Characterization of the Metal-Substituted Dipeptidyl Peptidase III (Rat Liver)†

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ABSTRACT: Dipeptidyl peptidase III (DPP III) (EC 3.4.14.4), which has a HELLGH-E (residues 450−455, 508) motif as the zinc binding site, is classified as a zinc metallopeptidase. The zinc dissociation constants of the wild type, Leu^{453}-deleted, and E508D mutant of DPP III at pH 7.4 were 4.5 (±0.7) × 10^{-13}, 5.8 (±0.7) × 10^{-12}, and 3.2 (±0.9) × 10^{-10} M, respectively. The recoveries of the enzyme activities by the addition of various metal ions to apo-DPP III were also measured, and Co^{2+}, Ni^{2+}, and Cu^{2+} ions completely recovered the enzyme activities as did Zn^{2+}. The dissociation constants of Co^{2+}, Ni^{2+}, and Cu^{2+} ions for apo-DPP III at pH 7.4 were 8.2 (±0.9) × 10^{-13}, 2.7 (±0.3) × 10^{-12}, and 1.1 (±0.1) × 10^{-14} M, respectively. The shape of the absorption spectrum of Co^{2+}-DPP III was very similar to that of Co^{2+}-carboxypeptidase A or Co^{2+}-thermolysin, in which the Co^{2+} is bound to two histidyl nitrogens, a water molecule, and a glutamate residue. The absorption spectrum of Cu^{2+}-DPP III is also very similar to that of Cu^{2+}-thermolysin. The EPR spectrum and the EPR parameters of Cu^{2+}-DPP III were very similar to those of Cu^{2+}-thermolysin but slightly different from those of Cu^{2+}-carboxypeptidase A. The five lines of the superfine structure in the perpendicular region of the EPR spectrum in Cu^{2+}-DPP III suggest that nitrogen atoms should coordinate to the cupric ion in Cu^{2+}-DPP III. All of these data suggest that the donor set and the coordination geometry of the metal ions in DPP III, which has the HExxxH motif as the metal binding site, are very similar to those of the metal ions in thermolysin, which has the HExxH motif.

Recently, we determined that dipeptidyl peptidase III (DPP III)[1] (EC 3.4.14.4) should be classified as a zinc metalloexopeptidase (*1, 2*). When the gene was first cloned and sequenced, we noted that there was a region in the predicted protein (HELLGH, residues 450−455) that was closely similar to the HExxH zinc binding motif of various zinc peptidases (*3*). Ohkubo et al. also confirmed our sequence results regarding DPP III (*4*). To determine the residues corresponding to the catalytic amino acids and the zinc ligands in the zinc metallopeptidase motif (HExxxH), in our previous paper we replaced Glu^{451}, His^{450}, and His^{455} with alanine or aspartic acid and tyrosine (*2*). We also employed site-directed mutagenesis for the conserved glutamic acid residue (Glu^{508}) in order to identify another residue important as the zinc binding site. These results regarding mutagenic DPP III indicate that residues His^{450}, His^{455}, Glu^{508}, and Glu^{451} on the HExxxH-52-E motif are involved in zinc coordination and the catalytic activity and that HExxxH is the zinc binding motif (*2*). Recently, Li et al. (*5*) confirmed these results and proposed that the Cys^{176} residue is involved in the catalytic activity of DPP III using a site-directed mutagenic method. Abramić et al. (*6*) also showed that rat erythrocyte DPP III is a zinc- and a thiol-dependent peptidase. In carboxypeptidase A (*7*) and thermolysin (*8*), which are typical zinc peptidases, the zinc ions were replaced by other transition metal ions such as Co^{2+} and Cu^{2+} in order to characterize the coordination geometries of the metal binding sites and to determine how the metal ions of these enzymes are involved in enzyme activities. Vallee et al. (*9*) replaced the zinc ion of carboxypeptidase A by various transition metals (Co^{2+}, Cu^{2+}, Ni^{2+}, etc.) and measured the binding constants of the metal ions. It was shown that the visible absorption of Co^{2+}-carboxypeptidase A (*7*) and the EPR spectra of Cu^{2+}-carboxypeptidase A (*10*) were influenced by the binding of the substrates or the inhibitors. Therefore, the replacement of the zinc ions in the zinc enzymes with the copper(II) or cobalt(II) ions gives more information about the metal binding geometry and the roles of the metal ions in enzyme catalytic activity (*11*−*13*).

In this paper, we replaced the zinc ions in DPP III with Co^{2+} and Cu^{2+} in order to characterize the coordination geometry of the metal ions and to determine how the metal ions are involved in the expression of the enzyme activity.

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[1] Abbreviations: DPP III, dipeptidyl peptidase III; EPR, electron spin resonance; PBS, phosphate-buffered saline; Arg-Arg-*β*-NA, Arg-Arg-*β*-naphthylamide; 2,6-PA, 2,6-pyridinedicarboxylate; 2-PA, 2-pyridinecarboxylate; GST, glutathione *S*-transferase.

