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Solution Conformation of *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-) and Interaction with Cu(II)

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Proton NMR spectra of a cyclic peptide, *cyclo*-(*S*-acetamidomethyl-L-cysteinyl)-D-leucyl-L-histidyl-(*S*-acetamidomethyl-L-cysteinyl)-D-leucyl-L-histidyl, were measured and analyzed in order to investigate its conformation and its interaction with Cu(II). A linear peptide, [*N*-(*t*-butoxycarbonyl)-*S*-acetamidomethyl-L-cysteinyl]-D-leucyl-L-histidyl-(*S*-acetamidomethyl-L-cysteinyl)-D-leucyl-L-histidine methyl ester, was also investigated for comparison. It was strongly suggested that the cyclic hexapeptide forms transannular hydrogen bonds between the cysteine residue peptide bonds and thus two β-turns exist in the D-leucyl-L-histidyl sequences. This finding is particularly noteworthy because the present cyclic peptide has no prolines which are frequently seen in β-turn structures of proteins, naturally occurring peptides or synthetic peptides and because the cyclic peptide we studied is composed of amino acids all bearing the large side chains which seemed likely to prevent β-turn formation due to their steric hindrance. The interactions of the cyclic peptide and the linear peptide with Cu(II) were also studied by observing ^{1}H NMR spectral changes on addition of $CuCl_2$. It was suggested that the imidazolyl groups in the peptides are directly coordinated to Cu(II).

Imidazolyl and carbamoyl groups of histidine and glutamine (or asparagine) residues or main-chain peptide bonds seem to play an important role for the coordination of proteins or naturally occurring peptides to metal cations. It seems particularly interesting to study the interaction of metal cation with the cyclic peptide having both imidazolyl groups and peptide bonds at the side chains. Few studies have been undertaken about the interaction of metal cations with imidazole and amide-containing cyclic peptide. Cyclic peptides are better ligands than linear peptides in the following respects: (i) In naturally occurring antibiotics or other biologically functional peptides, there are many cyclic peptides which have much higher activities than those of the corresponding linear peptides, such as gramicidin S.[1] Therefore, cyclic peptides are important from the biological point of view. (ii) Intramolecular rotations around the main-chain bonds are restricted by cyclization and thus overall conformations are relatively rigid. Contributions of the end groups to the metal ligations can be excluded for cyclic peptides. These features would make the NMR analyses much easier.

D-Leucine is incorporated into the cyclic peptide in order to place the imidazolyl and the acetamido groups on the same side of the cyclic molecular plane. This would make the imidazolyl and acetamido groups liable to coordinate to metal cations.

In this paper, the conformation of the cyclic peptide in the solution has been investigated in detail. We have also studied the effect of Cu(II) on the ^{1}H NMR spectra of the cyclic peptide.

Experimental

Materials. The syntheses of [*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 *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 the present study have been described elsewhere.[2] $CuCl_2 \cdot 2H_2O$ was of reagent grade. D_2O was the product of Commissariat à L'Energie Atomique of France, and dimethyl-d_6 sulfoxide (DMSO-d_6) was obtained from E. Merck, Japan.

Methods. Proton NMR spectra were recorded on a Bruker CXP-300 FT NMR spectrometer operating in the pulsed Fourier transform mode at 300.07 MHz using a spinning 5 mm tube. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference (0.00 ppm) in D_2O solution, and partially deuterated dimethyl sulfoxide (2.49 ppm from DSS) was used as a reference in DMSO-d_6 solution. The pD values given are the actual pH meter readings and have not been corrected for the deuterium isotope effect at the glass electrode and pK_a* refers to the pK_a value determined from the chemical shifts of imidazolyl C-2 proton in D_2O. Adjustment of pD was made by adding either NaOD or DCl. Observed NMR signals were assigned to the individual amino acid residue by comparing the chemical shifts of the peptides with those of model compounds and by using the technique of homonuclear spin decoupling.

