  Bull. Chem. Soc. Jpn., 55, 3817—3823 (1982)

Multiple Conformations and Conformational Change in the Acetonitrile of Cation-binding Cyclic Tetrapeptide, Cyclo[Gly-L-Cys(Bzl(OMe))-Sar-L-Pro]

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(Received February 1, 1982)

The conformations of the cyclic tetrapeptide, cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro] (CGCSP), in solution, were studied by means of ^{1}H-, ^{13}C-nuclear magnetic resonance (NMR), and circular dichroism (CD). The CGCSP equilibrates among three conformers, **M**, **m**, and **n**, in acetonitrile, providing three sets of resonances for each proton in the ^{1}H-NMR spectrum. An inspection of a CPK model indicated that the three structures were sterically allowed to occur by the *cis-trans* isomerism around the Cys–Sar and Sar–Pro peptide bond. The temperature dependence of the amide proton resonances in ^{1}H-NMR suggested that one γ-turn (1←3 hydrogen bond from the Cys to the Pro) is present in the two conformers, **M** and **m**. Thus, the three conformations in acetonitrile were deduced from all of the spectral data. This cyclic tetrapeptide is capable of binding a monovalent cation, Li^+, and divalent cations, Mg^{2+}, Ca^{2+}, and Ba^{2+}. Its CD titration curves demonstrated that at least three types of complexes with Ca^{2+} and Ba^{2+} were formed. In contrast, Li^+ and Mg^{2+} were shown to form the 1 : 1 complex with this peptide. The NMR data suggest that the conformational change of the cyclic backbone takes place by changing the geometry of the peptide bonds around the Cys–Sar–Pro sequence on the addition of a metal cation.

In the preceding paper,[1] the conformation of bis-(cyclic tetrapeptide), *S,S'*-Bis[cyclo(Gly–L-hemiCys–Sar–L-Pro)], in solution was investigated using such spectroscopic studies as ^{1}H-NMR, ^{13}C-NMR, Raman, and CD. The proposed conformation in dimethyl-d_6 sulfoxide, which takes a "castanet-type" structure, attracted our attention to the potential activity of this bis(peptide) as an ionophore.

In the present report, the conformations and ion-binding capabilities of the homodetic cyclic tetrapeptide, cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro], in solution, are described on the basis of the results from ^{1}H-NMR, ^{13}C-NMR, and CD measurements. The small cyclic peptides consisting of 4—5 amino acid units have somewhat rigid backbones of the ring. Therefore, a conformational change is unlikely to take place in these small cyclic peptides on the addition of metal cation to the solution. Actually, the ^{13}C-NMR spectrum of a cyclic tetrapeptide, cyclo(Sar–L-Pro–Sar–L-Pro), in acetonitrile afforded a single resonance for each carbon resulting from one conformer. This conformation is retained even on the addition of the magnesium cation.[2] Recent investigations of naturally occurring cyclic tetrapeptide have revealed the importance of the cyclic structure in the evolution of its biological activity.[3,4] The present study describes an investigation of the conformational change on binding to a metal cation by the cyclic tetrapeptide. This is closely connected with biological events by means of some small cyclic molecules in the membrane transport.[5]

Experimental

Synthesis. The synthesis of cyclo[Gly–L-Cys(Bzl(OMe))–Sar–L-Pro] (CGCSP) has been described in the previous paper.[1]

Procedures. The ^{1}H-NMR spectra were recorded at 360.06 MHz on a NICOLET NT-360 spectrometer with a NIC 1180 Computer Data System, operating in the Fourier transform mode. The ^{13}C-NMR spectra were obtained at 20.12 MHz on a Varian CFT-20 spectrometer, in the Fourier transform mode with proton noise decoupling. All the chemical shifts are given in ppm relative to the internal tetramethylsilane.

The circular dichroism (CD) spectra were recorded using a 0.1-cm path length quartz cell on a JASCO J-40A automatic recording spectropolarimeter equipped with a J-DPY data processor at 25 °C. The data were represented as molar ellipticities. The concentrations of the resulting peptide solutions containing salt were 10^{-3}—10^{-4} mol dm^{-3}. Spectrophotometric-grade acetonitrile was used as the solvent. Analytical reagent-grade perchlorates were used as the metal salts.

