Effects of Divalent Metal Cations on Circular Dichroism and ^1H Nuclear Magnetic Resonance Spectra of Linear and Cyclic Peptides Having Side-chain Imidazolyl and Acetamido Groups

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A linear peptide, *N-t*-butoxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidine methyl ester (**1**) was synthesized by the solid phase method. Subsequent cyclization by the azide method offered a cyclic peptide, *cyclo*-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidyl (**2**). The circular dichroism (CD) and ^1H NMR were observed for these peptides **1** and **2**, in the absence and presence of metal cations, Zn(II), Ca(II), Mg(II). Spectral results for Zn(II) complexes indicated striking contrasts to those of Ca(II) and Mg(II) complexes. Thus, in H_2O, the addition of Zn(II) caused CD spectral changes for both peptides, while Ca(II) and Mg(II) did not cause changes in any CD spectral region. In methanol, the additions of Zn(II) and Ca(II) caused CD changes, while the addition of Mg(II) did not cause any CD changes. The changes caused by adding Zn(II) were quite different from those by Ca(II), suggesting that Zn(II) is coordinated with the peptides in a mode different from that of Ca(II). ^1H NMR data also suggested the different coordination mode between these two metal complexes in methanol.

A number of studies have been undertaken about the complexation between metal cations and cyclic or linear oligopeptides in order to simulate the natural ionophores such as valinomycin[1] or the natural metalloproteins such as blue copper proteins.[2-4] In the former cases, the peptides studied are cyclic and the most cations used belong to alkali or alkaline earth metals, which are coordinated with the carbonyl oxygens of the main-chain peptide bonds.[5-8] In the latter cases, however, the peptides thus far investigated are primarily linear and metal cations belong to transition metals such as Cu(II), Ni(II), Pd(II), *etc.*, which are coordinated with imidazole, thiol, *etc.*, or deprotonated peptide-bond nitrogen anions.[9-11] There are in nature certain enzymes which contain Zn(II) at their active sites, such as carboxypeptidase A.[12] Since the properties of Zn(II) are intermediate between those of the diamagnetic metal ions and those of the paramagnetic transition metal ions, it seems to be interesting to investigate the interaction between Zn(II) and peptides. However, relatively few studies about Zn(II)–peptide complexes have been carried out. In the present study, therefore, the linear and the cyclic peptides having imidazolyl and acetamido groups at the side chains were synthesized, and circular dichroism (CD) spectra and ^1H NMR spectra of these peptides were measured in the presence or absence of Zn(II). Zn(II) is expected to be coordinated with the imidazolyl and the acetamido groups of the side chains in these complexes. Interactions with divalent diamagnetic metal ions, Ca(II) and Mg(II), were also investigated as well for comparison.

Experimental

Materials. The linear peptide, *N-t*-butoxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidine methyl ester (**1**) (Boc–L-Cys(*S*-Acm)–D-Leu–L-His–L-Cys(*S*-Acm)–D-Leu–L-His-OCH_3), and the cyclic peptide, *cyclo*-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-L-histidyl (**2**) (*cyclo*(–L-Cys(*S*-Acm)–D-Leu–L-His–L-Cys(*S*-Acm)–D-Leu–L-His–)), used in this study were synthesized by the solid phase method and subsequent cyclization as follows. *N-t*-Butoxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-N^{Im}-tosyl-L-histidyl-*S*-acetamidomethyl-L-cysteinyl-D-leucyl-N^{Im}-tosyl-L-histidine-resin was synthesized from *N-t*-butoxycarbonyl-N^{Im}-tosyl-L-histidine-resin (0.27 mmol His/g-resin) on 1 mmol scale by the solid phase procedure in a Beckman model 990B Peptide Synthesizer according to the schedule shown in Table 1. The tosyl group was removed by suspending the obtained resin (*ca.* 4 g) in a solution of 1-hydroxybenzotriazole (1 g) in DMF (30 ml) at room temperature for 8 h.[13] The peptide was then removed from the resin by treatment with 1 M (1 M = 1 mol dm^{-3}) triethylamine in methanol (300 ml) at room temperature for 20 h.[14] The peptide was purified by preparative thin-layer chromatography (preparative TLC) on silica gel in *n*-BuOH–AcOH–H_2O (10 : 1 : 3 in vol.) and by gel filtration on Sephadex LH-20 in methanol. The purified peptide (**1**) showed a single spot (Pauly test, positive; ninhydrin test, negative) at R_f 0.29 on TLC in *n*-BuOH–AcOH–H_2O (10 : 1 : 3 in vol.). ^1H NMR