Results

^{1}H NMR Signals of Acm-Methylene Protons. Proton NMR spectra of the linear and the cyclic peptides in D_2O are shown in Fig. 1. The chemical shift difference between the two protons of the Acm-CH_2 in the cyclic peptide (**2**) (each resonance is further split into two by geminal protons spin-spin coupling) is greater than those in the linear peptide (**1**), indicating that the former methylene protons are located in environments considerably different from each other in D_2O solution. The motional fluctuation of the molecule may apparently reduce the environmental difference of the two protons of the Acm-CH_2 for the linear peptide. In DMSO solution, however, only one resonance split into two by the spin-spin coupling with the amide NH proton (not shown in a figure) was seen for the cyclic peptide, suggesting that the environments of the two protons of the Acm-CH_2 in the cyclic peptide are similar to each other in DMSO solution. For the linear peptide, the two resonances corresponding to the Acm-CH_2 observed

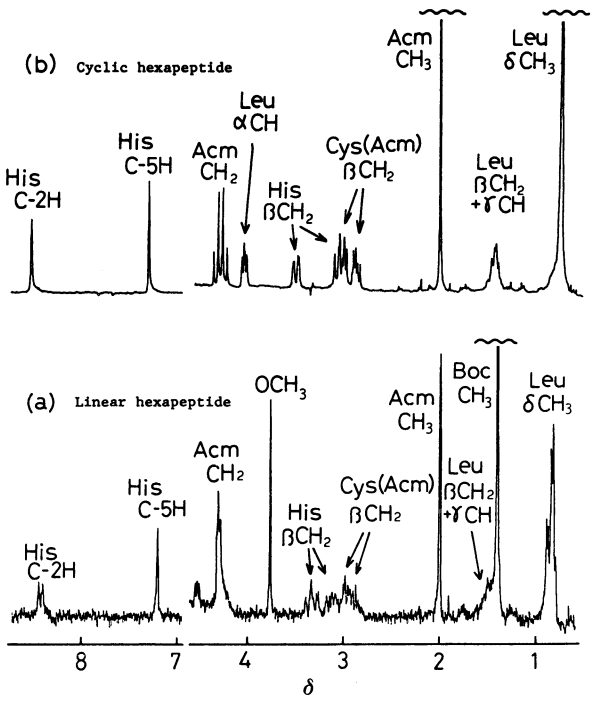


Fig. 1. The 300 MHz proton NMR spectra of linear or cyclic peptides in D_2O solution; peptide concentrations, 1.0×10^{-3} mol dm^{-3}; (a) Boc-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-OCH_3, (b) *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-).

in D_2O solution do not appear to be split significantly in DMSO solution, too (not shown in a figure).

pK_a* *Values.* The changes of the chemical shifts of the histidyl C-2 and C-5 protons in both the the linear and the cyclic peptides and the change of the chemical shift of one of the histidyl β-CH_2 protons in the cyclic peptide were measured as a function of pD as shown in Figs. 2 and 3. The observed histidyl C-2 proton chemical shifts (δ_{obsd}) were fitted by a nonlinear least-squares method to the equation (1)

$$\delta_{obsd} = \delta_0 + [(\delta_+ - \delta_0)\times 10^{(pK_a^*-pD)}/\{1+10^{(pK_a^*-pD)}\}] \quad (1)$$

in which δ_+ is the chemical shift of the fully protonated histidine and δ_0 that of the neutral histidine. The theoretical curves with the pK_a* values fitted are drawn as shown in Figs. 2 and 3. The pK_a* values of the linear peptide are 6.40 and 6.25, corresponding to those of the two different histidine residues in the molecule, and the pK_a* value of the cyclic peptide is 6.18. The chemical shifts of the histidyl C-5 and β-CH_2 protons were also fitted for comparison. As a whole the values obtained from the C-2 proton chemical shifts agree well with those obtained from the C-5 proton chemical shifts (6.25 and 6.33 for the linear peptide, and 6.19 for the cyclic peptide) and that obtained from the histidyl β-CH_2 chemical shift (6.15 for the cyclic peptide). These values are as a whole a little smaller than those reported.[3)]

