Proton Nuclear Magnetic Resonance Studies of the Complexation of Zn(II) with Histidine-Containing Peptides, L-histidyl-L-histidylglycylglycine and L-histidylglycyl-L-histidylglycine

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The complexation of Zn(II) with L-histidyl-L-histidylglycylglycine (HisHisGlyGly) and L-histidylglycyl-L-histidylglycine (HisGlyHisGly) was studied by proton nuclear magnetic resonance spectroscopy over the pH range from 3 to 11 at room temperature. In weakly acidic solutions below pH 6, both HisHisGlyGly and HisGlyHisGly coordinated to Zn(II) through amino and imidazole nitrogens at the *N*-terminal histidyl residue. The imidazole nitrogens of the histidyl residues in the second and third positions which are protonated at this pH could not coordinate to the Zn(II) ion. As the pH was raised, however, the imidazolium protons were released and associated with the metal ion. When the peptide bond between His–His in HisHisGlyGly was deprotonated over pH 7, the imidazole nitrogen of the histidyl residue in the second position was likely to coordinate to the metal ion in place of the imidazole nitrogen at the *N*-terminal histidyl residue. A kinetically stable complex in which the Zn(II) coordinated to three nitrogens from the terminal amino, deprotonated peptide-bond, and imidazole of the histidyl residue in the second position was newly produced. The fourth coordination site of Zn(II) was probably occupied with the imidazole nitrogen of the histidyl residue at the *N*-terminal of another HisHisGlyGly complex.

On the other hand, since HisGlyHisGly did not undergo deprotonation of the peptide bond between His–Gly at the *N*-terminal, the coordination mode with Zn(II) did not change over pH 6.

Key words proton nuclear magnetic resonance; zinc(II) ion; L-histidyl-L-histidylglycylglycine; L-histidylglycyl-L-histidylglycine; complexation

The side chain of the histidyl residue, which is one of the strongest metal-binding sites in proteins, plays an important role in constructing the active sites of metal-containing enzymes.[1–5] In zinc-enzymes, Zn(II) is located in the cluster composed of histidyl residues in the polypeptide chain.[6,7] For example, in carboxypeptidase A which functions to cleave peptides and ester bonds, Zn(II) coordinates to two imidazole nitrogens from histidyl residues, two oxygens from glutamate and coordinated water. In carbonic anhydrase C, Zn(II) is firmly bound with three imidazole nitrogens from histidyl residues. Recently, much attention has been paid to a novel property of Zn(II) in the putative formation of "zinc fingers,"[8–10] in which the metal ion associates with two imidazole nitrogens and two cysteinyl sulfurs to form a loop that can take part in protein–nucleic acid interactions.

To elucidate the coordination mode and environment in Zn(II)-containing proteins and enzymes, the interactions of Zn(II) with histidine-containing peptides have been studied.[10,11] Since Zn(II) has neither intrinsic color nor unpaired electrons, nuclear magnetic resonance (NMR) is a useful but limited technique for investigating metal–ligand coordination in zinc complexes.[12] In this context, we have been investigating the reactions of Zn(II) with histidine-containing peptides, *e.g.* L-histidylglycylglycine (HisGlyGly),[13] glycyl-L-histidylglycine (GlyHisGly),[14] and glycylglycyl-L-histidine (GlyGlyHis),[15] by ^{1}H-NMR spectroscopy. However, there have been few studies of the complexation of Zn(II) ions with peptides containing more than two histidyl residues.[16] Here, we examined the complexation of Zn(II) with L-histidyl-L-histidylglycylglycine (HisHisGlyGly) and L-histidylglycyl-L-histidylglycine (HisGlyHisGly) by ^{1}H-NMR spectroscopy.

Experimental

Materials HisHisGlyGly and HisGlyHisGly were synthesized by the conventional solution method.[17] $ZnSO_4 \cdot 7H_2O$ and D_2O (99.3%) were purchased from E. Merck (Darmstadt, Germany) and Nacalai Tesque Chemical Co. (Japan), respectively, and used as received.