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EXPERIMENTAL PROCEDURES

Materials. Arg-Arg-β-naphthylamide (Arg-Arg-β-NA) was obtained from Sigma Co. Ltd. All other reagents were of analytical grade and were purchased from Nakalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Measurement of Enzyme Activity. Fifty millimolar sodium phosphate buffer, pH 7.4, was used as the assay buffer (*1*). The incubation mixture contained 25 mM buffer, 0.2 mM Arg-Arg-β-NA, and an appropriate amount of enzyme plus water to attain a volume of 200 μL. After incubation at 37 °C for 10 min, 500 μL of 1 M acetate buffer, pH 4.0, containing 10% (v/v) Tween-20 and 200 μL of Fast Garnet GBC (0.2 mg/mL in water) was added to the reaction mixture. The absorbance of the resulting diazo dye was measured at 530 nm.

Measurement of Kinetic Parameters of Various Metal Derivatives of DPP III. To determine kinetic parameters at various pHs, the enzyme activities were measured in 50 mM acetate buffer (pH 4.5−6.0) and 50 mM phosphate buffer (pH 6.0−8.5). The substrate concentrations used in this experiment were 0.1−1 mM at all pHs.

Cloning of a Rat DPP III cDNA and Purification of Overexpressed Recombinant DPP III. The pBluescript phagemid containing cDNA encoding a rat DPP III was constructed as described previously (*1*, *2*). For expressing a rat DPP III as a glutathione *S*-transferase (GST) fusion protein, the DPP III cDNA isolated from the recombinant pBluescript phagemid was subcloned into pGEX-4T-3 (Amersham Pharmacia Biotech) digested with *Eco*RI−*Xho*I. The recombinant pGEX-4T-3 was named pGEX-III. *Escherichia coli* BL21 harboring a plasmid pGEX-III containing the gene for DPP III was cultured overnight in 2× YTA (1.6% trypton, 1.0% yeast extract, 0.5% NaCl) supplemented with 100 μg/mL ampicillin at 37 °C and then induced with 0.2 mM IPTG. After incubation for another 5 h at 20 °C, the cells were collected from centrifugation at 5000 rpm for 20 min. The cell pellet was resuspended in 1× PBS containing 1 mM DTT and lysed using French pressure and sonication at 4 °C. The lysate was spun at 12500 rpm for 30 min to remove cell debris. The supernatant was filtered through a 0.45 μm filter and loaded into a glutathione−Sepharose 4B column (1.4 × 4 cm, from Amersham Pharmacia Biotech) equilibrated with 1× PBS. After the column was washed with the same buffer, 4 mL of thrombin solution (100 units/mL in 1× PBS) was loaded into the column in order to cleave DPP III from GST. The column was incubated at room temperature for 1 h, and the proteins were eluted with 1× PBS at 4 °C. As indicated by SDS−PAGE, more than 90% of DPP III was recovered by this in-column thrombin digestion. After removal of thrombin from the DPP III solution by means of a benzamidine−Sepharose column (Amersham Pharmacia Biotech), enzyme fractions were dialyzed against 20 mM Tris-HCl, pH 7.3, and loaded into a Q-Sepharose column (1.7 × 15 cm, from Amersham Pharmacia Biotech). The column was washed with the same buffer, and the proteins were eluted using a linear gradient from 0 to 0.5 M NaCl in the same buffer. Peak DPP III fractions were pooled, concentrated, and subjected to a Superdex 200 Prep Grade gel filtration column equilibrated with 50 mM Tris-HCl, pH 7.3, containing 0.15 M NaCl. The purity of the protein after the above purification steps was typically greater than 95% homogeneous as analyzed by SDS−PAGE, and ~30 mg of pure DPP III was obtained from 4 L of 2× YTA broth. The purification yield for DPP III was about 40%, taking activity in the lysate as 100%. A reducing agent such as 2-mercaptoethanol did not influence the enzyme activity. The enzyme retained its activity even after storage of a few weeks at 4 °C. Enzyme concentrations were calculated using an extinction coefficient at 280 nm, $E_{280nm}^{1\%}$, of 15.8.

Preparation of Metal-Substituted DPP III. The zinc-free derivative of DPP III (apo-DPP III) was prepared by successive dialysis of DPP III against 50 mM Tris-HCl buffer (pH 7.4) containing 2.0×10^{-2} M 2,6-pyridinedicarboxylate (2,6-PA), then water, and 50 mM HEPES (pH 7.4). The zinc content of the apoenzyme was usually 1% of that of the native enzyme. The apoenzyme was fully reactivated by exactly 1 equiv of zinc (II) ions. The Co^{2+} derivative of DPP III was prepared by addition of 6.0 equiv of Co^{2+} ions for apo-DPP III in 50 mM HEPES buffer (pH 7.4) and then successive dialysis against water and 50 mM HEPES buffer (pH 7.4) to remove excess Co^{2+} ions. The Cu(II) derivative of DPP III was prepared by dialysis of apo-DPP III against 50 mM Tris-HCl buffer containing 10^{-4} M $CuSO_4$ and then 50 mM Tris-HCl (pH 7.4) in order to remove excess Cu^{2+} ions. The zinc, cobalt, and copper contents in metal derivatives of DPP III were measured using an atomic absorption spectrophotometer (Seiko Instrument SAS-7500 A).

