Proton Nuclear Magnetic Resonance Studies of the Complexation of Zinc(II) with Glycyl-L-histidylglycine

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The complexation of Zn(II) with glycyl-L-histidylglycine and its deuterated derivatives, glycyl-d_2-L-histidylglycine and glycyl-L-histidylglycine-d_2, was studied by proton nuclear magnetic resonance spectroscopy over the pD range, from 3.4 to 11.0, at 25 °C. Addition of Zn(II) to the peptide made the resonances of both imidazole C2-H and C4-H and both methylene-protons of glycyl residues at the amino-terminal and carboxylate end split to three lines in the pD range above 7.0. From the behavior of the C2-H and C4-H chemical shifts, formation of at least two species, in which the imidazole and amino nitrogens coordinated to the metal ion forming a chelate ring, was suggested. Those two species, the ratio of which varied depending on the total concentrations of complexes, seemed to be interconvertible; one referred to as B is a monomer and the other, referred to as C, may be a dimeric or polymeric complex.

Key words proton nuclear magnetic resonance; zinc(II) ion; glycyl-L-histidylglycine; deuterated peptides; complexation

There are physiologically active tripeptides which contain a histidyl residue in the second position from the amino terminus; for example, glycyl-L-histidyl-L-lysine functions as a liver-cell growth factor[1] and pyroglutamyl-L-histidyl-L-prolineamide is a thyrotropin-releasing hormone.[2] Those peptides exhibit strong chelating ability to heavy metal ions and the chelate formation with Cu(II) has been extensively studied by spectroscopic methods.[3–6] Nitrogen atoms derived from the terminal amino or imino group, the vicinal deprotonated amide and imidazole groups have been shown to take part in chelating to the metal ion. However, the interaction of Zn(II), which is a physiologically important metal, has received little attention.[7,8] Since Zn(II) has neither any intrinsic color nor unpaired electrons, the range of spectroscopic techniques available for investigating Zn(II)–peptide interactions is limited. Nuclear magnetic resonance (NMR) spectroscopy is a useful but limited technique for studying the coordination mode and environment of the Zn(II) site in such complexes.[7–13]

In an attempt to elucidate the metal-coordination mode and variety of environments in Zn(II)-containing proteins and enzymes, we have been studying the complex formation of Zn(II) with histidine-containing peptides. In our previous papers, we described the Zn(II) interactions with L-histidyl-glycylglycine[14] and glycylglycyl-L-histidine[15] studied by ^{1}H-NMR spectroscopy. In the present paper, we describe the complexation of Zn(II) with glycyl-L-histidylglycine, which is a skeletal peptide of many physiologically active tripeptides.

In order to ascertain the assignment of the NMR chemical shifts, deuterated peptides, glycyl-d_2-L-histidylglycine (Gly-d_2–His–Gly) and glycyl-L-histidyl–glycine-d_2 (Gly–His–Gly-d_2) were synthesized.

Experimental

Materials Glycyl–L-histidylglycine and Zn(II)$SO_4 \cdot 7H_2O$ were commercial products from Sigma Chemical Co. (St. Louis, MO) and E. Merck (Darmstadt, Germany), respectively, and used as received. The glycine-d_2 used was from Merck Sharp & Dohme of Canada Ltd. (Montreal, Canada). Deuterated peptides were synthesized by a conventional solution technique and purified by chromatography on a silica-gel column. The synthetic procedure is summarized in Chart 1.[16] Elemental analysis for those compounds were as follows:
Gly-d_2–L-His–Gly; Found: C, 41.52%; H, 5.60%; N, 24.04% (Calcd for $C_{10}H_{13}D_2N_5O_4 \cdot H_2O$: C, 41.52%; H, 5.92%; N, 24.21%).
Gly–L-His–Gly-d_2; Found: C, 42.26%; H, 5.69%; N, 24.53% (Calcd for $C_{10}H_{13}D_2N_5O_4 \cdot 2/3H_2O$: C, 42.50%, H, 5.83%; N, 24.78%).