Conformation of Histidyl Side Chain in D_2O. The rotamer populations of the histidyl side chain of the cyclic peptide were estimated from the H-C^α-C^β-H protons coupling constant ($J_{\alpha\beta}$) at acidic and basic regions in D_2O solution. In simplified approximation,

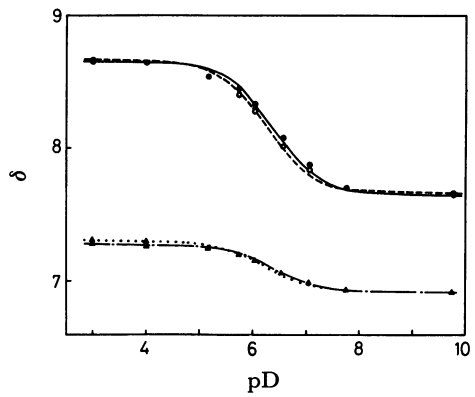


Fig. 2. Effects of pD on the chemical shifts of the histidyl C-2 and C-5 proton resonances of 1.0×10^{-3} mol dm^{-3} Boc-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-OCH_3. The symbols refer to C-2(●, ○) and C-5 (▲, △). The theoretical curves were fitted to the experimental data by a nonlinear least-squares analysis; pK_a*, 6.40 (——), 6.25 (------), 6.33 (·—·—·), 6.25 (·····).

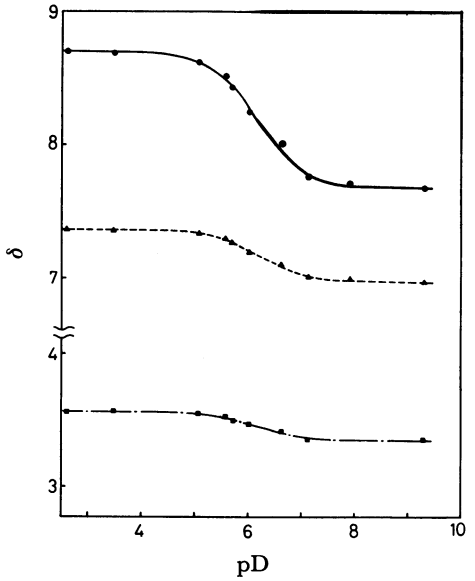
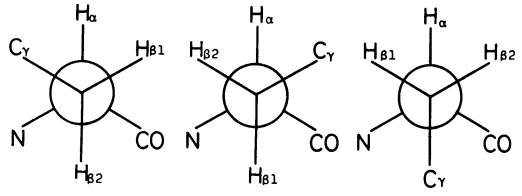


Fig. 3. Effects of pD on the chemical shifts of the histidyl C-2, C-5 and one of the histidyl β-CH_2 protons of 1.0×10^{-3} mol dm^{-3} *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-). The symbols refer to C-2 (●), C-5 (▲) and low-field resonance of the β-CH_2 (■). The theoretical curves were fitted to the experimental data by a nonlinear least-squares analysis; pK_a*, 6.18 (——), 6.19 (------), 6.15 (-·-·).



I II III

P_I P_{II} P_{III}

Fig. 4. Examples of rotamers possible for the histidine residue. P_I, P_{II}, and P_{III} denote the rotamer populations.

we have assumed that the $J_{\alpha\beta}$ values are weighted averages of different conformations in fast equilibrium and that a distribution is restricted to the three staggered rotamers in Fig. 4 and the two gauche coupling constants are equal. The $J_{\alpha\beta}$ values of the gauche and the trans rotamers were calculated as 3.2 Hz (J_g) and 12.4 Hz (J_t), respectively, from the Karplus-like equation (2) proposed by Kopple *et al.*,[4]

$$J_{\alpha\beta} = 11.0\cos^2\theta - 1.4\cos\theta + 1.6\sin^2\theta, \quad (2)$$

where θ denotes the dihedral angle of H–C$^\alpha$–C$^\beta$–H planes. Then, the following equations will hold.[5]