Determination of the Metal Binding Constant. A simple technique used to estimate the metal dissociation constants was based on the measurement of the enzyme activity in a series of metal buffer systems with known concentrations of free metal(II) ions (*1*). The metal buffers were solutions of the constant metal ions (10^{-4} M) and excess chelating agent [2,6-PA or 2-pyridinecarboxylate (2-PA)]. The available concentration of the metal ions in such a solution depends on the stability constants of the significant chelates, on the concentration ratio of metal ions to chelates, and on pH. The corresponding calculations, based on the data from the conditional stability constants of chelating agents at pH 7.4 [2,6-PA(Zn^{2+}), $\beta'_1 = 10^7$ and $\beta'_2 = 10^{13}$; 2,6-PA(Cu^{2+}), $\beta'_1 = 10^{10}$ and $\beta'_2 = 1.99 \times 10^{16}$; 2-PA ($Co^{2+}$), $\beta'_1 = 5.5 \times 10^5$, $\beta'_2 = 2.75 \times 10^{10}$, and $\beta'_3 = 1.23 \times 10^{14}$; 2-PA($Ni^{2+}$), $\beta'_1 = 2.51 \times 10^6$ and $\beta'_2 = 7.94 \times 10^{11}$] (*14*−*16*), were performed with the aid of a BASIC computer program (*17*, *18*). The chelating agent used for controlling free Cu^{2+} or Zn^{2+} was 2,6-PA, which is suitable for controlling Cu^{2+} or Zn^{2+} concentrations between 2.3×10^{-17} M and 2.2×10^{-13} M or 1.7×10^{-14} M and 3×10^{-9} M at pH 7.4. 2-PA was also selected as the suitable chelating agent to control Co^{2+} or Ni^{2+} concentration between 10^{-14} M and 2.8×10^{-7} M or 7.1×10^{-14} M and 10^{-8} M at pH 7.4. The concentration of free metal ions ($[M^{2+}]$) was calculated as described previously (*1*, *17*).

The dissociation of the metal ions (M^{2+}) from DPP III is described by

$$EM \rightleftarrows E + M^{2+} \quad (1)$$

$$K_d = ([E][M^{2+}])/[EM] \quad (2)$$

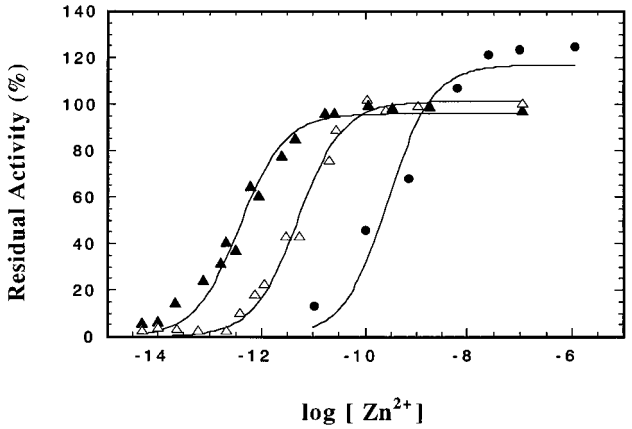

FIGURE 1: Relationship between residual enzyme activities (wild type and point mutated DPP III) and log [Zn^{2+}] at pH 7.4. The free zinc concentrations were controlled by metal buffers containing 10^{-4} M $ZnSO_4$ and the chelating agent (2,6-PA for wild type and Leu^{453}-del; 2-PA for $Glu^{508} \rightarrow Asp$). The assays were controlled in 100 μL of 50 mM phosphate buffer, pH 7.4, containing the metal buffer. After equilibration between apo-DDP III and the chelating agent for the Zn^{2+} (60 min incubation at 25 °C), reactions for the enzyme activity were performed using a standard enzyme assay described in Experimental Procedures. Symbols: (▲) wild type; (△) Leu^{453}-del; (●) $Glu^{508} \rightarrow Asp$.

In the addition of the metal buffer to the enzyme, the enzyme activity is shown by

$$\text{enzyme activity} = V_{\text{lim}}([\text{EM}]/([\text{E}] + [\text{EM}])) = V_{\text{lim}}([\text{M}^{2+}]/(K_{\text{d}} + [\text{M}^{2+}])) \quad (3)$$

where V_{lim} is the limit value of the enzyme activity and the free M^{2+} ions in the metal buffer is calculated as described in the previous paper (*1*, *17*).