Preparation of Solutions A stock solution of Zn(II) was prepared by adding Zn(II)$SO_4 \cdot 7H_2O$ to 99.3% D_2O to give a final Zn(II) concentration of 4.0×10^{-2} M. The solution was standardized by titration with EDTA using Eriochrome Black T indicator. Stock solutions of the peptides were prepared in 99.3% D_2O. Deuteration of those solutions was carried out by a freeze and thaw method. For the ^{1}H-NMR measurements, aliquots of the deuterated Zn(II) and peptide solutions were placed in a vessel and titrated with either DNO_3 or NaOD solution. At selected pD values, an aliquot was withdrawn from the solutions

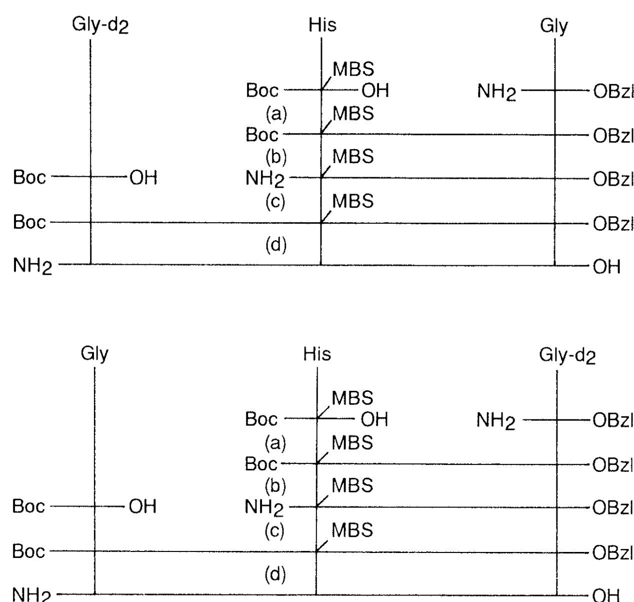

Chart 1. Synthetic Procedure of Deuterated Gly–L-His–Gly

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and transferred to an NMR tube for spectral recording. Under 1 : 1 (metal : ligand) conditions, the Zn(II) was likely to be precipitated above pH 6, probably because of the low coordinating ability of Zn(II). Thus the ratio of metal to ligand, 1 : 2.1, was used throughout the NMR recording and the concentration of Zn(II) routinely used was 4.0×10^{-3} M.

^{1}H-NMR Measurements ^{1}H-NMR spectra were obtained with a JEOL FX-270 FT spectrometer at 25 °C. Chemical shifts were reported in parts per million (ppm) relative to the resonance signal of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

pD Measurements Measurements of pD were made on a TOA Denpa HM-5A pH meter equipped with a TOA HGS-2005 glass electrode and a TOA HS-305D double-junction, saturated calomel, reference electrode. The outer liquid junction solution of the reference electrode was replaced by saturated KCl in 99.3% D_2O. Meter readings from measurements in D_2O solution were converted to pD using the relationship pD = pH + 0.40.[17]

Results

^{1}H-NMR Spectra of Gly-L-His-Gly and Its Deuterated Compounds The ^{1}H-NMR spectra of Gly–His–Gly, Gly-d_2–His–Gly, and Gly–His–Gly-d_2 at pD 4.7 in D_2O are shown in Fig. 1. The chemical shifts at 8.60 ppm and 7.34 ppm, which had been assigned to the imidazole C2-H and C4-H resonances respectively, shifted upfield with increasing pD. The quadruplet resonances centered at 3.86 ppm in Gly–His–Gly were not observed in Gly-d_2–His–Gly and that at 3.80 ppm was absent in Gly–His–Gly-d_2. This clearly indicates that the quadruplets at 3.86 and 3.80 ppm are assignable to the methylene protons of glycines at the amino-terminal and carboxylate-ends, respectively. The octet centered at 3.23 ppm was assigned to the β-methylene protons of the histidyl residue. The chemical shift of the methylene protons of the N-terminal glycyl residue and those of the C2-H and C4-H of the histidyl residue shifted upfield due to deprotonation of the ammonium and imidazolium groups, respectively. The methine protons of the histidyl residue were overlapped by a large HDO signal and could not be observed. The chemical shifts of the C2-H, C4-H, and other resonances at pD 4.7 and pD 8.5 are summarized in Table 1.