Results

Backbone of Cyclic Peptide. ^{1}H-NMR. The ^{1}H-NMR spectrum at 360 MHz of CGCSP in acetonitrile-d_3 is presented in Fig. 1. The assignments were confirmed by the spin-decoupling method and by comparisons with the precursor linear tetrapeptides. It may be seen that the spectrum contains three sets of ABMX-spin systems for the cysteinyl residue and three sets of the ABX systems for the glycyl residue. This finding demonstrates that three species (**M**, **m**, and **n**), each having well-defined, highly populated conformational states, exist on the NMR time scale. From the relative intensities of the Cys and the Gly amide proton signals, the populations of **M**, **m**, and **n** are estimated to be 50, 30, and 20% respectively. The chemical shifts for the three sets of signals and related coupling constants are summarized in Table 1. All the coupling constants were experimentally obtained and optimized by computer simulations. In order to determine the accessibility of each amide NH proton to the solvent, the temperature dependences of the Gly and the Cys NH resonances in **M**, **m**, and **n** (a total of six NH resonances) were measured. The results are shown in Fig. 2. The temperature coefficients of the three cysteinyl amide NH signals, A, B, and F, are zero or small ($d\delta(dT)^{-1}$=0.00(A), 0.00(B), and 2.33 ppm deg^{-1} (F)). In contrast, those of the remaining three glycyl amide NH resonances, C, D, and E, are larger than those of the A, B, and F signals

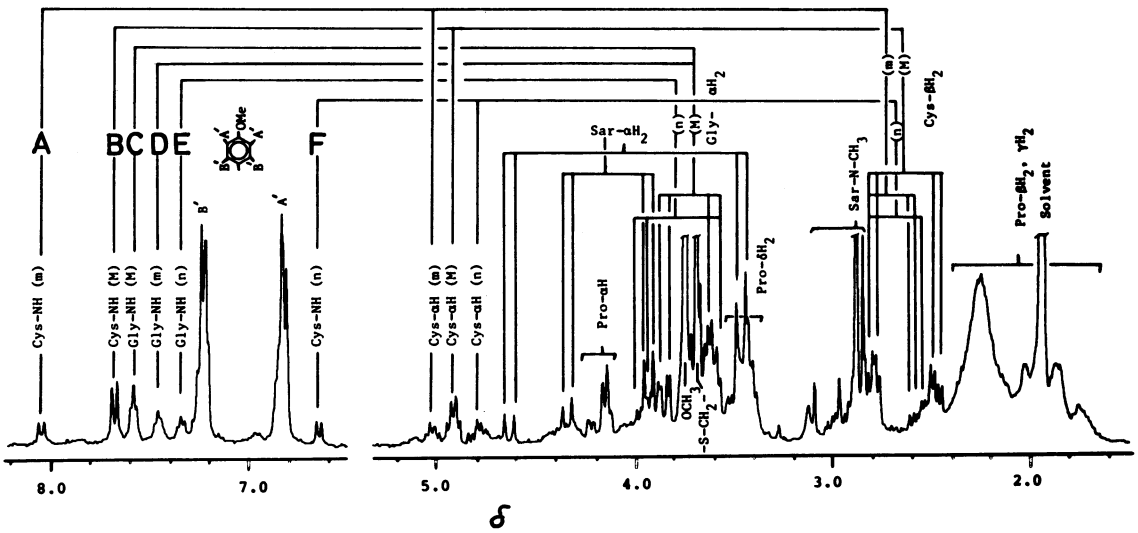

Fig. 1. ^{1}H-NMR spectrum (360 MHz) of CGCSP in CD_3CN at 20°C. Concentration; 25 g dm^{-3}

TABLE 1. PARAMETERS FOR THE THREE DIFFERENT CONFORMERS (**M**, **m**, AND **n**) OBSERVED IN A SOLUTION OF CGCSP IN CD_3CN AT 20 °C[a)]