Measurement of Absorption and EPR Spectra. Near-infrared and visible absorption spectra were measured using a Beckman DU-640 spectrophotometer employing a microcell (100 μL, 10 mm path length). Wild-type DPP III was used as a blank. The X-band EPR spectra were measured at 77 K (in liquid N_2) using an EPR spectrophotometer (JES-TE200, JEOL Tokyo) interfaced with a microcomputer system to accumulate EPR data. Spectral *g* values were calibrated using Mn^{2+} as a standard.

RESULTS

Zinc Binding Constant in Wild-Type and Mutated DPP IIIs. Wild-type (recombinant DPP III) and mutated DPP IIIs were incubated with the zinc buffers containing various concentrations of 2,6-PA or 2-PA and 10^{-4} M $ZnSO_4$ for 1 h at 25 °C, and the residual activities of the enzymes were measured. In Figure 1, the residual activities of wild-type and mutated DPP IIIs were plotted against the logarithm of the free zinc ion concentrations. The relationships between the logarithm of free Zn^{2+} and the residual activities of the enzymes gave sigmoidal curves.

By use of eq 3, the zinc dissociation constants (K_d) of apo-DPP III and the mutated apo-DPP IIIs were calculated from the relationships between the free Zn^{2+} ions and the residual enzyme activities. In Figure 1, the theoretical curves obtained from eq 3 in wild-type and mutated DPP IIIs are consistent with the results obtained from the residual enzyme activities. The zinc dissociation constants of wild-type and mutated DPP IIIs at pH 7.4 are shown in Table 1.

Table 1: Zinc Dissociation Constants of Various Point-Mutated Dipeptidyl Peptidase IIIs at pH 7.4

DPP III	zinc dissociation constant (M)	zinc content (atom/mol of protein)
wild type	$4.5\ (\pm 0.7) \times 10^{-13}$	0.80 ± 0.1
Leu^{453}-del	$5.8\ (\pm 0.7) \times 10^{-12}$	0.96 ± 0.04[a]
$Glu^{508} \rightarrow Asp$	$3.2\ (\pm 0.9) \times 10^{-10}$	0.43 ± 0.10[a]

[a] Reference 2.

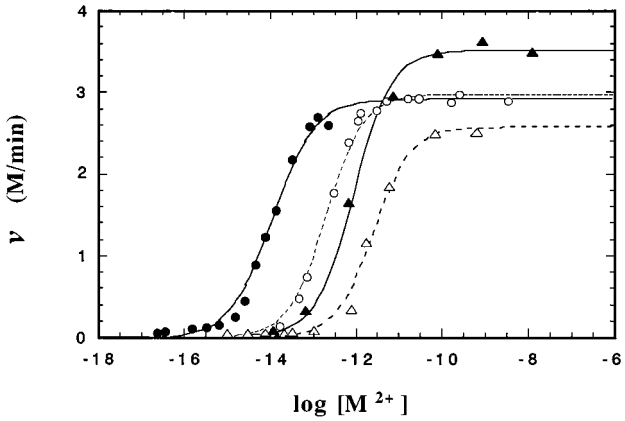

FIGURE 2: Recovering the enzyme activity of apo-DPP III in the presence of various concentrations of metal ions at pH 7.4. The free metal concentrations were controlled by metal buffers containing 10^{-4} M metal ions and various concentrations of chelating agent. 2,6-PA and 2-PA were used to control the free Zn^{2+} or Cu^{2+} concentrations and free Co^{2+} or Ni^{2+} concentrations, respectively. The final concentration of apo-DPP III used in metal binding experiments was 4.6×10^{-7} M. The assays were controlled in 100 μL of 50 mM phosphate buffer, pH 7.4, containing the metal buffer. After equilibration between apo-DDP III and the chelating agent for the metal ions (60 min incubation at 25 °C), reactions for the enzyme activity were performed using a standard enzyme assay described in Experimental Procedures. Symbols: (●) Cu^{2+}; (○) Zn^{2+}; (▲) Co^{2+}; (△) Ni^{2+}.

The zinc dissociation constant [$4.5\ (\pm 0.7) \times 10^{-13}$ M] obtained from recombinant rat liver DPP III was completely consistent with that [$2.5\ (\pm 0.5) \times 10^{-13}$ M] of human placental DPP III obtained in the previous paper (*1*). The zinc dissociation constant of Leu^{453}-deleted DPP III having the motif (HExxH- -E), similar to that of thermolysin, was slightly larger than that of wild-type DPP III. The zinc dissociation constant of the E508D mutant of DPP III, in which Asp^{508} may be involved in binding to the zinc ion, is much larger than that of the wild type.

Enzyme Activities of Metal-Substituted DPP III. Apo-DPP III obtained by dialysis against 2,6-PA was incubated with various metal buffers (Zn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} buffer) for 1 h at 25 °C, and the recoveries of the enzyme activities in apo-DPP III were measured. In Figure 2, the recoveries of the enzyme activities in apo-DPP III were plotted against the logarithm of the free metal concentrations (log [M^{2+}]). The relationships between the logarithm of free M^{2+} and the enzyme activities gave sigmoidal curves.