TABLE 1. SCHEDULE FOR THE SOLID PHASE SYNTHESIS

Step	Operation and reagent	Mixing time / min
1	$CHCl_3$, 45 ml (3 times)	1.5
2	25% trifluoroacetic acid/$CHCl_3$, 45 ml (once)	1.5
3	25% trifluoroacetic acid/$CHCl_3$, 45 ml (once)	30
4	$CHCl_3$, 45 ml (6 times)	1.5
5	10% triethylamine/$CHCl_3$, 45 ml (3 times)	1.5
6	$CHCl_3$, 45 ml (3 times)	1.5
7	CH_2Cl_2, 45 ml (3 times)	1.5
8	Boc-amino acid (3 equivalents) in CH_2Cl_2,[a] 22 ml, and 0.25 M DCC in CH_2Cl_2, 10 ml (once)	120
9	CH_2Cl_2, 45 ml (3 times)	1.5
10	$CHCl_3$, 45 ml (3 times)	1.5

a) The solvent of Boc–L-Cys(*S*-Acm)–OH was the mixture of CH_2Cl_2 (20 ml) and DMF (2 ml).

(D_2O, DMSO-d_6) and field desorption mass spectrometry (FDMS) spectral data were in good agreement with those of the expected structure. Amino acid analysis: Leu : His= 1.00 : 0.93, Cys was not determined correctly. Found: C, 51.20; H, 7.23; N, 16.22; S, 6.13%. Calcd for $C_{42}H_{68}N_{12}O_{11}S_2 \cdot 1/2C_2H_4O_2$: C, 51.09; H, 6.93; N, 16.63; S, 6.34%. The cyclization procedure was as follows. Boc-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-$NHNH_2$ was prepared from **1** (100 mg) by treatment with hydrazine hydrate (0.7 ml) in methanol (2 ml) for 12 h, followed by the removal of *t*-butoxycarbonyl (Boc) group with trifluoroacetic acid (1 ml) in the presence of dimethyl sulfide (0.2 ml) for 10 min. To a solution of H-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-$NHNH_2 \cdot 4CF_3COOH$ (*ca.* 100 mg) in DMF (1 ml) were added 6 M HCl in dioxane (0.07 ml) and isopentyl nitrite (0.02 ml) at −50 °C, and the mixture was stirred in an ice–salt bath until hydrazine test became negative (*ca.* 10 min).[15] The reaction mixture was then added drop by drop into cold pyridine (*ca.* 500 ml) in an ice–salt bath with vigorous stirring.[16] After stirring for an additional 30 min, it was allowed to stand at 4 °C for 48 h and then at room temperature for 12 h. After the solvent was evaporated under reduced pressure at 30—40 °C, the residue was purified by preparative TLC on silica gel in *n*-BuOH–AcOH–H_2O (10 : 1 : 3 in vol.) and by gel filtration on Sephadex LH-20 in methanol. The purified cyclic peptide (**2**) showed a single spot (Pauly test, positive; ninhydrin test, negative) at R_f 0.40 on TLC in *n*-BuOH–AcOH–H_2O (4 : 1 : 2 in vol.). ^1H NMR (D_2O, DMSO-d_6) and FDMS (*m/e*; 848) spectral data agreed well with those of the expected structure. Found: C, 47.68; H, 6.41; N, 17.50; S, 7.50%. Calcd for $C_{36}H_{56}N_{12}O_8S_2 \cdot 1/2\ C_2H_4O_2 \cdot 3H_2O$: C, 47.64; H, 6.87; N, 18.03; S, 6.87%.