Preparation of Solutions A stock solution of Zn(II) was prepared by adding $ZnSO_4 \cdot 7H_2O$ to D_2O to give a final Zn(II) concentration of 4.0×10^{-2} M. Stock solutions of the peptides were prepared in D_2O. These solutions were deuterated in 99.3% D_2O by the freeze-thawing method. The peptide solutions in the presence and absence of Zn(II) were titrated with either DNO_3 or NaOD solution. At selected pH values, aliquots were withdrawn from the solutions and transferred to NMR tubes for spectroscopic analysis. Unless otherwise noted, the ratio of concentrations of Zn(II) to the peptides was 1 : 2 and the concentration of Zn(II) was fixed as 2.0×10^{-3} M at the start of titration. The pH values given in the text are the readings from a TOA Denpa HM-5A pH meter.

NMR Measurements ^{1}H-NMR spectra were obtained using a JEOL JNM FX-270 FT NMR spectrometer at 25 °C. Chemical shift values are quoted in parts per million (ppm) downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) used as an internal standard.

Results and Discussion

Interaction of HisHisGlyGly with Zn(II) The ^{1}H-NMR spectra of HisHisGlyGly at pH 4.9 and pH 10.4 are shown in Fig. 1. Assignments of resonances were made by comparison with published chemical shifts of constituent amino acids,[18] and by combined use of proton decoupling as shown in Fig. 1 and pH titration methods as shown in Fig. 2. At pH 10.4, the irradiation of the resonance at 2.86 ppm, assignable to histidyl β-methylene proton, affected resonance at 3.68 ppm due to the histidyl α-methine proton as shown in Fig. 1C. These two resonances were assignable to the same histidyl

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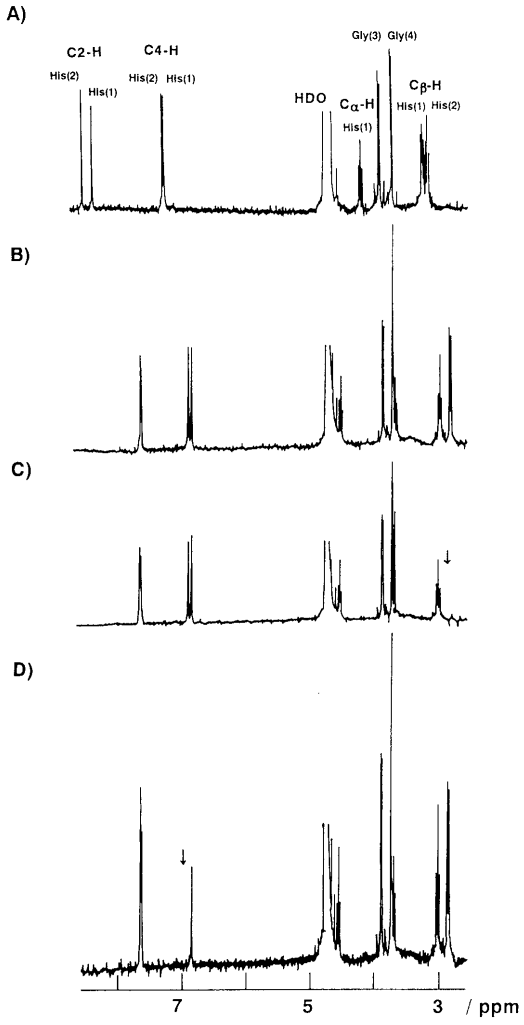

Fig. 1. ^{1}H-NMR Spectra of 4.2 mM HisHisGlyGly in D_2O Solution at pH 4.9 and 10.4

No irradiation was detected in (A) or (B). The arrow indicates irradiation at 2.86 ppm (C) and 6.92 ppm (D).