The maximal recovery of the enzyme activity in the addition of Co^{2+} was slightly larger than that of Zn^{2+}, but those obtained by the addition of other metal ions (Cu^{2+} and Ni^{2+}) were almost consistent with that of Zn^{2+}. By use of eq 3, the metal dissociation constants of apo-DPP III were

Table 2: Dissociation Constants of Various Metal Ions for Apodipeptidyl Peptidase III at pH 7.4

metal ion	dissociation constant (K_d) (M)
Zn^{2+}	$1.9\ (\pm 0.2) \times 10^{-13}$
Co^{2+}	$8.2\ (\pm 0.9) \times 10^{-13}$
Cu^{2+}	$1.1\ (\pm 0.1) \times 10^{-14}$
Ni^{2+}	$2.7\ (\pm 0.3) \times 10^{-12}$

Table 3: Kinetic Parameters for Arg-Arg-β-NA and Metal Contents of Various Metallodipeptidyl Peptidase IIIs at pH 7.4[a]

	K_m ($\times 10^{-5}$ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^4$ M^{-1} s^{-1})	metal content (atom/mol of protein)
Zn^{2+}-DPP III	8.1 ($\pm$1.0)	7.2 ($\pm$0.2)	8.8	0.8 ($\pm$0.10)
Co^{2+}-DPP III	8.2 ($\pm$0.9)	7.0 ($\pm$0.1)	8.5	1.0 ($\pm$0.1)
Cu^{2+}-DPP III	9.9 ($\pm$1.1)	10.1 ($\pm$0.3)	10.2	1.1 ($\pm$0.1)

[a] The reactions for the enzyme activity were performed using a standard enzyme assay described in Experimental Procedures.

calculated on the basis of the relationships between the free M^{2+} ions and the recoveries of the enzyme activities in apo-DPP III. In Figure 2, the theoretical curves calculated using eq 3 are consistent with the results obtained from the recoveries of the enzyme activities with the addition of various metal ions to apo-DPP III. The dissociation constants of Zn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} in apo-DPP III at pH 7.4 are shown in Table 2.

The order of the dissociation constants in various metal derivatives of DPP III is Zn^{2+}-DPP III > Cu^{2+}-DPP III < Co^{2+}-DPP III < Ni^{2+}-DPP III.

At very high concentrations of free metal ions, inhibition of enzyme activities was observed in Zn^{2+}, Cu^{2+}, and Ni^{2+} buffer (data not shown). Recently, Abramić et al. (*6*) also found that excess zinc ions inhibit the activity of DPP III. These inhibition behaviors were also observed in thermolysin and carboxypeptidase A (*19*, *20*).

Kinetic Parameters of Various Metal Derivatives of DPP III. Co^{2+}- and Cu^{2+}-DPP III were isolated in order to characterize the kinetic parameters of their enzyme activities (substrate: Arg-Arg-β-NA) and to compare them to those of wild-type DPP III. The metal contents and the kinetic parameters of Co^{2+}-, Cu^{2+}-, and wild-type DPP III are shown in Table 3.

In Table 3, the isolated Co^{2+}- or Cu^{2+}-DPP III contains almost 1 mol of the metal per enzyme mole. Kinetic parameters of Co^{2+}- and Cu^{2+}-DPP III were very similar to those of wild-type DPP III. The copper(II) derivative of carboxypeptidase A (*7*) or thermolysin (*8*, *12*), which is a well-known zinc peptidase, has no or very low peptidase activity, but Cu^{2+}-DPP III shows high peptidase activity for Arg-Arg-β-NA (see Table 3). The pH dependences of the kinetic parameters in wild-type DPP III, Co^{2+}-DPP III, and Cu^{2+}-DPP III were also measured between pH 5.0 and pH 8.0 and shown in Figure 3.

In Figure 3, the pH dependences of kinetic parameters in wild-type DPP III, Co^{2+}-DPP III, and Cu^{2+}-DPP III show similar behavior. These results indicate that the metal substitution of DPP III shows highly similar behavior.

Various spectrophotometric methods are very useful in characterizing the properties of the metal binding site in DPP III. Therefore, the absorption and EPR spectra of Co^{2+}-DPP III and Cu^{2+}-DPP III were measured.