Methods. CD spectra were measured on a JASCO J-20 or J-500 spectropolarimeter using 0.1 or 1 mm cells at room temperature. In the case of aqueous solutions, 0.1 M NaCl was contained. Proton NMR spectra were recorded on a Bruker CXP-300 FT NMR spectrometer operating in the pulsed Fourier transform mode at 300.07 MHz in a spinning 5 mm tube at room temperature. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (0.00 ppm) was used as an internal reference in D_2O solution. In the case of methanol-d_4 solution, the solvent (4.78 ppm from DSS) was used as a reference.

Results

Figure 1 shows CD spectra of the linear peptide at

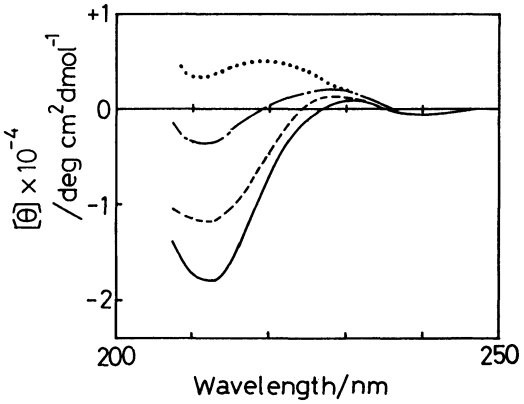


Fig. 1. CD spectra of Boc-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$-CCH_3 in H_2O: peptide concentration, 2.8×10^{-4} M; pH, 3.57 (——), 5.52 (------), 6.40 (—·—), 7.55 (……).

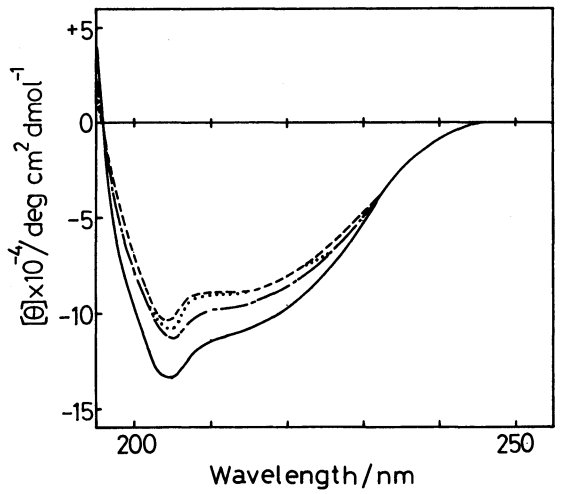


Fig. 2. CD spectra of *cyclo*-(L-Cys(*S*-Acm-D-Leu-L-His)$_2$ in H_2O: peptide concentration, 4.0×10^{-4} M; pH, 3.06 (——), 6.25 (—·—), 8.59 (----); in the presence of $ZnCl_2$ (4.0×10^{-3} M) at pH 6.25 (……).

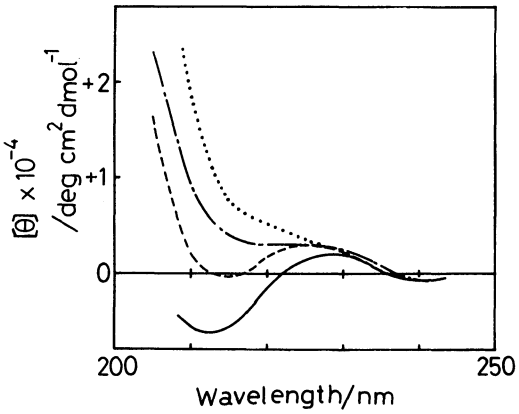


Fig. 3. CD spectra of Boc-L-(Cys(*S*-Acm)-D-Leu-L-His)$_2$-OCH_3 in H_2O: peptide concentration, 2.8×10^{-4} M; pH 6.25; $ZnCl_2$ concentration, 0 M (——), 2.8×10^{-4} M; (------), 5.6×10^{-4} M (—·—), 1.1×10^{-3} M (……).