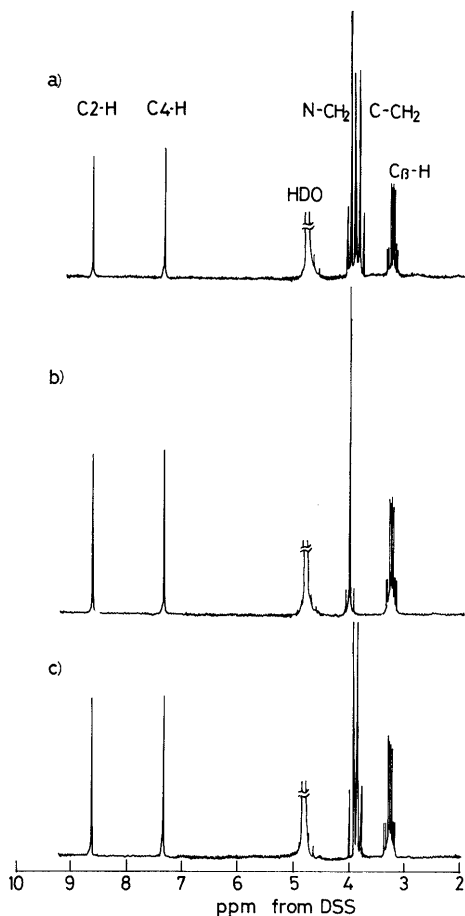

Fig. 1. ^{1}H-NMR Spectra of Gly–His–Gly and Its Deuterated Derivatives at pD 4.7

a: Gly–His–Gly, b: Gly-d_2–His–Gly, c: Gly–His–Gly-d_2, [peptide] = 8.4×10^{-3} M.

^{1}H-NMR Spectra of the Zn(II) Complexes of Gly-L-His-Gly The spectra of Gly–His–Gly and its deuterated derivatives in the presence of Zn(II) at pD 8.5 are shown in Fig. 2, where the Gly–His–Gly/Zn(II) ratio is 2.1. Addition of Zn(II) at pD 4.7 did not affect the resonance of the peptide as far as both line shape and chemical shift were concerned. This indicates that Zn(II) does not

Table 1. ^{1}H-NMR Chemical Shifts of GlyHisGly in the Absence and Presence of Zn(II) at pD 4.7 and at pD 8.5

	pD 4.7		pD 8.5			
	Peptide alone	Peptide with Zn(II)	Peptide alone	Peptide with Zn(II)		
				A[d]	B[d]	C[d]
C2-H	8.60	8.63	7.72	7.68	7.82	7.42
C4-H	7.34	7.37	6.98	6.98	6.98	6.53
N-CH_2[a]	3.86	3.87	3.61	3.61	3.33	3.33
C-CH_2[b]	3.80	3.83	3.75	3.72	3.55	3.99
C_β-CH_2[c]	3.23		3.10			

a) Amino-terminal CH_2, *b*) carboxylate-terminal CH_2, *c*) histidyl-CH_2, *d*) described in the text; see Chart 2.

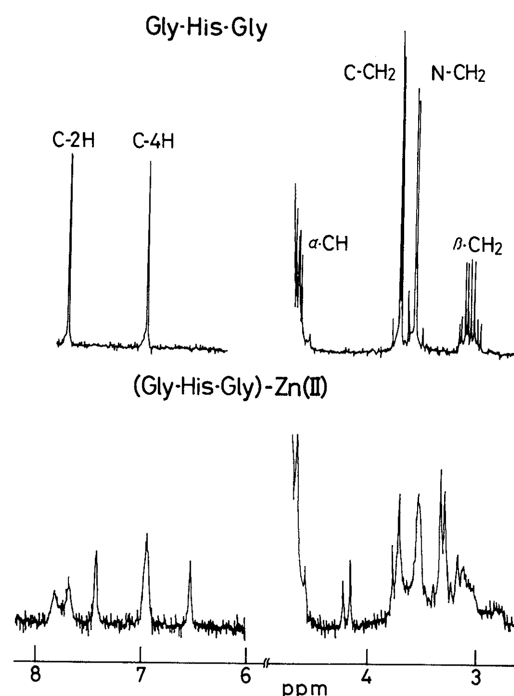

Fig. 2. ^{1}H-NMR Spectra of Gly–His–Gly in the Presence and Absence of Zn(II) at pD 8.5

[Zn(II)] = 4.0×10^{-3} M, [peptide] = 8.4×10^{-3} M.