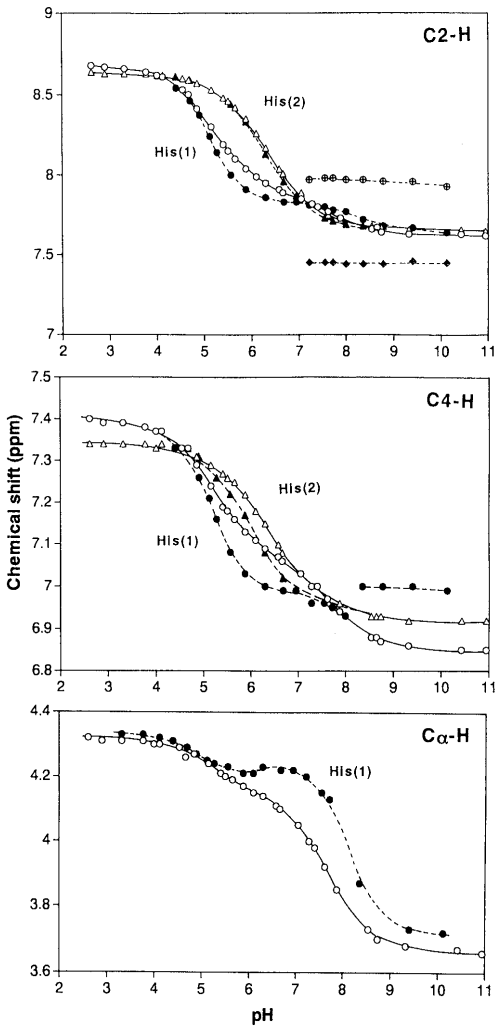

Fig. 2. NMR Titration Curves of Imidazole C-2 and C-4 Protons, and His-Cα Proton of HisHisGlyGly in the Absence (○, △) and Presence (●, ▲) of Zn(II) Ions

Peptide = 4.2 mM, Zn(II) = 2.0 mM, ⊕; species A, ◇; species B. 1, 2, 3 and 4 in parenthesis indicate the positions of amino acid residues from the N-terminal.

residue. Another histidyl residue exhibited resonances at 4.56 (α-methine proton) and 3.04 ppm (β-methylene proton). The irradiation of the imidazole C2-H resonance at 6.92 ppm caused the increase in intensity of the C4-H resonance at 7.65 ppm as shown in Fig. 1D, suggesting that these two resonances belonged to the same imidazole ring. Therefore, resonances at 7.62 and 6.85 ppm were attributed to imidazole C2-H and C4-H, respectively, of another imidazole ring. These two histidyl residues, as well as two glycyl residues, could be distinguished from each other by the NMR titration method. Plots of the chemical shifts of imidazole C2-H and C4-H protons, and α-methine proton (Cα-H) against pH are presented in Fig. 2. The pK_a values of imidazolium groups of histidyl residues at the *N*-terminal and in the second position were determined as 5.6 and 6.3, respectively, from the inflection points of their NMR titration curves. These pK_a values were similar to those for imidazole groups of HisGlyGly ($pK_a = 5.52$)[19] and GlyHisGly ($pK_a = 6.63$).[19] The assignments of methylene protons of glycine at the carboxylate-end were made by the procedure described above. The chemical shifts of the C2-H and C4-H protons, and other resonances at pH 4.9 and pH 10.4 are summarized in Table 1.

In the presence of an equimolar concentration of Zn(II), the peptide solution became cloudy over pH 6, probably because of the precipitation of Zn(II) hydroxides. Then, the measurements had to be carried out in the 1:2 (Zn(II)/peptide) solution. The ^{1}H-NMR spectra of HisHisGlyGly at three different pH values, *i.e.* pH 5.3, 5.9 and 7.6, in the presence of a half molar equivalent of Zn(II) are shown in Fig. 3. The imidazole C2-H proton of the histidyl residue at the *N*-terminal was broadened at pH 5.3. As the pH increased, it shifted upfield and became a sharp peak, while the α-methine proton of the same histidyl residue at the *N*-terminal shifted downfield as compared with that of free peptide observed in the spectrum at pH 5.9. This suggests the formation of a labile Zn(II) complex with histidine-like coordination structure.[13] At pH 7.6, both the resonances of imidazole C2-H protons at the *N*-terminal and in the second position split into two lines, which were referred as A and B, respectively, as shown in Table 1. A set of C2-H resonances provided chemical shifts at 7.80 ppm and at 7.73 ppm, which were similar to those of the free peptide at 7.78 ppm. This was assignable to either the deprotonated peptide, *i.e.* free

Table 1. ^{1}H-NMR Chemical Shifts of HisHisGlyGly in the Absence and Presence of Zn(II)

	pH 4.9	pH 5.3		pH 5.9		pH 7.6			pH 10.4
	Peptide	Peptide	with Zn(II)	Peptide	with Zn(II)	Peptide	with Zn(II) A	with Zn(II) B	Peptide
His(1)C2-H	8.50	8.24	8.14	8.04	7.91	7.78	7.80	7.98	7.62
His(1)C4-H	7.31	7.21	7.16	7.13	7.03	6.98	6.96	7.00(sh)	6.85
His(2)C2-H	8.59	8.51	8.51	8.35	8.33	7.78	7.73	7.45	7.65
His(2)C4-H	7.31	7.28	7.26	7.22	7.17	6.98	6.96	6.96	6.92
His(1)Cα-H	4.27	4.22	4.24	4.17	4.21	3.94	ND	4.15	3.67
Gly(3)CH_2	3.98	3.97	3.93	3.96	3.87	3.92	ND	ND	3.91
Gly(4)CH_2	3.78	3.77	3.74	3.77	3.67	3.76	ND	ND	3.76

ND: The assignment was not determined.