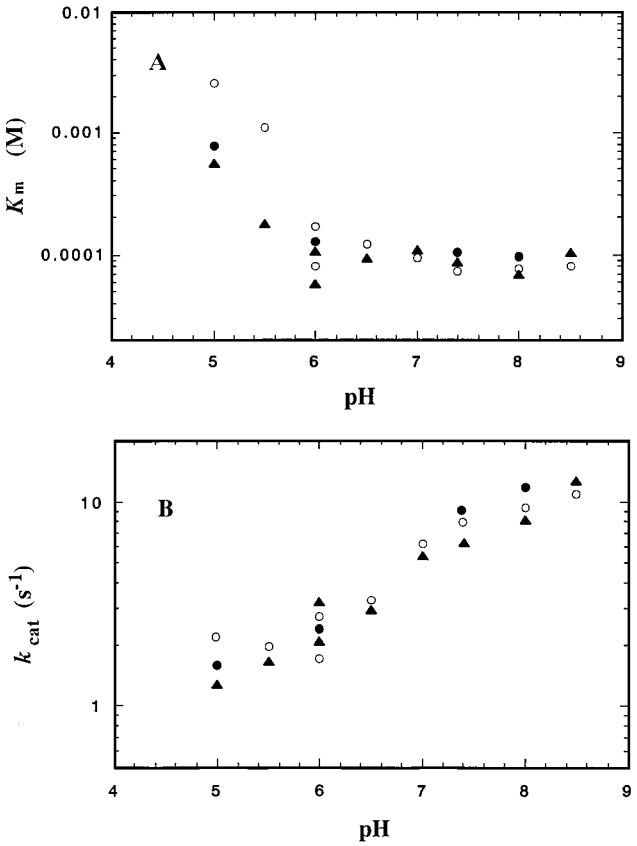

FIGURE 3: K_m and k_{cat} of Co^{2+}-, Cu^{2+}-, and Zn^{2+}-DPP III for Arg-Arg-β-NA at various pHs. (A) K_m of Co^{2+}-, Cu^{2+}-, and Zn^{2+}-DPP III. (B) k_{cat} of Co^{2+}-, Cu^{2+}-, and Zn^{2+}-DPP III. Symbols: (●) Cu^{2+}-DPP III; (○) Zn^{2+}-DPP III; (▲) Co^{2+}-DPP III.

Absorption Spectra and EPR Spectra of the Metal-Substituted DPP III. The absorption spectrum of Co^{2+}-DPP III is shown in Figure 4A. The shape of the spectrum of Co^{2+}-DPP III was very similar to that of Co^{2+}-carboxypeptidase A or Co^{2+}-thermolysin (*8*, *21*) in which the Co^{2+} is bound to two histidyl nitrogens, a water molecule, and a glutamate residue. X-ray crystallographic data regarding Co^{2+}-carboxypeptidase A or Co^{2+}-thermolysin (*22*, *23*) show a five coordination geometry of the cobalt(II) ion in Co^{2+}-carboxypeptidase A or Co^{2+}-thermolysin.

The spectrum of Cu^{2+}-DPP III is also shown in Figure 4B and is very similar to that of Cu^{2+}-thermolysin (*24*). The λ_{max} of the spectrum of Cu^{2+}-DPP III was slightly shorter than that of Cu^{2+}-thermolysin but about 80 nm shorter than that of Cu^{2+}-carboxypeptidase A (see Table 4) (*10*, *24*). The EPR spectra of Cu^{2+}-DPP III were also measured and shown in Figure 5.

In Figure 5A, the EPR spectrum shape is very similar to that of Cu^{2+}-thermolysin but slightly different from that of Cu^{2+}-carboxypeptidase A. The EPR parameters obtained from Figure 5A are shown in Table 4. In Table 4, the EPR parameters closely resemble those of Cu^{2+}-thermolysin. The perpendicular region of the EPR spectrum in Figure 5A is shown in Figure 5B. In Figure 5B, the five lines of the superfine structure are observed in the perpendicular region. This result suggests that these superfine structures result from the coordination of nitrogen atoms to the cupric ion in Cu^{2+}-DPP III.