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associate with Gly–His–Gly. However, at pD 8.5, both the imidazole C-2H and C-4H resonances were broadened and split into three lines. In both C-2H and C-4H resonances, the resonance at the center of those three lines showed the same chemical shift as those of the free peptide. A set of resonances at the center is assignable to either the deprotonated peptide or a complex which is kinetically unstable. Other two sets of lines were at 7.82 and 7.42 ppm for the C2-H and at 6.98 and 6.53 ppm for the C4-H. The resonance at 6.98 ppm was overlapped by that of the C4-H of the free peptide. The resonances at 7.42 and 6.53 ppm, which were of similar intensity, were assignable to a Zn(II) (Gly–His–Gly) complex. Similarly, the resonances at 7.82 and 6.98 ppm were assignable to another Zn(II) complex. The resonances of the methylene protons of the glycyl residues at the amino-terminal and carboxylate-ends were also split into three lines accompanied by splitting of the C2-H and C4-H. This suggests the formation of at least two types of kinetically stable complexes.

NMR Titration of Gly–L-His–Gly in the Presence and Absence of Zn(II) ^{1}H-NMR spectra were measured as a function of pD for the peptide alone in D_2O and in solutions containing Zn(II). The NMR titration curves of the imidazole C2-H and C4-H in the presence and absence of Zn(II) are shown in Fig. 3. The chemical shift of the imidazole C2-H and C4-H *vs.* pD plots were sigmoid in shape with an inflection point, from which the pK_a of the imidazolium was estimated to be approximate 6.9. Addition of Zn(II) did not affect the chemical shifts of both the C-2H and C-4H below pD 7.0, but above pD 7.0. This caused them to split into three lines. A set of resonances at the center shifted upfield with increasing pD and the pD profile of the chemical shifts displayed a sigmoid shape resembling that of the peptide alone and is thereby assignable to either exchange-averaged resonance between the ligand and a kinetically labile complex or between the protonated and deprotonated ligands. The chemical shifts of the other two sets, referred to as B and C respectively, did not shift irrespective of the variation of pD over the range 7.0—10.5, being attributed to kinetically stable complexes, in which the Zn(II) coordinates with the imidazole. The resonances B and C appeared at pD 7 and the intensity increased with increasing pD, reaching a maximum as shown in Fig. 4. Then, the intensity of both resonances decreased gradually above pD 10 and disappeared, probably because of hydrolysis of the Zn(II) complexes.

^{1}H-NMR spectra of the Gly–His–Gly/Zn(II) solutions, in which the metal ion concentration varied from 1.0×10^{-3} to 8.0×10^{-3} M, are presented in Fig. 5. The intensities of the three resonance lines due to the C2-H, as well as the C4-H, appeared to depend on the total concentration of Zn(II). Relative amounts of three species, A, B and C, were estimated by integration of the corresponding resonances. Plots of the relative amounts of species A, B and C against concentration are shown in Fig. 6. This indicates that the amount of species A remains constant, approximately 50%, irrespective of the variation in the total concentration of Gly–His–Gly/Zn(II). The other half was composed of species B and C and the ratio of these species appeared to decrease as the concentration increased.

The NMR titration curves of the methylene-proton of glycyl residues in the presence and absence of Zn(II) are shown in Fig. 7. Addition of Zn(II) split the resonances of the methylene-proton of glycyl residues into three lines above pD 7, and the relative intensities of the three lines