Residue	Type of NMR spectrum	Conformation		
		M	**m**	**n**
Gly	ABX	$\delta_A=3.62$, $J_{AB}=-16.11$	$\delta_A=3.64$, $J_{AB}=-16.11$	$\delta_A=3.62$, $J_{AB}=-16.14$
		$\delta_B=3.87$, $J_{AX}=4.65$	$\delta_B=3.87$, $J_{AX}=5.90$	$\delta_B=3.97$, $J_{AX}=4.65$
		$\delta_X=7.62$, $J_{BX}=6.32$	$\delta_X=7.50$, $J_{BX}=7.01$	$\delta_X=7.37$, $J_{BX}=6.16$
Cys(Bzl(OMe))[b)]	ABMX	$\delta_A=2.48$, $J_{AB}=-13.76$	$\delta_A=2.59$, $J_{AB}=-13.53$	$\delta_A=2.55$, $J_{AB}=-13.88$
		$\delta_B=2.79$, $J_{AM}=6.83$	$\delta_B=2.79$, $J_{AM}=7.07$	$\delta_B=2.80$, $J_{AM}=5.96$
		$\delta_M=4.93$, $J_{BM}=6.95$	$\delta_M=5.04$, $J_{BM}=6.90$	$\delta_M=4.80$, $J_{BM}=6.90$
		$\delta_X=7.69$, $J_{MX}=9.53$	$\delta_X=8.05$, $J_{MX}=9.78$	$\delta_X=6.66$, $J_{MX}=8.35$
Sar	A_3PQ	$\delta_A=2.89$, $J_{PQ}=-16.87$	$\delta_A=2.89$, $J_{PQ}=-16.60$	$\delta_A=2.85$, $J_{PQ}=-16.87$
		$\delta_P=3.47$	$\delta_P=3.94$	$\delta_P=3.47$
		$\delta_Q=4.66$	$\delta_Q=4.36$	$\delta_Q=4.66$
Pro	ABCDPQX	$\delta_A=$, $\delta_B=$, $\delta_C=$ 1.7—2.1	$\delta_A=$, $\delta_B=$, $\delta_C=$ 1.7—2.1	$\delta_A=$, $\delta_B=$, $\delta_C=$ 1.7—2.1
		$\delta_D=2.15$—2.40	$\delta_D=2.15$—2.40	$\delta_D=2.15$—2.40
		$\delta_P=$, $\delta_Q=$ 3.35—3.55	$\delta_P=$, $\delta_Q=$ 3.35—3.55	$\delta_P=$, $\delta_Q=$ 3.35—3.55
		$\delta_X=4.15$	$\delta_X=4.16$	$\delta_X=4.1$—4.25

a) Chemical shift, δ, coupling constant, J/Hz. The coupling constants, J, are to within $\pm$0.5 Hz. b) Signals for the protons of the *p*-methoxybenzyl group were omitted.

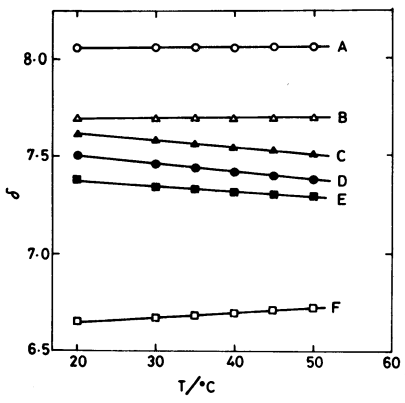

Fig. 2. Temperature dependence of the Gly and Cys amide proton chemical shifts of CGCSP in CD_3CN.

($d\delta(dT)^{-1}=-3.97$(C), -4.00(D), and -3.00 ppm deg^{-1} (E)). According to the usual criteria of accessibility determined from the temperature dependence of the NH's, the Cys NH proton in **M** and **m** is probably buried in the molecule or is hydrogen-bonded. The Gly NH proton is assumed to be exposed to the solvent in all three species.