$$P_I = (J_{\alpha\beta 2}-J_g)/(J_t-J_g) = (J_{\alpha\beta 2}-3.2)/9.2 \quad (3)$$

$$P_{II} = (J_{\alpha\beta 1}-J_g)/(J_t-J_g) = (J_{\alpha\beta 1}-3.2)/9.2 \quad (4)$$

$$P_{III} = 1 - P_I - P_{II}, \quad (5)$$

where $J_{\alpha\beta 1}$ and $J_{\alpha\beta 2}$ signify the H–C$^\alpha$–C$^\beta$–H$_{\beta 1}$ and the H–C$^\alpha$–C$^\beta$–H$_{\beta 2}$ protons coupling constants, respectively, and P_I, P_{II}, and P_{III} denote the rotamer populations as shown in Fig. 4. P_I, P_{II}, and P_{III} were determined from Eqs. 3—5 by using the experimental values of $J_{\alpha\beta 1}$ and $J_{\alpha\beta 2}$, assuming that the resonance at higher field was that of H$_{\beta 2}$.[6] The observed coupling constants and the estimated rotamer populations are summarized in Table 1.

Temperature Dependence of Chemical Shifts in DMSO-d_6. The temperature coefficients ($-d\delta/dT$) of the main-chain amide and the side-chain Acm amide protons were measured in the temperature range 292—332 K in DMSO-d_6 solution as shown in Table 2, in order to know which protons were shielded from the solvent. One can recognize easily that only the cysteinyl peptide bond proton of the cyclic peptide has the fairly small temperature coefficient (0.0014 ppm deg^{-1}), as compared with the others (0.0028—0.0043 ppm deg^{-1}). In addition, the chemical shift of this proton at 292 K (7.43 ppm) is significantly smaller than that found in an ordinary random-coiled peptide (8.29 ppm for cystine).[7]

Conformation of Cyclic Peptide **2** *in DMSO.* As the cyclic peptide in this study is presumed to take a fairly rigid skeleton conformation, the observed H–N–C$^\alpha$–H protons coupling constant ($J_{HN\alpha}$) would be interpreted in terms of a single conformation. Therefore, the dihedral angle (θ') of H–N–C$^\alpha$–H planes can be estimated from the $J_{HN\alpha}$ value by using the following Karplus-like equation (6) proposed by Ramachandran *et al.*,[8]

$$J_{HN\alpha} = 7.9\cos^2\theta' - 1.5\cos\theta' + 1.3\sin^2\theta'. \quad (6)$$

The conventional ϕ values ($\theta'=|\phi-60°|$ for L-amino acid and $\theta'=|\phi+60°|$ for D-amino acid) can be obtained from the θ' values.[9] In general, two θ' values are possible for a given $J_{HN\alpha}$ value and two ϕ values are possible for a given θ' value, and thus at most four ϕ values are possible. Accordingly, one must estimate the θ' and ϕ values by building a model of the cyclic peptide consistent with the requirement of the Corey-Pauling-Koltun (CPK) molecular model, assuming that all the peptide bonds take the trans conformation ($\omega=+180°$[9]). Thus, the experimentally obtained $J_{HN\alpha}$ values are shown in Table 3.

Effect of Cu(II) on ^1H *NMR Spectra.* In the present study, the effect of Cu(II) addition has been also examined. As Cu(II) has an unpaired electron extremely fast longitudinal and transverse relaxations of the protons situated near Cu(II) will be expected. Accordingly, the signals which are broadened on addition of trace amounts of Cu(II) will be those of the protons located near Cu(II). Of course, the broadenings will be somewhat ascribed to the conformational changes of the molecules caused by the complexation with Cu(II) in the high concentration range of Cu(II). Figure 5 indicates ^{1}H NMR spectral change of the cyclic peptide caused by adding Cu(II) in D_2O solution. There is a tendency that the histidyl C-2, C-5 and β-CH_2 proton signals are markedly broadened on addition of Cu(II) while those of the Acm–CH_2 and Acm–CH_3

