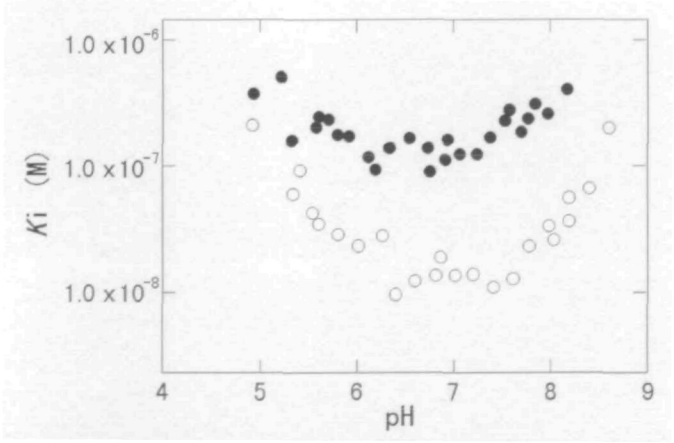

Fig. 4. **Effects of pH on the inhibitor constant K_i of zincov for native (●) and cobalt-substituted (○) thermolysins at 25°C.** The initial concentrations of thermolysin, cobalt-substituted thermolysin, FAGLA, and zincov were 100 nM, 30 nM, 130–750 μM, and 0–800 nM, respectively, at the indicated pH. The buffers (pH 4.9–9.0) were as described in the legend to Fig. 2.

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*, 3rd ed. (Boyer, P.D., ed.) Vol. 3, pp. 721–795, Academic Press, New York
3. Latt, S.A., Holmquist, B., and Vallee, B.L. (1969) Thermolysin: A zinc metalloenzyme. *Biochem. Biophys. Res. Commun.* **37**, 333–339
4. Feder, J., Garrett, L.R., and Wildi, B.S. (1971) Studies on the role of calcium in thermolysin. *Biochemistry* **10**, 4552–4555
5. Tajima, M., Urabe, I., Yutani, K., and Okada, H. (1976) Role of calcium ions in the thermostability of thermolysin and *Bacillus subtilis* var. *amylosaccharticus* neutral protease. *Eur. J. Biochem.* **64**, 243–247
6. Morihara, K. and Tsuzuki, H. (1970) Thermolysin: Kinetic study with oligopeptides. *Eur. J. Biochem.* **15**, 374–380
7. Titani, K., Hermodson, M.A., Ericsson, L.H., Walsh, K.A., and Neurath, H. (1972) Amino-acid sequence of thermolysin. *Nature* **238**, 35–37
8. 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
9. Holmes, M.A. and Matthews, B.W. (1982) Structure of thermolysin refined at 1.6 Å resolution. *J. Mol. Biol.* **160**, 623–639
10. 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
11. 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
12. 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
13. 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
14. 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
15. 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
16. 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
17. 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
18. 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
19. Holmquist, B. and Vallee, B.L. (1974) Metal substitutions and inhibition of thermolysin: Spectra of the cobalt enzyme. *J. Biol. Chem.* **249**, 4601–4607
20. 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
21. Feder, J. (1968) A spectrophotometric assay for neutral protease. *Biochem. Biophys. Res. Commun.* **32**, 326–332
22. Uchida, T., Isoyama, H., Oda, H., and Wada, H. (1993) Determination of ultratrace metals in biological standards by inductively coupled plasma atomic emission spectrometry with ultrasonic nebulisation. *Anal. Chim. Acta* **283**, 881–886
23. Bieth, J. (1974) Some kinetic consequences of the tight binding of protein-proteinase-inhibitors to proteolytic enzymes and their application to the determination of dissociation constants in *Bayer-Symposium V "Proteinase Inhibitors"* (Fritz, H., Tschesche, H., Greene, L.J., and Truscheit, E., eds.) pp. 463–469, Springer-Verlag, Berlin
24. Kitagishi, K. and Hiromi, K. (1984) Binding between thermolysin and its specfic inhibitor, phosphoramidon. *J. Biochem.* **95**, 529–534
25. Pauling, L. (1960) The sizes of ions and the structure of ionic crystals in *The Nature of the Chemical Bond*, 3rd ed., pp. 505–562, Cornel University Press, Ithaca
26. Inouye, K., Tonomura, B., Hiromi, K., Sato, S., and Murao, S. (1977) The states of tyrosyl and tryptophyl residues in a protein proteinase inhibitor (*Streptomyces* subtilisin inhibitor). *J. Biochem.* **82**, 1207–1215
27. Matthews, W.L. (1988) Structural basis of the action of thermolysin and related zinc peptidases. *Acc. Chem. Res.* **21**, 333–340
28. Mock, W.L. and Stanford, D.J. (1996) Arazoformyl dipeptide substrates for thermolysin. Confirmation of a reverse protonation catalytic mechanism. *Biochemistry* **35**, 7369–7377
29. Holmes, M.A. and Matthews, B.W. (1981) Binding of hydroxamic acid inhibitors to crystalline thermolysin suggests a pentacoordinate zinc intermediate in catalysis. *Biochemistry* **20**, 6912–6920
30. Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., Umezawa, H., Fujimoto, K., and Umezawa, S. (1975) Studies on inhibitory effect of phosphoramidon and its analogs on thermolysin. *Arch. Biochem. Biophys.* **171**, 727–731
31. Weaver, L.H., Kester, W.R., and Matthews, B.W. (1977) A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substrates. *J. Mol. Biol.* **114**, 119–132