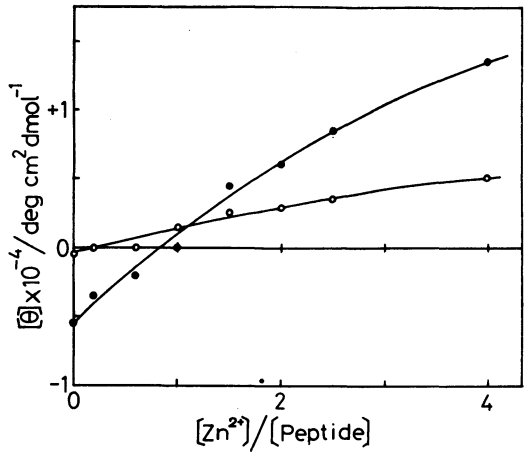


Fig. 4. Relation between molar ellipticity [θ] (deg cm^2 dmol^{-1}) and [Zn^{2+}]/[peptide] in H_2O: concentration of Boc-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$-OCH_3, 2.8×10^{-4} M; at 212 nm (—●—) and 220 nm (—○—).

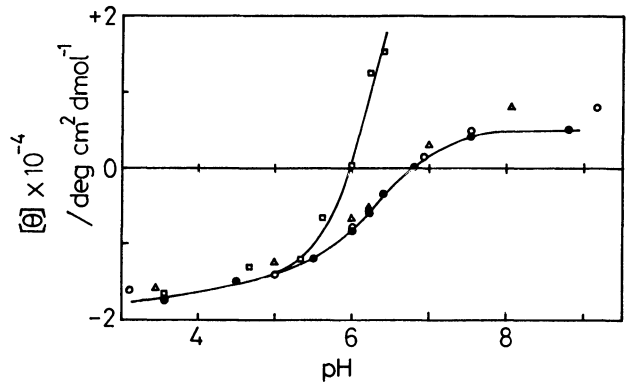
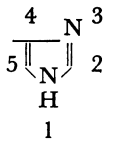


Fig. 5. Titration curves of molar ellipticity [θ] (deg cm^2 dmol^{-1}) at 212 nm of Boc-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$-OCH_3 in H_2O: peptide concentration, 2.8 $\times 10^{-4}$ M; metal salt free (-●-), 5.6×10^{-3} M $MgCl_2$ (-○-), 5.6×10^{-3} M $CaCl_2$ (-△-), 1.1×10^{-3} M $ZnCl_2$ (-□-).

Table 2. Chemical shift (ppm) of imidazolyl C-2 and C-5 protons of linear or cyclic peptides in D_2O at pD 6.22[a)]

[Zn^{2+}]/[Peptide]	Linear peptide		Cyclic peptide	
	C-2	C-5	C-2	C-5
0	8.22	7.11	8.28	7.20
	8.15			
3	8.08	7.14	—	—
		6.98		
10	8.05	7.11	8.22	7.17
		6.95		

a) Peptide concentration, 1.0×10^{-3} M; sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference (0.00 ppm); C-2 and C-5 are numbered as in the following figure:



neutral and acidic pH regions. Remarkable CD changes dependent upon the pH values are noted. Thus, the CD peak around 218 nm observed at pH 7.55 is decreased when the pH value is decreased. Instead, a CD trough at 212 nm is to be seen at acidic pH regions.

In contrast to the pH-dependent CD spectra of the linear peptide, the CD trough observed from 205 nm to 230 nm of the cyclic peptide is much less pH-dependent as can be seen in Fig. 2. The CD magnitude of the cyclic peptide at pH 8.59 is increased by 20—30% when the pH is decreased to pH 3.06.

Effects of metal salts added to the peptide solutions were investigated at pH 6.25. Typical CD spectrum in the presence of Zn(II) is shown in Fig. 2 (dotted line) for the cyclic peptide. As can be seen, no remarkable CD spectral change is observed by adding Zn(II) to the cyclic peptide solution. Figure 3 shows the effects of Zn(II) on the CD spectra of the linear peptide. CD spectra of the linear peptide are markedly changed by adding Zn(II). The CD magnitudes of the linear peptide are correlated with the Zn(II) concentrations as is shown in Fig. 4 where the molar ellipticity [θ] at ordinate is the values at 212 and 220 nm. Additions of Ca(II) and Mg(II) to the linear peptide solution did not cause any marked change in the CD spectra. In Fig. 5 are shown the pH-titration curves of [θ] at 212 nm of the linear peptide in the presence or absence of the metal salts ($ZnCl_2$, $CaCl_2$, $MgCl_2$). In this case, $ZnCl_2$ brings about a characteristic titration curve in contrast with that of free form, while $CaCl_2$ and $MgCl_2$ essentially do not cause change in the titration curve.