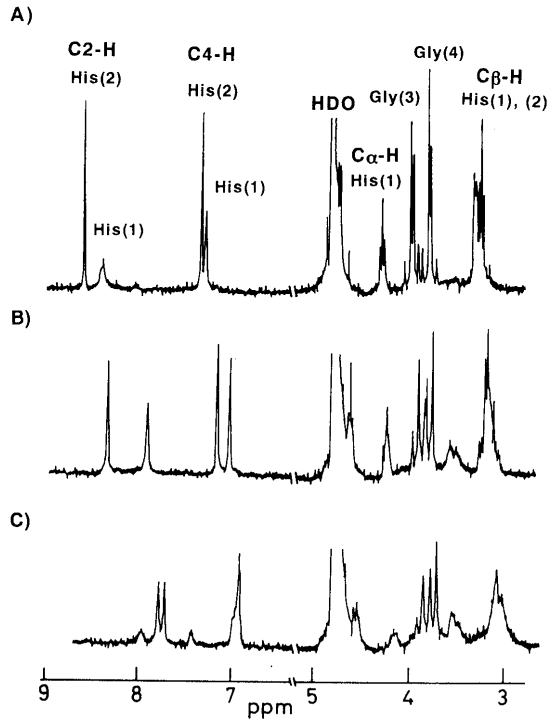

Fig. 3. ^{1}H-NMR Spectra of 4.2 mM HisHisGlyGly in the Presence of 2.0 mM $ZnSO_4$ in D_2O Solution at pH 5.3 (A), 5.9 (B) and 7.6 (C)

ligand, or labile complexes. Another set of C2-H resonances was observed at 7.98 and 7.45 ppm. A downfield shift of the C2-H resonances of the histidyl residue at the *N*-terminal, from 7.78 to 7.98 ppm, could be elucidated by electron delocalization from the imidazole ring to Zn(II) on complex formation, and upfield shift of the imidazole C2-H proton of the histidyl residue at the second position, from 7.78 to 7.45 ppm, resulting from Zn(II)–imidazole bonding. The complex thus produced was fairly stable based on the NMR time scale. Similar results were observed in the Zn(II) interaction with peptides containing a histidyl residue in the second position, *e.g.* GlyHis,[20] GlyHisGly,[21] and GlyHisLys.[21]

The NMR titration curves of the imidazole C2-H and C4-H, and His Cα-H of HisHisGlyGly in the presence and absence of Zn(II) are shown in Fig. 2. In species B, the chemical shifts of these imidazole C2-H protons at the *N*-terminal and in the second position did not change irrespective of the variation of pH over the range from 7.3 to 10.2. This is evidence of formation of a kinetically stable complex with Zn(II)–imidazole bonding, which causes C2-H proton to shift downfield. It has been shown that peptides with the sequence His–His at the *N*-terminal such as HisHisGlyGly[17] and HisHis[22] coordinate firmly with Cu(II) to form a binuclear complex, in which each Cu(II) coordinates to four nitrogens derived from a terminal amino group, an adjacent deprotonated peptide and an imidazole of the histidyl residue in the second position, and from the imidazole of the *N*-terminal of another peptide. Provided that the Zn(II) complex has a similar coordination mode to the Cu(II) complex, the ^{1}H-NMR spectra of HisHisGlyGly coordinated with Zn(II) could be explained as follows. The Zn(II) complex formed below pH 7, possessing a histidine-like coordination structure, is kinetically unstable on the NMR time scale. As the pH increased, the peptide bond between His–His would be deprotonated and consequently the ligand would be changed from the imidazole nitrogen of the *N*-terminal to the deprotonated peptide nitrogen. The new complex thus formed would be dimeric and kinetically stable with slow exchange of the Zn(II) between the complex and the peptide on the NMR time scale. One HisHisGlyGly molecule probably coordinates to Zn(II) through an amino nitrogen, an adjacent deprotonated peptide nitrogen, and an imidazole nitrogen in the second position. The fourth coordination site of Zn(II) may be ligated from the imidazole nitrogen of the histidyl residue at the *N*-terminal of the other complex.