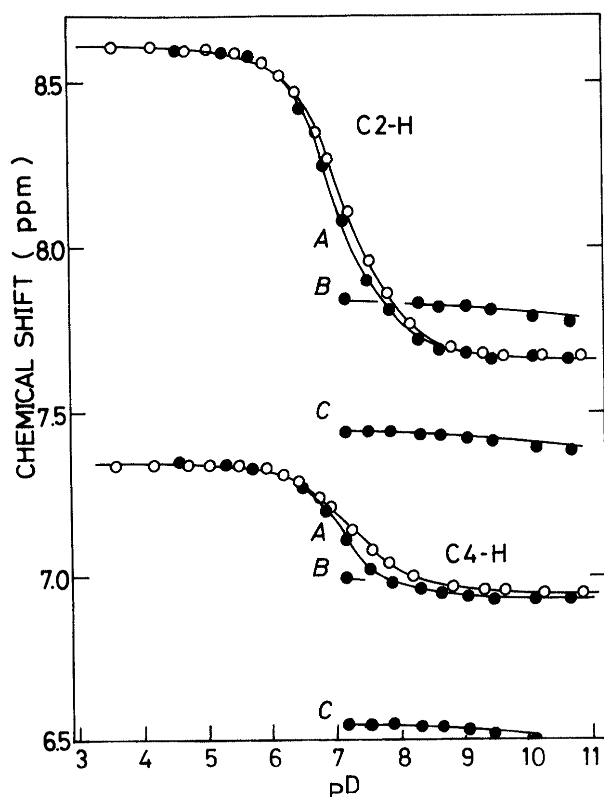

Fig. 3. ^{1}H-NMR Titration Curves of the Imidazole C2-H and C4-H
Conditions as in Fig. 2

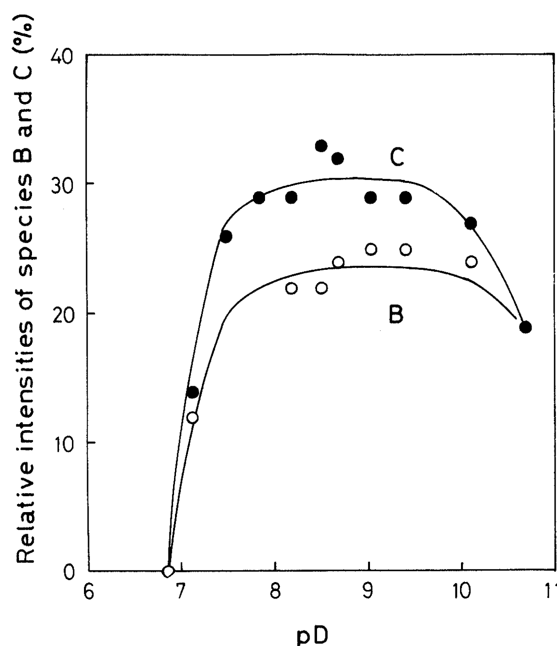

Fig. 4. pD Profile of the Intensities of Species B and C in the Imidazole C-2H Resonance

○, species B; ●, species C.

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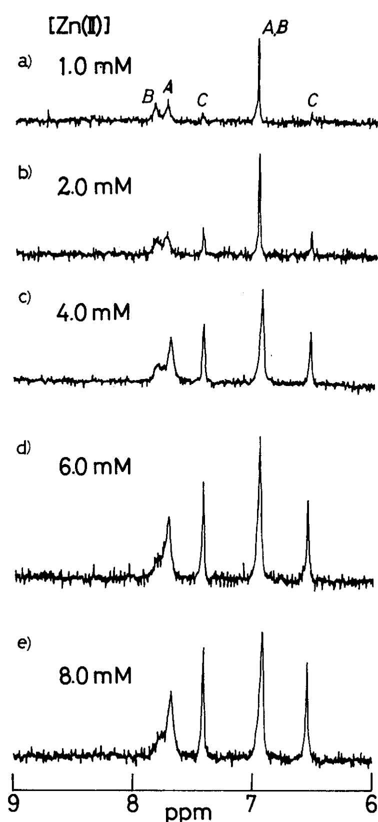

Fig. 5. ^{1}H-NMR Spectra in the 6—9 ppm Region at Various Concentrations of Zn(II) Ion

[Gly–His–Gly]/[Zn(II)] = 2.1, pD 8.5.

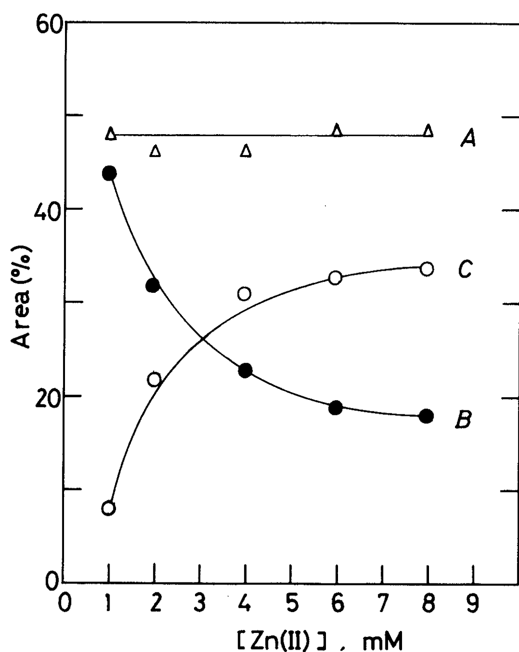

Fig. 6. Plots of the Integrated Intensities of Species A, B, and C against the Concentration of Zn(II)

Conditions as in Fig. 5.