Table 2 summarizes ^{1}H NMR signals of imidazole C-2 and C-5 protons of the linear and the cyclic peptides in D_2O at pD 6.22 (not corrected for isotope effects). The C-2 and C-5 signals of the both peptides as a whole move to high field by adding Zn(II). However, the C-5 signal at 7.11 ppm of the linear peptide splits to two signals at 7.11 and 6.95 ppm when the Zn(II) concentration is large excess against that of the linear

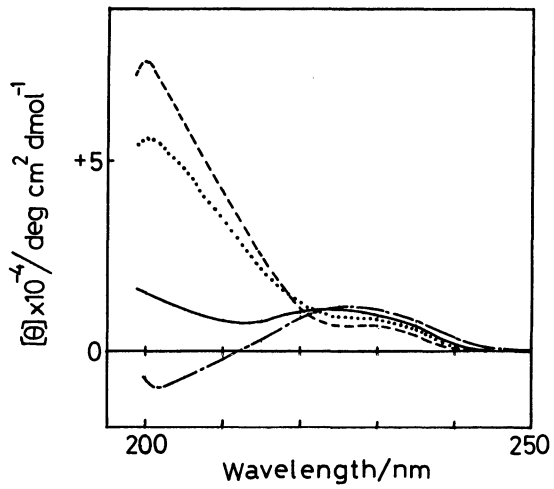


Fig. 6. CD spectra of Boc-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$-OCH_3 in MeOH: peptide concentration, 2.8×10^{-3} M ;metal salt free or 5.6×10^{-2} M $MgCl_2$ (——), 2.0×10^{-3} M $ZnCl_2$ (----), 2.8×10^{-2} M $ZnCl_2$ (……), 5.6×10^{-2} M $CaCl_2$ (—·—).

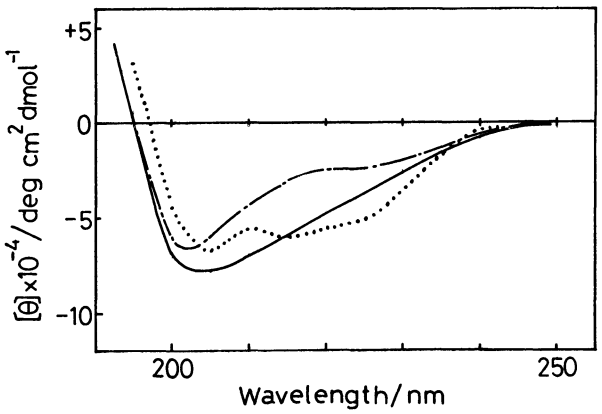


Fig. 7. CD spectra of *cyclo*-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$ in MeOH: peptide concentration, 4.0×10^{-4} M; metal salt free or 8.0×10^{-3} M $MgCl_2$ (——), 8.0×10^{-3} M $CaCl_2$ (……), 8.0×10^{-3} M $ZnCl_2$ (—·—).

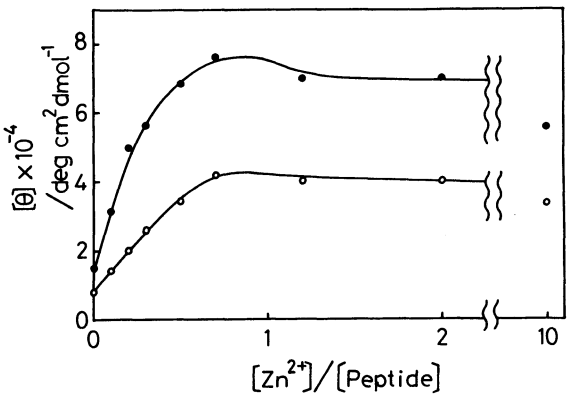


Fig. 8. Relation between molar ellipticity [θ] (deg cm^2 dmol^{-1}) and [Zn^{2+}]/[peptide] in MeOH: concentration of Boc-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$-OCH_3, 2.8×10^{-3} M; at 200 nm (—●—) and 210 nm (—○—).