Table 1. Conformational parameter of histidyl side chain of *cyclo*(–L-Cys(*S*-Acm)–D-Leu–L-His–L-Cys(*S*-Acm)–D-Leu–L-His–) in D_2O

pD	$J_{\alpha\beta 1}$[a] / Hz	$J_{\alpha\beta 2}$[a] / Hz	P_I[b]	P_{II}[b]	P_{III}[b]
3.28	3.2	11.7	0.92	0.00	0.08
8.40	3.6	9.9	0.73	0.04	0.23

a) H–C$^\alpha$–C$^\beta$–H coupling constants. $\beta 1$ and $\beta 2$ denote H$_{\beta 1}$ and H$_{\beta 2}$ as shown in Fig. 4. b) Rotamer populations as shown in Fig. 4, assuming that histidyl imidazole is in fast equilibrium among three staggered rotamers.

Table 2. Main chain amide and side chain acetamido proton resonances of linear and cyclic pertides in dimethyl sulfoxide

	Linear peptide[a]			Cyclic peptide[a]	
	δ[b]	$-d\delta/dT$[c] / ppm deg^{-1}		δ[b]	$-d\delta/dT$[c] / ppm deg^{-1}
His_I	8.37	0.0040	His	8.65	0.0033
His_{II}, Cys_{II}	8.24	0.0040	Leu	8.43	0.0043
Leu_I, Leu_{II}	8.01	0.0028	Cys	7.43	0.0014
Cys_I	6.93	0.0035	Acm	8.53	0.0035
Acm_I, Acm_{II}	8.55	0.0035			

a) Linear and cyclic peptides are represented as Boc–L-Cys_I(*S*-Acm_I)–D-Leu_I–L-His_I–L-Cys_{II}(*S*-Acm_{II})–D-Leu_{II}–L-His_{II}–OCH_3 and *cyclo*(–L-Cys(*S*-Acm)–D-Leu–L-His–L-Cys-(*S*-Acm)–D-Leu–L-His–), respectively. b) Chemical shifts-referred to the partially deuterated dimethyl sulfoxide (2.49 ppm from DSS) at 292 K. c) Temperature coefficients in the temperature range 292—332 K.

Table 3. Conformational parameters of main chain of *cyclo*(–L-Cys(*S*-Acm)–D-Leu–L-His–L-Cys(*S*-Acm)–D-Leu–L-His–) in dimethyl sulfoxide

	$J_{HN\alpha}$[a] / Hz	θ'[b] / deg	ϕ[c] / deg
His	6.7	143	−83
Leu	4.3	125	+65
Cys	6.7	143	−157

a) H–N–C$^\alpha$–H coupling constant at 322 K. b) Dihedral angle of H–N–C$^\alpha$–H planes estimated from $J_{HN\alpha}$ value. c) Conventional angle obtained from θ' according to the IUPAC-IUB convention.[9]

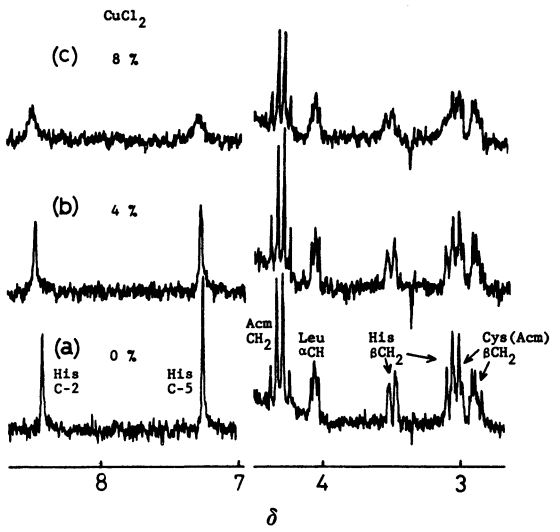


Fig. 5. Effect of $CuCl_2$ addition on the 300 MHz ^1H NMR spectrum of 1.0×10^{-3} mol dm^{-3} *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-) in D_2O solution at pD 5.70. Contents of $CuCl_2$ (mol%) are (a) 0%, (b) 4%, and (c) 8%.