Next, two resonances for the Sar N–CH_3 protons occur at 2.85 ppm and 2.89 ppm, with the relative intensity of 4 : 1 in acetonitrile-d_3. Solvent-titration experiments were performed in an attempt to distinguish the Sar N–CH_3 resonances derived from a trans Cys–Sar peptide bond from that derived from a cis Cys–Sar bond. First, the ^{1}H-NMR spectra of the cyclic tetrapeptide in a chloroform-*d* solution were examined, while benzene-d_6 was added to bring the total up to 30% (by

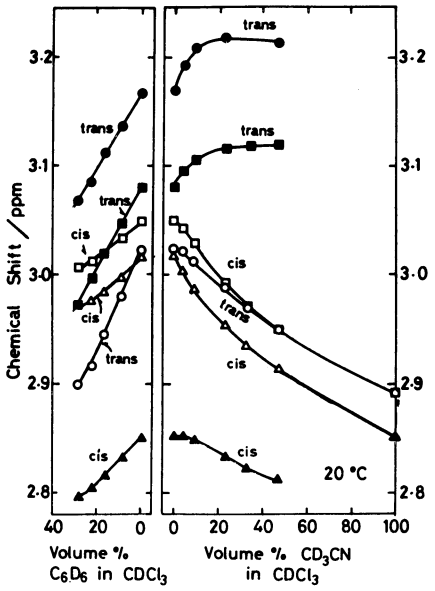

Fig. 3. Upfield shifts for N–CH_3 signals of CGCSP as a function of the volume fraction of C_6D_6 in $CDCl_3$–C_6D_6 solvent mixtures (left) and the shift of N–CH_3 signals as a function of the volume fraction of CD_3CN in $CDCl_3$–CD_3CN solvent mixtures (right). —●—: a, —■—: b, —□—: c, —○—: d, —△—: e, —▲—: f.

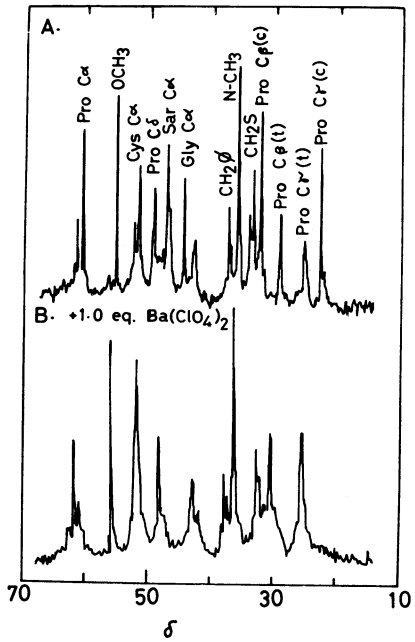

Fig. 4. ^{13}C-NMR spectrum (20 MHz) of CGCSP in CD_3CN. The peptide concentration was 0.12 mol dm^{-3}. The spectra are (A) free peptide and (B) barium perchlorate added to a concentration of 0.12 mol dm^{-3} (molar ratio of peptide: salt, 1: 1).

volume). The addition of benzene gave rise to neither any significant change in the vicinal coupling constants of the Gly or the Cys residue nor that of the intensity of six Sar N–CH_3 resonances. At the same time, the chemical shifts of N–CH_3 signals changed monotonically with the increase in the volume fraction of benzene. These observations, therefore, established that no conformational changes in CGCSP were induced by benzene. The addition of benzene to chloroform induces six N–CH_3 signals to move upfield. The profiles of the upfield shifts for the N–CH_3 signals are shown in Fig. 3 as a function of the volume fraction of benzene-d_6. The a, b, and d signals have larger benzene-induced upfield shifts than those of the c, e, and f signals. These two different tendencies of upfield shifts for N–CH_3 signals are considered to reflect the different local geometries of the Sar N–CH_3 group and the Cys carbonyl group, namely, a cis Cys–Sar peptide bond or a trans Cys–Sar bond. Therefore, an established method based on the benzene-induced shift concerning the assignment of the N–CH_3 signal of cyclo(Sar)$_4$[6] or cyclo(L-Pro–Sar–Gly)$_2$[7] is applicable in this case. Thus, the a, b, and d resonances in a chloroform solution are assigned to the N–CH_3 protons involved in the trans Cys–Sar peptide bond, while the remaining resonances, c, e, and f, are attributable to those involved in the cis Cys–Sar bond.