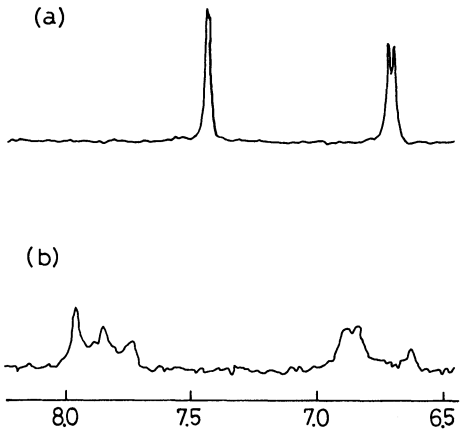


Fig. 9. ^1H NMR spectra of Boc-(L-Cys(*S*-Acm)-D-Leu-L-His)$_2$-OCH_3 in methanol-d_4: peptide concentration, 2.0×10^{-3} M; (a) in the absence of $ZnCl_2$, (b) in the presence of 2.0×10^{-3} M $ZnCl_2$.

peptide. In addition, the C-2 signals at 8.22 and 8.15 ppm of the linear peptide coalesce into a signal at 8.05 ppm by the Zn(II) addition.

In less polar solvent, such as methanol, Ca(II) or Mg(II) seems to coordinate with the linear and the cyclic peptides. Figures 6 and 7 show effects of metal cations on the CD spectra of those peptides in methanol. For the linear peptide, Ca(II) as well as Zn(II) brought about the spectral changes, while Mg(II) did not. However, the changes caused by Ca(II) are different from those by Zn(II), possibly because of the differences of the coordination structures between these two metal complexes. For the cyclic peptide, no remarkable CD spectral changes are noted by adding Mg(II), Ca(II) or Zn(II) (Fig. 7). The molar ellipticities [θ] at 200 and 210 nm are plotted against Zn(II)-linear peptide concentration ratios as shown in Fig. 8. One can see that [θ] increases rapidly until [Zn^{2+}]/[peptide] is *ca.* 0.7 and then decreases gradually.

Proton NMR spectra were measured in methanol-d_4, too. As Fig. 9 shows, Zn(II) causes the splitting and the broadening of the imidazolyl resonances of the linear peptide. For the cyclic peptide, the imidazolyl resonances are shifted to lower magnetic field without any significant signal broadenings (Table 3). Effects of Mg(II) and Ca(II) on the peptide NMR signals in methanol-d_4 are summarized in Table 3 for comparison.

TABLE 3. CHEMICAL SHIFT (ppm) OF LINEAR OR CYCLIC PEPTIDES IN METHANOL-d_4 IN THE PRESENCE OR ABSENCE OF METAL SALT[a)]

Linear peptide[b)]					
Metal salt	His		OCH_3	Acm-CH_3	Boc-CH_3
	C-2	C-5			
Free	7.46	6.74	3.57	1.85	1.29
	7.45	6.71		1.83	
$MgCl_2$ 20 mM	7.52	6.75	3.57	1.85	1.29
	7.49			1.83	
$CaCl_2$ 20 mM	7.54	6.78	3.58	1.86	1.30
	7.51	6.76		1.85	
$ZnCl_2$ 2 mM[c)]	7.96	6.89	3.64	1.84	1.30
	7.85	6.85	3.59		
	7.74	6.64			

Cyclic peptide[d)]						
Metal salt	His		Acm-CH_2		Leu[e)] α-CH	Acm-CH_3
	C-2	C-5				
Free	7.45	6.72	4.31	4.11	3.92	1.84
			4.26	4.06		
$MgCl_2$ 24 mM	7.54	6.77	4.32	4.11	3.92	1.85
			4.28	4.07		
$CaCl_2$ 24 mM	7.57	6.81	4.30	4.17	3.99	1.87
			4.26	4.12		
$ZnCl_2$ 2.4 mM	7.79	7.42	4.35	4.27	4.08	1.85
			4.30	4.22		

a) Methanol-d_4 was used as a reference (4.78 ppm). b) Concentration, 2.0×10^{-3} M. c) Histidyl C-2 and C-5 proton resonances were broadened and split, so the chemical shift values described here are those of the representative resonances. d) Concentration, 2.4×10^{-3} M. e) Triplet resonances apparently.