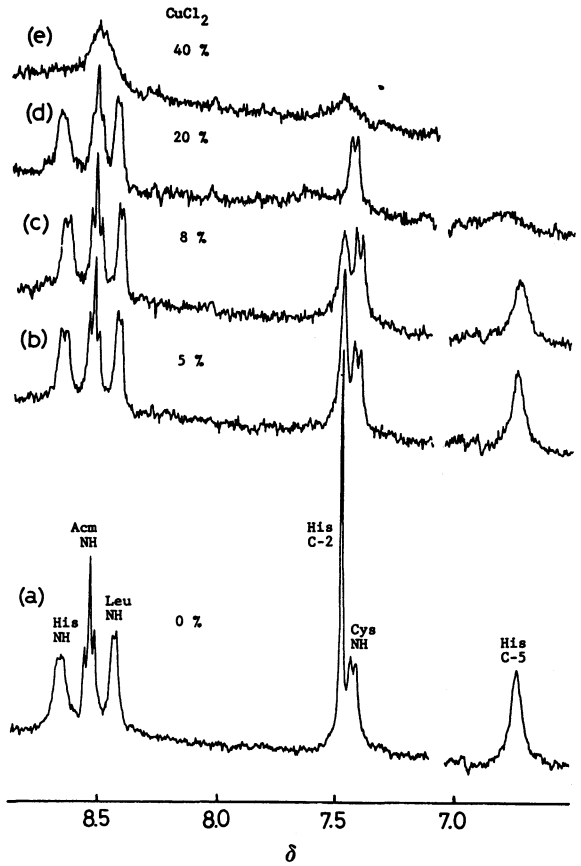


Fig. 6. Effect of $CuCl_2$ addition on the 300 MHz ^1H NMR spectrum of 2.4×10^{-3} mol dm^{-3} *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-) in DMSO-d_6 solution. Contents of $CuCl_2$ (mol%) are (a) 0%, (b) 5%, (c) 8%, (d) 20%, and (e) 40%.

(not shown in Fig. 5) proton signals are not broadened significantly compared to the histidyl proton signals. Figure 6 shows the change of ^1H NMR spectrum on addition of Cu(II) in DMSO-d_6. The signals of the histidyl C-2 and C-5 protons are broadened or disappear, whereas those of the Acm amide proton and the Acm-CH_2 and the Acm-CH_3 protons (not shown in Fig. 6) are affected much less compared with those of the histidyl protons.

Discussion

The ^1H NMR spectrum of the cyclic hexapeptide (Fig. 1 (b)) clearly indicated that it takes a C_2 symmetric conformation on the NMR time scale, since the same kind of amino acid residues in the molecule shows the same chemical shift. In contrast to this, the spectrum of the linear peptide (Fig. 1 (a)) is more complex, because the two identical amino acid residues are situated in different environments in the molecule. The large difference of the chemical shifts between the two protons of the Acm-CH_2 in the cyclic peptide can be attributed to the environmental difference of these protons probably caused by the particular orientation of the Acm-group in D_2O. This is in striking contrast with the behavior of the Acm-CH_2 protons in DMSO-d_6 solution, where these protons appear to have the same chemical shift (not shown in a figure).

The difference of the pK_a* values of the histidyl imidazolyl groups between the linear (6.25 and 6.40) and the cyclic (6.18) peptides seems to be due to the conformational difference between these two peptides. The two pK_a* values, 6.25 and 6.40, in the linear peptide must reflect the environmental difference between the two histidyl imidazoles, one situated in the C-terminal and the other in the middle of the molecule. In the present study, however, it is impossible to determine which of the histidines corresponds to each of the pK_a* values. On the other hand, the cyclic peptide has only one pK_a* value, 6.18, indicating the identical environment of the two histidine residues.