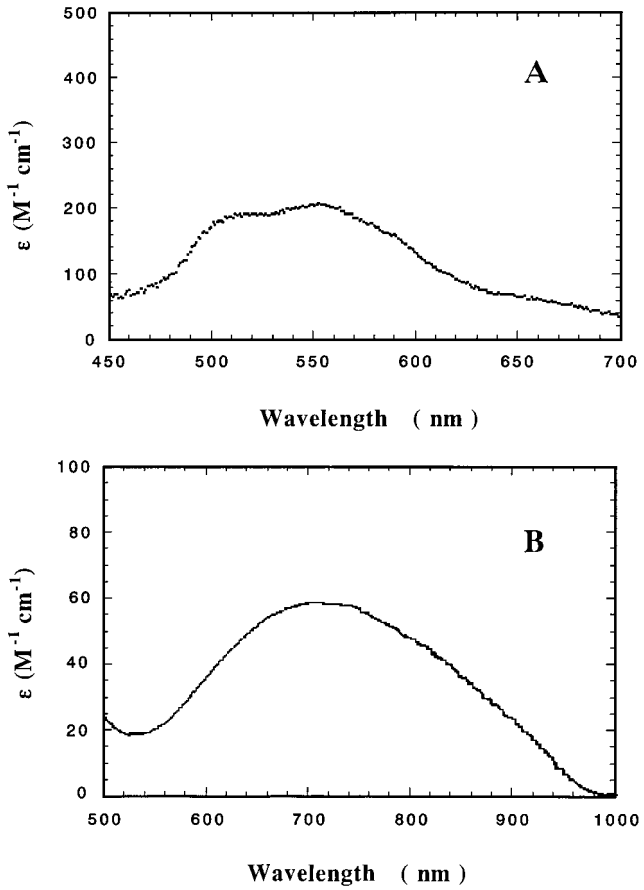

FIGURE 4: Absorption spectra of metallo derivatives of DPP III at pH 7.4. (A) Absorption spectrum of Co^{2+}-DPP III in 0.05 M HEPES buffer (pH 7.4), Co^{2+}-DPP III, 8.8×10^{-5} M. (B) Absorption spectrum of Cu^{2+}-DPP III in 0.05 M Tris-HCl buffer (pH 7.4); Cu^{2+}-DPP III, 3.7×10^{-4} M.

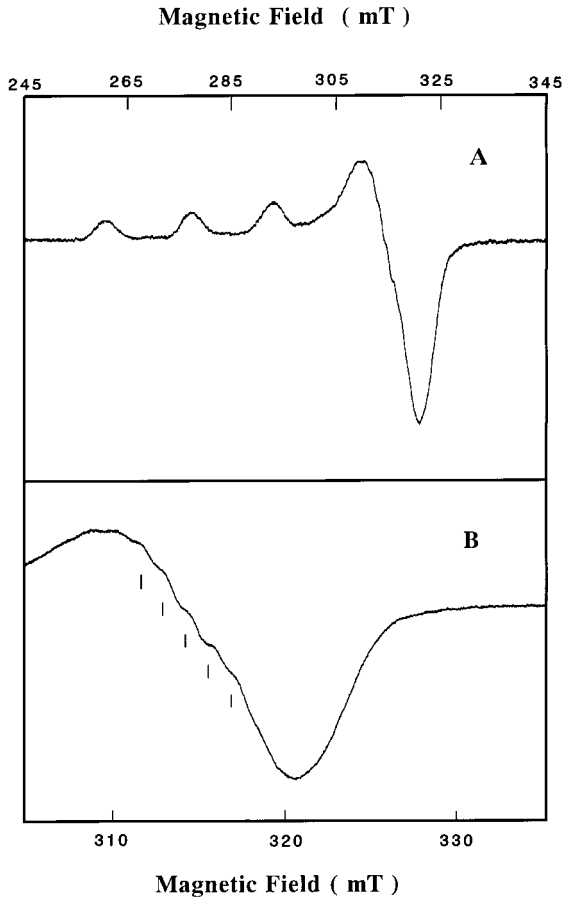

FIGURE 5: EPR spectra of a frozen solution of Cu^{2+}-DPP III (9.4×10^{-5} M) in 0.05 M Tris-HCl buffer (pH 7.4) at liquid nitrogen temperature. (A) EPR spectrum between 245 and 345 mT. (B) EPR spectrum between 305 and 335 mT. The peak positions of the superfine structure in the perpendicular region of the EPR spectrum in Cu^{2+}-DPP III are represented as short lines in panel B.

Table 4: EPR Parameters and Electronic Absorption Bands of Copper(II) DPP III, Copper(II) Thermolysin, and Copper(II) Carboxypeptidase A at pH 7.4

	$g_\perp$	$g_\parallel$	$A_\parallel$ ($\times 10^{-4}$ cm^{-1})	λ_{max} [ϵ (M^{-1} cm^{-1})] (nm)
copper(II) DPP III	2.06	2.27	167	710 (~60)
copper(II) thermolysin[a]	2.06	2.26	163	730 (~90)
copper(II) carboxypeptidase A[b]	2.05	2.33	115	787 (~125)

[a] Reference 24. [b] Reference 10.