Second, the ^{1}H-NMR spectra of the cyclic tetrapeptide were examined in a chloroform-*d*–acetonitrile-d_3 mixed solvent system. In Fig. 3, the chemical shifts of the Sar N–CH_3 signals are plotted as a function of the acetonitrile fraction in a volume. Two signals, a and b, move lowfield, while the f signal reveals an upfield shift, as the concentration of acetonitrile increases. At the same time, the three signals were accompanied by a decrease in the intensity. Finally, in the 1 : 1 mixture of a chloroform–acetonitrile solvent system by volume, these three signals, a, b, and f, almost disappeared. On the other hand, the c signal, associated with the cis Cys–Sar peptide bond, shifts upfield with an increase in the intensity. At the same time, the d signal, attributed to the trans Cys–Sar bond, and the e signal, attributed to the cis Cys–Sar bond, weakened in intensity with the upfield shifts. On the basis of these observations, it is confirmed that the signal at 2.89 ppm in acetonitrile contains two N–CH_3 resonances involved in the cis and trans Cys–Sar peptide bonds, while the resonance at 2.85 ppm is associated with the cis Cys–Sar peptide bond. From the relative intensity of the Gly and the Cys NH proton resonances, it is assumed that the conformers, **M**, **m**, and **n**, contain cis, trans, and cis Cys–Sar peptide bonds respectively.

^{13}C-NMR. The upfield regions of the ^{13}C-NMR spectrum at 20 MHz of CGCSP in acetonitrile-d_3 are shown in Fig. 4A. The assignments of the resonances were confirmed by examining ^{1}H-coupled and selectively ^{1}H-decoupled spectra and by comparisons with the spectra of related compounds. Further conformational features of **M**, **m**, and **n** other than those from ^{1}H-NMR observations can not be inferred from this ^{13}C-NMR spectrum. However, rotational isomers around the Sar–Pro peptide bond can be discriminated by the resulting separate chemical shifts of the Pro C_β and Pro C_γ carbons.[8] The two chemical shifts of the signal at 32.8 ppm for the Pro C_β and at 23.1 ppm for the Pro C_γ are indicative of the presence of the cis Sar–Pro bond. Moreover, the other two signals, at 29.6 ppm for the Pro C_β and at 25.6 ppm for the Pro C_γ, are within the region associated with the trans Sar–Pro bond. The

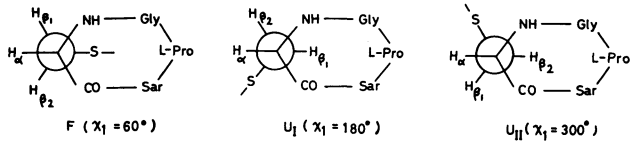

Fig. 5. Schematic representation of three rotational isomers about C_α–C_β bond of side chain of CGCSP.

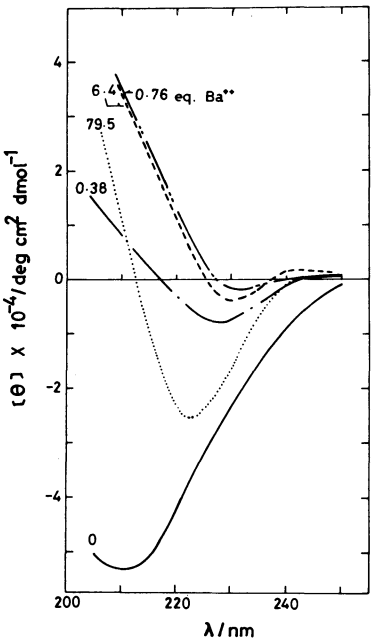

Fig. 6. CD spectral change of CGCSP in CD_3CN as barium perchlorate added.
Molar ratio of $Ba(ClO_4)_2$ to CGCSP; ——: 0, —·—: 0.38, —··—: 0.76, – – –: 6.4, ·····: 79.5. Peptide concentration; 3.37×10^{-4} mol dm^{-3}.