We have estimated the rotamer populations of the histidyl side chain for the cyclic peptide from the H-C^α-C^β-H protons coupling constant, $J_{\alpha\beta}$. One can see readily from Table 1 that the population of the rotamer I is predominant in both acidic and basic solutions, which indicates that the histidyl imidazole is not folded on the cyclic molecular plane but extended outward. It is generally known that in cyclic dipeptides aromatic side chain is frequently folded but in larger cyclic peptides such folding is rare[10] and that histidyl imidazole has a tendency to orient itself toward the adjacent amide NH group of the peptide backbone.[6] These tendencies are consistent with the results in the present study. It has been also reported that in the case of histidyl side chain the torsion angle χ_2[9] is apt to be around 90°.[6] The CPK molecular model built according to all these results shows that the histidyl imidazolyl plane faces toward the adjacent leucyl side chain, and so the resonances of the leucyl side chain are expected to be shifted to higher field compared with those found in ordinary peptides owing to the imidazole ring current field effect. This inference is supported by the experimental results that the chemical shifts of the leucyl $\beta CH_2+\gamma CH$ (*ca.* 1.47 ppm) and δCH_3 (*ca.* 0.76

ppm) at pD 6.22 of the cyclic peptide are smaller than the chemical shifts (*ca.* 1.6 ppm and *ca.* 0.9 ppm, for the leucyl $\beta CH_2+\gamma CH$ and δCH_3, respectively) of random-coil proteins and peptides reported by McDonald *et al.*[11]

As mentioned in the Results section, the cysteinyl peptide bond proton of the main chain in the cyclic peptide has the temperature coefficient (0.0014 ppm deg^{-1}) strikingly lower than the others (0.0028—0.0043 ppm deg^{-1}) and the chemical shift of this proton (7.43 ppm) is located at the magnetic field much higher than that (8.29 ppm for cystine) of an ordinary peptide.[7] The abnormal chemical shifts of amide protons in a strong hydrogen-bonding solvent such as DMSO may be ascribed to the shielding of the amide protons from the solvent by intramolecular hydrogen bonds or other causes.[10,12-17] Some cyclic hexapeptides form transannular intramolecular hydrogen bonds,[10,12-17] and thus it seems very likely that the cyclic hexapeptide in the present study forms the similar transannular hydrogen bonds between the cysteinyl peptide bonds. This will suggest that two β-turns exist in the D-leucyl-L-histidy sequences. In proteins and naturally occurring or synthetic peptides, proline residues are frequently contributed to β-turn conformations.[18] The cyclic hexapeptide we studied has no proline residues. This finding is thus in contrast of our expectation and noteworthy. The cyclic peptide we studied is composed of the amino acid residues all having the large side chains, which seem to prevent β-turn formation owing to steric hindrance. The orientations of the side chains may happen to be favorable for β-turn formation in the D-leucyl-L-histidyl sequence.

Table 4. Chemical shift of leucyl side chain (δCH_3) in various environment[a]

	δ
Boc-D-Leu-OH	0.88
	0.86
	0.85
	0.82
F_3C-CO-Gly-Gly-L-Leu-L-Ala-OCH_3[b]	0.89
	0.85
Boc-D-Leu-L-His-OCH_3	0.83
	0.81
	0.80
	0.79
Boc-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-	0.81
D-Leu-L-His-OCH_3	0.79
	0.77
cyclo(-L-Cys(*S*-Acm)-D-Leu-L-His-L-	0.78
Cys(*S*-Acm)-D-Leu-L-His-)	0.76
	0.73
	0.71

a) Chemical shifts corresponding to all signals observed apparently are described here, except for the tetrapeptide. b) Random coiled tetrapeptide investigated by Bundi *et al.*[7]

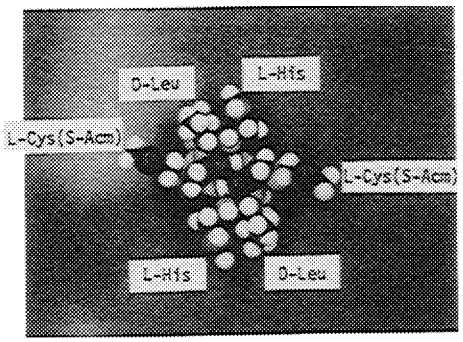


Fig. 7. Corey-Pauling-Koltun (CPK) model of the conformation proposed for *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-) in DMSO.