Discussion

The CD spectra of both the cyclic and the linear peptides in aqueous solution shown in Figs. 1 and 2 can tell us conformational and/or electronic differences between the two types of peptides we studied. It is generally known that β-turn conformation can be seen in certain cyclic hexapeptides.[17,18] Therefore, there is a possibility that the cyclic peptide studied here may take a β-turn. However, the absorptions due to the low-energy band of the split amide π-π^* and the amide n-π^* transitions of the main chain seem to overlap with those due to the imidazolyl π-π^* and the Acm-amide π-π^* or n-π^* transitions of the side chains, and thus the detailed conformational analysis is difficult in the present study. It is shown that the CD spectra of the linear peptide vary remarkably with pH while those of the cyclic one change a little. This indicates that the conformation of the cyclic peptide is fairly rigid, while that of the linear peptide is flexible. For the linear peptide, the spectra at lower pH value might be ascribed to relatively extended conformations caused by electro-

static repulsion force between the charged imidazolyl groups, while those at high pH value might be attributed to less perturbated conformations. From Figs. 3—5, it is concluded that in aqueous solution Zn(II) is much more liable to be coordinated with the linear peptide compared with Ca(II) or Mg(II). On the other hand, the CD spectrum of the cyclic peptide is affected very little by $ZnCl_2$ as shown in Fig. 2, which also suggests the rigid conformation of the cyclic peptide. Since Zn(II) is a medium soft acid, it seems likely that Zn(II) is bound to histidyl imidazoles.

The binding of Zn(II) to histidyl imidazole is supported by the results of ^1H NMR spectra in deuterium oxide (D_2O) at pD 6.22 in that the imidazolyl C-2 and C-5 proton resonances in both the cyclic and the linear peptides were split or shifted on addition of Zn(II) as Table 2 shows. These resonances are shifted to higher magnetic field in general. The splitting in the linear peptide must be due to the environmental difference between the two imidazoles in the molecule.

The CD spectral changes of the linear peptide observed by adding Zn(II) in methanol are correlated with the Zn(II)/peptide concentration ratios as shown in Fig. 8. The first increasing stage of the titration trace observed at 200 or 210 nm is considered to be due to the formation of 1 : 2 ($[Zn^{2+}]$/[peptide]) complex and the next decreasing stage due to that of 1 : 1 or other complexes.

The NMR spectra changes of the linear peptide by adding Zn(II) (Fig. 9) will suggest that there are various populations of conformational isomers interconverting at or near the coalescence state on the NMR time scale. In contrast to this, the corresponding resonances of the cyclic peptide are shifted to lower magnetic field without significant broadening. This will suggest that the population of a certain isomer is predominant or that the interconversion between isomers is sufficiently rapid on the NMR time scale. In view of the conformational rigidity of the cyclic peptide, the former interpretation seems to be more reasonable. For both of the peptides, the magnitude of the induced shifts is as a whole in the order: Zn(II)> Ca(II)>Mg(II), except for the Acm-CH_3. From the Acm–CH_3 chemical shift, an appreciable effect of Ca(II) ion addition is noted. From these results, it seems likely that Ca(II) is bound to the Acm–amide carbonyl, but Zn(II) does not interact with the Acm–CONH group. These NMR findings suggest the structural differences between Zn(II) and Ca(II) peptide complexes, which have been implied by the CD findings.

In concluding remarks, CD and NMR findings offered convincing evidences that the cyclization of the peptide we studied makes the peptide conformation rigid, and the coordination ability of the peptide to metal cations markedly changes. It seems very likely, in addition, that the cyclization of the peptide reduces the differences in the coordination abilities among metal cations to the peptide. As has been shown in the present study, it seems to be very helpful to use the techniques of both CD and NMR, since CD offers the information about the overall structure of a molecule, while NMR offers that about the local structure of a molecule. Although ^1H NMR of the peptides was investigated in the present study, the NMR of the metal cations, such as ^{67}Zn(II),[19,20] ^{43}Ca(II),[19] or ^{25}Mg(II),[21] would be also very useful for the present study.

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