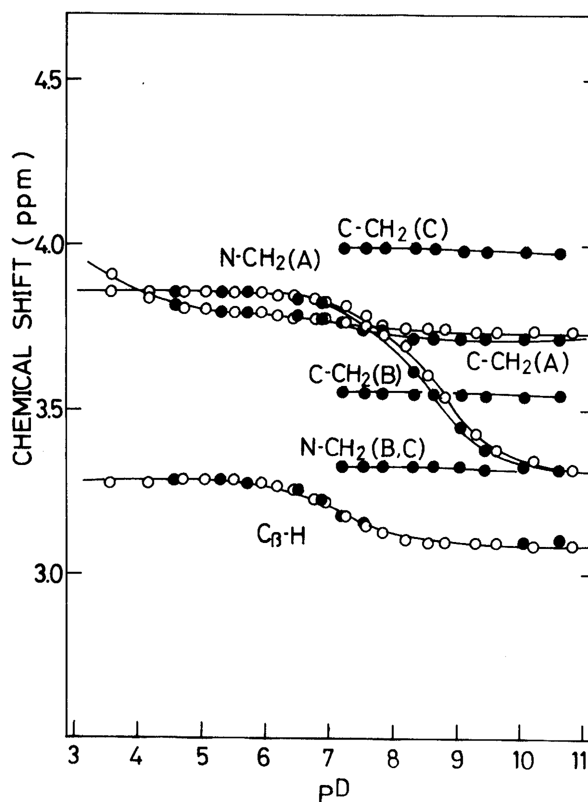

Fig. 7. ^{1}H-NMR Titration Curves of the Methylene-Protons of Glycyl and Histidyl Residues

Conditions as in Fig. 2.

appeared to depend on the total concentration of Zn(II), as observed in the C2-H and C4-H chemical shifts. The spectra of Gly–His–Gly and its deuterated derivatives over the 3—5 ppm range at $[Zn(II)] = 1.0 \times 10^{-3}$ and 8.0×10^{-3} M, where [Gly–His–Gly]/[Zn(II)] = 2.1, are shown in Figs. 8A and B. At $[Zn(II)] = 1.0 \times 10^{-3}$ M, where species A and B was predominant, the chemical shifts of the methylene-proton of the glycine at the amino-terminal and carboxylate-ends were observed at 3.33 and 3.55 ppm, respectively. As the concentration increased, species C became predominant. The spectra at $[Zn(II)] = 8.0 \times 10^{-3}$ M are shown in Fig. 8B. The chemical shift at the carboxylate end shifted downfield, while that at the amino-terminal remained unchanged. The chemical shift, observed at 3.33 ppm, of the methylene-proton of the glycyl residue at the amino-terminal is identical to that of the deprotonated glycyl residue which was observed at pD 10.5. This suggests that Zn(II) associates with the amino nitrogen in both species B and C.

Discussion

The complexation reaction of peptides with metal ions, such as Cu(II) or Ni(II), has been extensively studied by potentiometric and spectroscopic methods. The metal ion coordinates initially with the peptide through the amino nitrogen and the oxygen of the neighboring peptide-bond to form a five-membered chelate complex, abbreviated as ML. As the pH increases, the peptide-bond coordinated to the metal ion undergoes deprotonation and subsequent prompt ligand-exchange to form another stable complex, abbreviated as $M(H_{-1}L)$, in which the donor atom from the peptide bond changes from oxygen to nitrogen. Since the complex Zn(II)L is relatively unstable ($\log K_{ML} = 3.10$ for glycylglycylglycine[18]), it is liable to be hydrolyzed to ZnL(OH) or $Zn(OH)_2$. However, as far as the peptide involving the histidyl residue in the second position from