TABLE 2. SIDE-CHAIN CONFORMATIONS OF THE CYSTEIN RESIDUE IN CGCSP IN CD_3CN

Species	Proportion[a]/%		
	F	U_I (or U_{II})	U_{II} (or U_I)
M	21.7	39.7	38.6
m	20.0	40.8	39.2
n	30.1	30.7	39.2

a) Calculated from observed J_{AM} and J_{BM} using the following relationship; $U_I=[J_{AM}(\text{or } J_{BM})-J_g]/(J_t-J_g)$, $U_{II}=[J_{BM}(\text{or } J_{AM})-J_g]/(J_t-J_g)$, $F=1-(U_I+U_{II})$ where $J_t=13.56$, $J_g=2.60$ Hz.

relative intensity of the two Pro C_β and two Pro C_γ resonances show clearly the presence of 70—80% of the cis Sar–Pro peptide bond and that of 20—30% of the trans Sar–Pro bond.

Side Chain of Cyclic Peptide. ^1H-NMR. It is assumed that the orientation of the cysteinyl side chain from C_β carbon on (C_β–S–Bzl(OMe)) occurs in an extended chain with a lower potential energy. Therefore, the conformation of the side-chain group C_α–C_β–S–Bzl(OMe), is essentially determined by the rotation about the C_α–C_β bond of the cysteinyl residue. This implies that the side-chain group exists in the three energetically stable rotational isomers (Fig. 5). One is

TABLE 3. CD EXTREMA FOR CGCSP AT 25 °C

Solvent	M_θ[a] / deg cm^2 dmol^{-1}	λ / nm
Water–methanol (9 : 1, by volume)	−47500	217
Methanol	−71700	213
Acetonitrile	−53400	212
Acetonitrile+$Ba(ClO_4)_2$(0.5 : 1)[b]	1200	244
	−2500	232
	>43000	<205
Acetonitrile+$Ba(ClO_4)_2$ (1 : 1)[b]	600	244
	−2500	232
	>44000	<205
Acetonitrile+$Ba(ClO_4)_2$ (79.5 : 1)[b]	−25300	223
	>35000	<205
Acetonitrile+$Ca(ClO_4)_2$ (0.5 : 1)[b]	−20200	215
Acetonitrile+$Ca(ClO_4)_2$ (1 : 1)[b]	−14000	222
Acetonitrile+$Ca(ClO_4)_2$ (163 : 1)[b]	−67400	211
Acetonitrile+$Mg(ClO_4)_2$ (0.44 : 1)[b]	−42600	214
Acetonitrile+$Mg(ClO_4)_2$ (1 : 1)[b]	−27600	217
Acetonitrile+$LiClO_4$ (324 : 1)[b]	−25000	219
Acetonitrile+$NaClO_4$ (225 : 1)[b]	−23800	220

a) M_θ: Molar ellipticity. b) Salt to peptide molar ratio.

the folded conformation (F), while the others are unfolded ones (U_I and U_{II}), which rapidly interconvert on the NMR time scale. From the Pachler equation[9] using the vicinal coupling constants, J_{AM} and J_{BM}, in Table 1, the ratio of the presence of the three rotamers was evaluated; it is shown in Table 2. A folded conformation (F) in which the aromatic ring of the side chain is positioned over the cyclic backbone of peptide, is more favored in **n** than in **M** and **m**.

Ion Binding. Circular Dichroism (CD): A solution of CGCSP in acetonitrile was titrated with alkali ions (Li^+ and Na^+) and alkaline earth ions (Mg^{2+}, Ca^{2+}, and Ba^{2+}) while the circular dichroism (CD) spectra were examined. The extrema of the CD spectrum of free cyclotetrapeptide in different solvents and that of complexed peptide with various cations in acetonitrile are summarized in Table 3. The CD spectrum of the free cyclic peptide shows a negative band at 212 nm in acetonitrile (Fig. 6). Common features are observed for the CD spectra of the free peptide in water–methanol (9 : 1, by volume) and in methanol. This finding suggests that there are no significant differences among the conformations of the peptide backbone in these solvents. Upon the addition of metal ions to the solution of the cyclic peptide in acetonitrile, remarkable changes take place, particularly when the divalent cations are added. In the case of Ba^{2+} addition (Fig. 6), the minimum observed for the free peptide increases until the molar ratio of peptide to salt is 2 : 1 stoichiometry ("peptide-sandwich type"). After that, the curves display no drastic change upon the addition of salt until the molar ratio is *ca.* 10. This second step of complexing corresponds to the formation of a 1 : 1 complex (cyclopeptide–Ba^{2+}). At a high salt concentration above this point, the CD spectrum shows a decrease in the minimum which appeared in the presence of 10 equiv. of Ba^{2+}. This finding suggests the appearance of the third complex of Ba^{2+}–cyclopep-