Interaction of HisGlyHisGly with Zn(II) The ^{1}H-NMR spectra of HisGlyHisGly at pH 4.9 and 9.7 are shown in Fig. 4. Assignments of resonances were made by the procedures described in the section on HisHisGlyGly. At pH 4.9, the irradiation of resonance at 8.59 ppm, which was assignable to the imidazole C2-H, caused an increase in intensity of the C4-H resonance at 7.34 ppm as shown in Fig. 4B. This suggests that these two resonances were assignable to the same imidazole ring. The resonances at 8.51 and 7.38 ppm were assignable to the imidazole C2-H and C4-H, respectively, of another histidyl residue. The irradiation of resonance at 4.33 ppm, which was centered at the triplet due to the histidyl α-methine proton, affected the histidyl β-methylene resonance at 3.37 ppm as shown in Fig. 4C. This suggests that these two resonances were assignable to the same histidyl residue. The chemical shifts of the C2-H and C4-H protons, and other resonances at pH 4.9 and 9.7 are summarized in Table 2.

Two histidyl residues, as well as two glycyl residues, of the peptides could be distinguished by NMR titration. The pK_a values of imidazole groups of the two histidyl residues were estimated as 5.6 and 6.8 from the pH values at the inflection point of their NMR titration curve as shown in Fig. 5. These pK_a values were attributed to deprotonation of imidazolium groups of the histidyl residues at the *N*-terminal and in the third position, respectively, and were similar to those for imidazolium groups of HisGlyGly ($pK_a = 5.52$),[19] and GlyGlyHis–*N*-methylamide ($pK_a = 6.39$)[23] and GlyGlyHisGly ($pK_a = 6.47$).[24] The assignment of the methylene protons of the glycyl residue at the C-terminal end was made on the basis of its titration behavior reflecting protonation of the C-terminal carboxylate group.

The ^{1}H-NMR spectra of HisGlyHisGly at three different pH values, *i.e.* 5.0, 5.7 and 7.1, in the presence of a half molar equivalent of Zn(II) are shown in Fig. 6. The imidazole C2-H resonance of the histidyl residue at the *N*-terminal were broadened at pH 5.0. This suggests that the peptide would undergo medium exchange on the NMR time scale with its Zn(II) complexes, in which the peptide coordinated to Zn(II) through an amino nitrogen and/or an imidazole nitrogen of the histidyl residue at the *N*-terminal. Thus, the Zn(II) complex with the histidine-like coordination structure would be formed at pH 5.0. We reported previously that a tripeptide containing one histidyl residue such as HisGlyGly was in medium exchange with the Zn(II) complex as studied by NMR spectroscopy.[13] As pH increased over 5, however, the imidazole C2-H underwent an upfield shift sharpening a

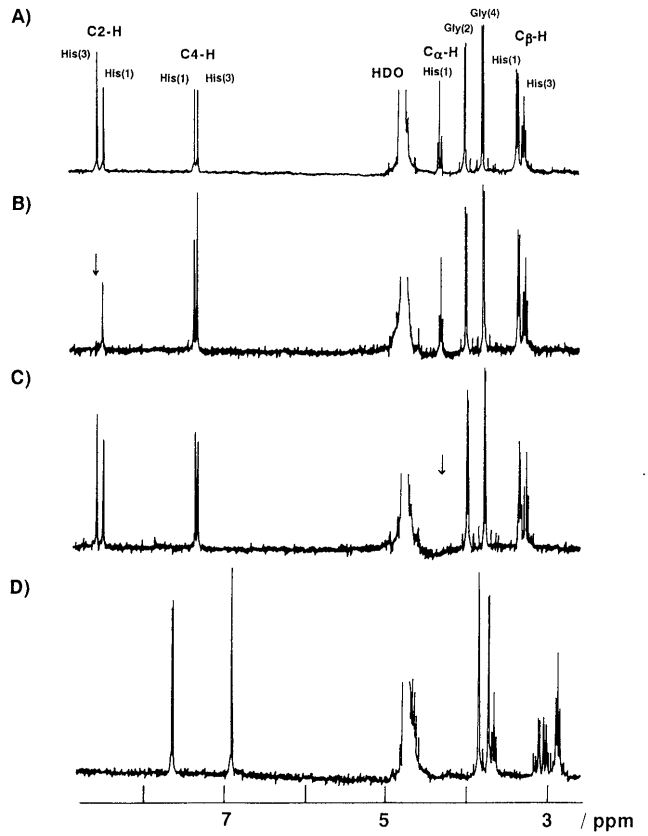

Fig. 4. ^{1}H-NMR Spectra of 4.2 mM HisGlyHisGly in D_2O Solution at pH 4.9 and 9.7

No irradiation was detected in (A) or (D). The arrows indicate irradiation at 8.59 ppm (B) and 4.33 ppm (C).