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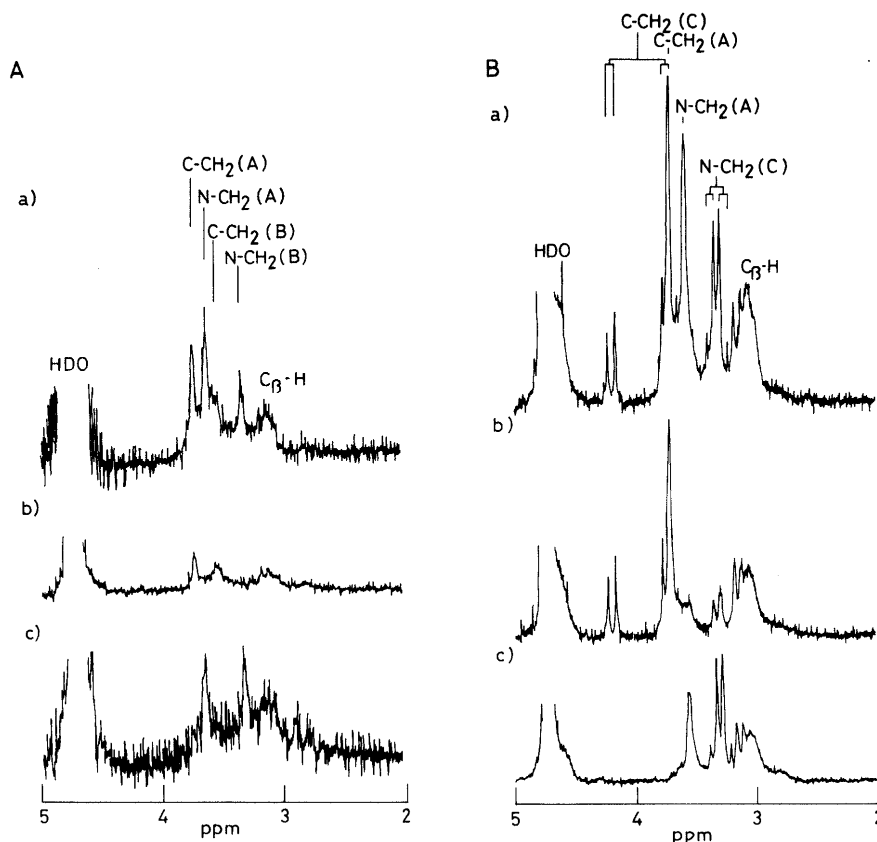

Fig. 8. ¹H-NMR Spectra in the 3—5 ppm Region at Various Concentrations of Zn(II) Ion
[Gly–His–Gly]/[Zn(II)] = 2.1, pD 8.5, A; [Zn(II)] = 1.0×10^{-3} M, B; [Zn(II)] = 8.0×10^{-3} M, a; Gly–His–Gly, b; Gly-d_2–His–Gly, c; Gly–His–Gly-d_2.

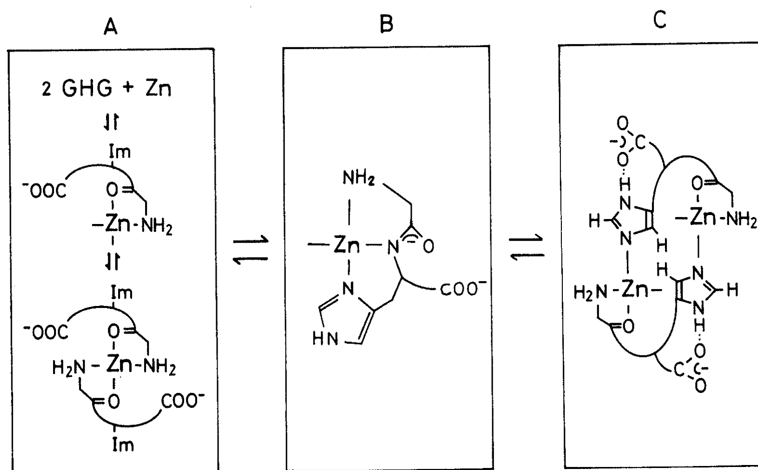

Chart 2

the N-terminal is concerned, such as Gly–His–Gly, the Zn(II)(H_{-1}L) species shown in Chart 2 is a stable one.[8,19,20)]

Both the C2-H and C4-H resonances of Gly–His–Gly shifted upfield on deprotonation of imidazolium (ImH^+). This suggests that the positive charge on the imidazole ring, which results from either protonation or metal-coordination, affords the C2-H and C4-H downfield shift. The change in the C2-H chemical shift due to deprotonation was 2.5-fold larger than that in the C4-H, because the C2-H is markedly affected by deprotonation. Then, the C2-H signal at 7.8 ppm is assignable to a monomeric Zn(II) (H_{-1} Gly–His–Gly), in which the metal ion coordinates to three nitrogens from the terminal-amino group, the deprotonated peptide bond and the imidazole group.