The CPK molecular model of the cyclic peptide was built in view of the θ' values derived from the Eq. 6, the transannular hydrogen bonds between the two cysteinyl peptide bonds and the conformational maps proposed by Venkatachalam.[19] We have assumed here that all the peptide bonds take trans conformation ($\omega = +180°$[9]). This assumption seems to be reasonable for the present study, although some deviations from the standard planar trans conformation due to steric hindrance are possible. We have assumed further that the histidyl imidazolyl plane in DMSO solution is apt to be orientated toward the same direction as that in D_2O solution (Table 1). This assumption also seems to be reasonable, since the leucyl δCH_3 proton signals in DMSO-d_6 are shifted to higher field when the leucine residue is situated in D-leucyl-L-histidyl sequence as is shown in Table 4. It is considered that θ' obtained in this way represents an approximate value, since there is some uncertainty in the analysis of $J_{HN\alpha}$. Thus, the error in θ' seems to be about $\pm 10°$.[20,21] The conformation proposed in the present study is shown in Fig. 7, and the estimated θ' and ϕ values are summarized in Table 3.

As is indicated in the Results section, the histidyl imidazoles seem to be mainly coordinated to Cu(II). In order to investigate the complexation quantitatively we have further estimated the dissociation rate constant of the Cu(II)-peptide complex. Figure 8 shows the relation between the reciprocal of the transverse relaxation time ($1/T_{2A}$) of the histidyl C-2 and C-5 protons

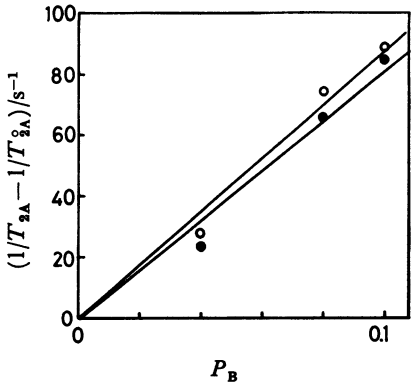


Fig. 8. Paramagnetic effect of Cu(II) on the transverse relaxation rates of the histidyl C-2 (—○—) and C-5 (—●—) proton resonances of *cyclo*(-L-Cys(*S*-Acm)-D-Leu-L-His-L-Cys(*S*-Acm)-D-Leu-L-His-). P_B denotes the mole fraction of the Cu(II)-peptide complex, and T_{2A} and T_{2A}° denote the transverse relaxation times in the presence and absence of Cu(II), respectively.

and the mole fraction of the complex (P_B) in D_2O solution at pD 5.7. $1/T_{2A}$ is obtained experimentally from the following equation (7),

$$1/T_{2A} = \pi\Delta\nu_{1/2}, \quad (7)$$

where $\Delta\nu_{1/2}$ denotes half-band-width of a signal. In the present experimental condition the following equation (8) holds approximately,[22]

$$1/T_{2A} - 1/T^{\circ}_{2A} = P_B/\tau_B, \quad (8)$$

where T_{2A} is the transverse relaxation time of the histidyl C-2 or C-5 proton in the presence of Cu(II), T°_{2A} is that in the absence of Cu(II), and τ_B is the lifetime of the complex. From Fig. 8 we can obtain the dissociation rate constant ($k_d = 1/\tau_B$) of the Cu(II)–peptide complex as to be *ca.* $1 \times 10^3\ s^{-1}$.

In conclusion, the findings we obtained are significantly noteworthy in that D-leucyl-L-histidyl sequence is liable to form β-turn in a cyclic hexapeptide, since this type of peptide sequence in β-turn has never been found. It was also found that Cu(II) interacts with histidyl imidazoles but not with Acm groups, perhaps owing to the conformational rigidity of the cyclic peptide. We are now continuing to investigate other cyclic hexapeptides having sequences such as X-D-leucyl-L-histidyl, X-glycyl-L-histidyl, *etc.* in order to confirm the formation of β-turn by D-leucyl-L-histidyl sequence.

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