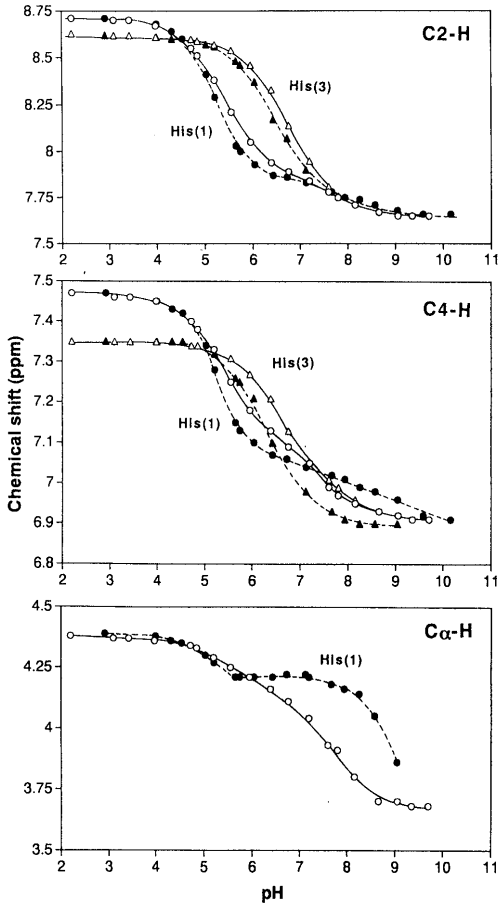

Fig. 5. NMR Titration Curves of Imidazole C-2 and C-4 Protons, and His-Cα Proton of HisGlyHisGly in the Absence (○, △) and Presence (●, ▲) of Zn(II) Ion

Peptide = 4.2 mM, Zn(II) = 2.0 mM. 1, 2, 3 and 4 in parenthesis indicate the positions of amino acid residues from the N-terminal.

Table 2. ^{1}H-NMR Chemical Shifts of HisGlyHisGly in the Absence and Presence of Zn(II)

	pH 4.9	pH 5.0		pH 5.7		pH 7.1		pH 9.7
	Peptide	Peptide	with Zn(II)	Peptide	with Zn(II)	Peptide	with Zn(II)	Peptide
His(1)C2-H	8.51	8.45	8.41	8.13	8.00	7.85	7.83	7.66
His(1)C4-H	7.38	7.34	7.34	7.21	7.13	7.05	7.04	6.91
His(3)C2-H	8.59	8.58	8.57	8.50	8.46	7.96	7.90	7.66
His(3)C4-H	7.34	7.34	7.34	7.29	7.25	7.05	6.98	6.91
His(1)Cα-H	4.33	4.31	4.31	4.23	4.21	4.05	4.21	3.68
Gly(2)CH_2	4.01	4.01	4.01	3.98	3.95	3.93	3.88	3.87
Gly(4)CH_2	3.80	3.79	3.79	3.79	3.77	3.76	3.71	3.75