The integrated intensity of species A remained constant, approximately 50% of the total peptides, when the total concentration increased. However, the intensities of both species B and C varied depending on the concentration. The intensity of species C increased with an increase in the concentration of Zn(II) ion, while that of species B decreased as shown in Fig. 6. Provided that the main species for the Zn(II) complex is Zn(II) (H_{-1} Gly–His–Gly), species A corresponds to the free peptide which is 50% of the total and species B and C, the sum of which

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is the remaining half of the total peptide, is considered to be interconvertible. Species B is probably a monomeric species and assignable to the kinetically stable Zn(II) (H_{-1} Gly–His–Gly) (B in Chart 2), in which the C2-H and C4-H resonances probably shift downfield. Species C could be a dimeric or polymeric species and the imidazole groups bridge the monomeric species (C in Chart 2). The nature of the Zn(II) binding in species C cannot be elucidated from the present data; however its relative amount increases with the concentration.

References

1) Pickart L., Thaler M. M., *Nature* (London), **243**, 85 (1973).
2) Burgus R., Dunn T. F., Desiderio D., Ward D. N., Vale W., Guillemin R., *Nature* (London), **226**, 321 (1970); Nair R. M. G., Barrett J. F., Boweres C. Y., Schally A. V., *Biochemistry*, **9**, 1103 (1970).
3) Freedman J. H., Pickart L., Weinstein B., Mimsand W. B., Peisach J., *Biochemistry*, **21**, 4540 (1982).
4) Laussac J.-P., Haran R., Sarkar B., *Biochem. J.*, **209**, 533 (1983).
5) Kozlowska G. F., Kozlowski H., Kupryszewski G., *Inorg. Chim. Acta*, **46**, 29 (1980).
6) Sigel H., Martin R. B., *Chem. Rev.*, **82**, 385 (1982).
7) Rainer M. J. A., Rode B. M., *Inorg. Chim. Acta*, **93**, 109 (1984).
8) Daignault S. A., Arnold A. P., Isab A. A., Rabenstein D. L., *Inorg. Chem.*, **24**, 3984 (1985).
9) Lakusta H., Deber C. M., Sarkar B., *Can. J. Chem.*, **58**, 757 (1980).
10) Dabrowiak J. C., Greenaway F. T., Longo W. E., van Husen M., Crooke S. T., *Biochim. Biophys. Acta*, **517**, 517 (1978).
11) Oppenheimer N. J., Rodriguez L. O., Hecht S. M., *Biochemistry*, **18**, 3439 (1979).
12) Lenkinski R. E., Dallas J. L., *J. Am. Chem. Soc.*, **101**, 5902 (1979).
13) Cass A. E. G., Galdes A., Hill H. A. O., McClelland C. E., *FEBS Lett.*, **89**, 187 (1978).
14) Ueda J.-I., Hanaki A., Yoshida Y., Nakajima T., *Chem. Pharm. Bull.*, **33**, 3096 (1985).
15) Ueda J.-I., Hanaki A., Yoshida N., Nakajima T., *Chem. Pharm. Bull.*, **34**, 1315 (1986).
16) Abbreviation used are as follows; Boc: *tert*-butoxycarbonyl, Bzl: benzyl, MBS: *p*-methoxybenzenesulfonyl, DEPC: diethylphosphorocyanidate, DMF: dimethylformamide, TFA: trifluoroacetic acid, HF: anhydrous hydrogen fluoride.
17) Bates R. G., "Determination of pH," Wiley-Interscience, New York, 1973.
18) Rabenstein D. L., Libich S., *Inorg. Chem.*, **11**, 2960 (1972).
19) Agarwal R. P., Perrin D. D., *J. Chem. Soc., Dalton Trans.*, **1975**, 1045.
20) Rabenstein D. L., Daignault S. A., Isab A. A., Arnold A. P., Shoukry M. M., *J. Am. Chem. Soc.*, **107**, 6435 (1985).

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