DISCUSSION

In Figure 4, the shape of the spectrum of Co^{2+}-DPP III was very similar to that of Co^{2+}-carboxypeptidase A or Co^{2+}-thermolysin, in which the Co^{2+} is bound to two histidyl nitrogens, a water molecule, and a glutamate residue. The spectrum of Cu^{2+}-DPP III is also very similar to that of Cu^{2+}-thermolysin. In Figure 5 and Table 4, the EPR spectrum shape and the EPR parameters of Cu^{2+}-DPP III are very similar to those of Cu^{2+}-thermolysin (*24*) but slightly different from those of Cu^{2+}-carboxypeptidase A (*10*). The five lines of the superfine structure in the perpendicular region of the EPR spectrum of Cu^{2+}-DPP III suggest that nitrogen atoms should coordinate to the cupric ion in Cu^{2+}-DPP III. All of these data suggest that the donor set and the coordination geometry of the metal ions in DPP III, which has the HELLGH motif, are very similar to those of the metal ions in thermolysin, which has the HELTH motif in the metal binding site. The donor atom set proposed from the absorption and EPR spectra of the metal derivatives of DPP III shown in this paper is completely consistent with that proposed on the basis of the site-directed mutagenic method in our previous paper (*2*). In our previous paper (*2*), His^{331} and His^{336} coordinating geometry to the iron ion in the HELLGH motif of tyrosine hydroxylase (*25*), which forms a part of the α-helix, is very similar to the coordination geometry of His^{142} and His^{146} in relation to the zinc ion in the HELTH motif of thermolysin which also forms a part of the α-helix (*23*). In Table 1, the zinc dissociation constant of Leu^{453}-deleted DPP III having the motif HELGH like that of thermolysin (HELTH) still has a very low value. This shows that the deletion of Leu in the HELLGH motif of wild-type DPP III does not induce a large change in the coordination geometry in the zinc binding site of DPP III. The Gly in the HELLGH motif of DPP III may allow flexibility in the α-helix part of the motif.

In Table 1, the zinc dissociation constant of the E508D mutant DPP III is much larger than that of wild-type DPP III. Between the carbon of carboxylate and the α-carbon, Asp has one carbon atom less than does Glu, so that the carboxylate of Asp in the E508D mutant may not sufficiently coordinate to the zinc ions of the enzyme.

The order of the dissociation constants in various metal derivatives of DPP III is Zn^{2+}-DPP III > Cu^{2+}-DPP III < Co^{2+}-DPP III < Ni^{2+}-DPP III. The dissociation constant of the nickel(II) ion in Ni^{2+}-DPP III was larger than that of the cobalt(II) ion in Co^{2+}-DPP III. This is an order opposite that of the Irving−Williams order. The limitations of the recoveries of the enzyme activities of apo-DPP III in the additions of Co^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} ions gave almost the same values. In Table 3 and Figure 3, the kinetic parameters of the enzyme activity in isolated Co^{2+}- and Cu^{2+}-DPP III are also very similar to those of wild-type DPP III. The complete recovery of the peptidase activity by the addition of the Cu^{2+} ion to apo-DPP III is particularly surprising in view of the fact that the addition of the Cu^{2+} ion to apocarboxypeptidase A and apothermolysin fails to restore their enzyme activities (*7*, *8*). However, it is recently known that apoastacin, which has HExxH- -H motif as the zinc binding site, is reactivated by cupric ions (37% of the native enzyme activity) (*26*). In aminopeptidase Ey (*27*) and aminopeptidase (*28*) from the edible basidiomycete *Grifola frondosa*, which contain one zinc atom per enzyme molecule, the apoenzymes were also reactivated by the cupric ions.

The presence of the excess metal ions inhibited the enzyme activity for DPP III. This was also observed in thermolysin (*19*, *23*) and carboxypeptidase A (*20*), because the excess metal ions bind to the second binding site. Recently, Abramić et al. (*6*) showed that excess zinc ions inhibit the erythrocyte DPP III activity. Our preliminary experiments regarding the inhibition of the excess zinc ions for DPP III indicate that the excess zinc ions inhibit the peptidase activity by means of an uncompetitive mechanism [K_i (the inhibition constant) $= \sim 10^{-5}$ M at pH 8.8].

In this paper, the donor atom set and the coordination geometry of the zinc binding site in DPP III are very similar to those of thermolysin and carboxypeptidase A. On the basis of the site-directed mutagenic method, it is shown that Glu^{451} on the HELLGH motif (residues 450−455) should be involved in the catalytic activity of the enzyme shown in our previous paper (*2*). These behaviors indicate that the zinc ions on the HELLGH in DPP III should be involved in the expression of the enzyme activity like thermolysin and carboxypeptidase A. However, the peptidase activity (∼100%) of Cu^{2+}-DPP III in this paper was very different from those of Cu^{2+}-carboxypeptidase A (0%) and Cu^{2+}-thermolysin (2%). Li et al. (*5*) proposed that Cys^{176} is also involved in the catalytic activity of DPP III, and Abramić et al. (*6*) showed that DPP III is a metallo- and thiol-dependent peptidase. Therefore, both Cys^{176} and the zinc ion on the HELLGH motif in DPP III may be involved in the catalytic activity, and the catalytic mechanism of DPP III may be different from that of thermolysin or carboxypeptidase A. Cu^{2+}-astacin is enzymatically active like Cu^{2+}-DPP III, but the EPR shape and the electronic absorption spectrum of Cu^{2+}-astacin are very different from those of Cu^{2+}-DPP III (*26*).

Is the reaction mechanism of the peptidase activity in DPP III different from that of carboxypeptidase A or thermolysin? Are the zinc ions in DPP III directly involved in the expression of the enzyme activity? To answer these questions, we are now using the EPR, NMR, and kinetic methods to investigate the interaction between Cu^{2+}- or Co^{2+}-DPP III and the competitive inhibitors.

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