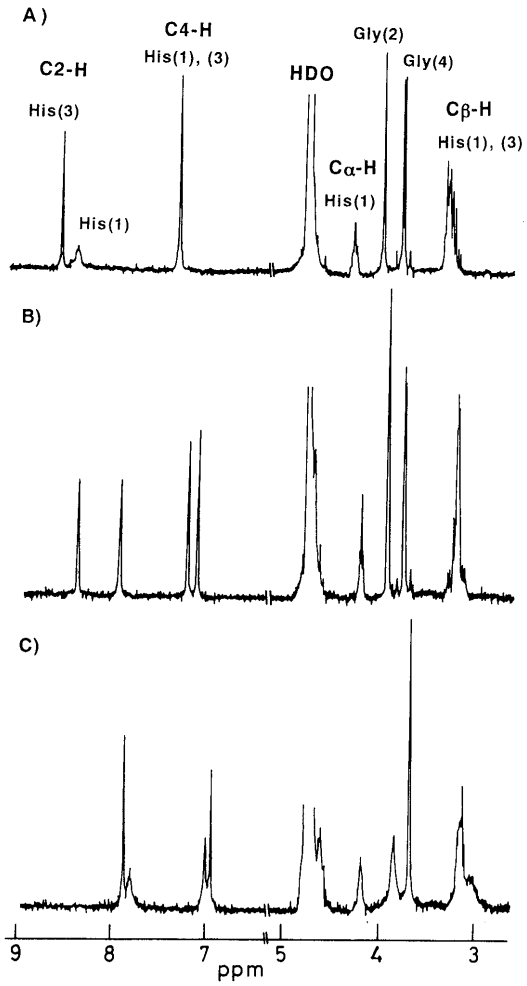

Fig. 6. ^{1}H-NMR Spectra of 4.2 mM HisGlyHisGly in the Presence of 2.0 mM $ZnSO_4$ in D_2O Solution at pH 5.0 (A), 5.7 (B), and 7.1 (C)

little, and the His-Cα resonance concomitantly shifted downfield as compared with that of the free peptide. This suggests the formation of an alternative new Zn(II) complex which is a little more inert than that produced below pH 5. The peptide could coordinate to Zn(II) through amino and imidazole nitrogens of the histidyl residue at the *N*-terminal. As pH increased further, line-widths of both the imidazole C2-H and C4-H resonances, and of the His-Cα resonance of the histidyl residue at the *N*-terminal were again broadened as observed at pH 7.1. This suggests that the fairly stable Zn(II) complex is transformed to a third complex with different coordination mode from the former two. These three complexes are probably in medium equilibrium on the NMR time scale. The third Zn(II) complex is probably hydrolyzed to a Zn(II)–hydroxo complex in the alkaline pH region above pH 8.

Addition of Zn(II) to HisGlyHisGly caused both the C2-H and C4-H resonances to shift upfield in the region over pH 5.0. The NMR titration curves of the imidazole C2-H and C4-H, and His Cα-H protons in the presence and absence of Zn(II) are shown in Fig. 5. Upfield shifts of the imidazole C2-H and C4-H protons may be explained by the proximal effect of the positively charged Zn(II), which causes a decrease in the pK_a of the imidazolium group. Above pH 7, however, the NMR chemical shifts were not affected by the addition of Zn(II), and were close to those of the deprotonated peptide. This suggests that the Zn(II) complex, in which HisGlyHisGly coordinates to Zn(II) through amino and imidazole nitrogens of the histidyl residue at the *N*-terminal in the region below pH 7, undergoes a significant structural modification to be hydrolyzed probably to a Zn(II)–hydroxo complex and peptide. Similar results were observed in the NMR titration of HisGlyGly in the presence of Zn(II).

Coordination Modes of the Histidyl Side Chains in Different Positions on the Peptide Chain Coordination modes in the metal site for the Zn(II) complexes with HisGlyGly, GlyHisGly, and GlyGlyHis have been reported previously.[13-15] HisGlyGly and GlyGlyHis coordinate to Zn(II) through the nitrogens from terminal amino and imidazole groups, while GlyHisGly coordinates to Zn(II) through nitrogen of the deprotonated peptide bond between Gly–His in addition to amino and imidazole nitrogens. The histidyl residue in the second position appeared to promote deprotonation of the peptide bond between His–His. Thus, HisHisGlyGly coordinates to Zn(II) through an amino nitrogen, an adjacent deprotonated peptide nitrogen, and an imidazole nitrogen of the histidyl residue in the second position. The fourth coordination site of Zn(II) is occupied by the imidazole nitrogen of histidyl residue at the *N*-terminal position of another complex. HisGlyHisGly coordinates to Zn(II) through an amino nitrogen and an imidazole nitrogen of the histidyl residue at the *N*-terminal position as in the Zn(II)–HisGlyGly complex. The histidyl residue in the second position appeared to promote deprotonation of the peptide bond nitrogen at the amino terminal and then its imidazole nitrogen coordinated with Zn(II) forming kinetically stable complexes. The histidyl residue in the third position, which coordinated firmly to Cu(II) and Ni(II), did not help to deprotonate the second peptide bond and thereby was not associated with Zn(II) binding.

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