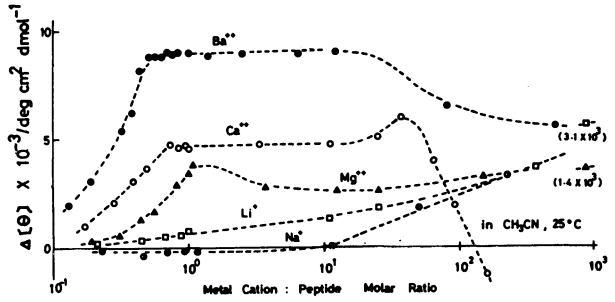

Fig. 7. Titration Curves of CGCSP for several salts in CH_3CN.
Peptide concentration: 3.37×10^{-4} mol dm^{-3}.

Table 4. Equilibrium constants of CGCSP to metal cations in acetonitrile at 25 °C

Cation[a)]	Ionic diameter/Å	K_1[b)] mol^{-1} dm^3	$K_{1/2}$[b)] mol^{-2} dm^6
Li^+	1.20	$<5\times10^2$	
Na^+	1.90	≈0	
Mg^{2+}	1.30	1×10^5	
Ca^{2+}	1.98		9×10^7
Ba^{2+}	2.70		1.7×10^8

a) Anion: perchlorate. b) Estimated error in equilibrium constants, ±20%.

tide–Ba^{2+} with 1 : 2 stoichiometry ("metal cation-sandwich type"). Titration curves were, therefore, depicted in Fig. 7 for the several divalent cations (Ba^{2+}, Ca^{2+}, and Mg^{2+}) and the monovalent cation (Li^+ and Na^+). Then, $\Delta[\theta]$ at 210 nm was plotted against the molar ratio of peptide to the metal cation, where $\Delta[\theta]$ indicates the difference between the molar ellipticity, $[\theta]$, of a peptide plus an ion and that of a free peptide. The curve obtained for Ca^{2+} clearly indicates at least three inflections. This finding demonstrates that at least three types of complexing occurred with the variation of the salt-to-peptide molar ratio. On the other hand, judging from the monotonous increment of $\Delta[\theta]$, only one type of complex with 1 : 1 stoichiometry is assumed to be formed when Li^+ or Mg^{2+} is added.

Equilibrium constants for the first complexed species with several cations were calculated from the CD titration data. For the "peptide-sandwich type" complexes with Ca^{2+} and Ba^{2+}, the following equilibrium constants were defined in terms of the concentrations;

$$K_{1/2} = [P_2C][P]^{-2}[C]^{-1}.$$

For 1 : 1 complexes in the presence of Li^+, Na^+, and Mg^{2+}, K_1 is defined as:

$$K_1 = [PC][P]^{-1}[C]^{-1},$$

where P_2C denotes a sandwich complex; PC, a 1 : 1 complex; P, a peptide, and C, cation. The degree of complexation was determined from the molar ellipticity at a certain wavelength and at a certain salt concentration, on the assumption that the value of ellipticity is the sum of that for free cyclic peptide and that for the complexed peptide present. The calculation of the equilibrium constants were based on the principle reported by Deber *et al.*[10] The results are shown in Table 4. One characteristic feature of the cation-binding properties of this peptide is that it binds divalent cations in preference to monovalent cations. The constant for the formation of a 1 : 1 complex with Mg^{2+} is about 10^3—10^5 fold those of Li^+ or Na^+.

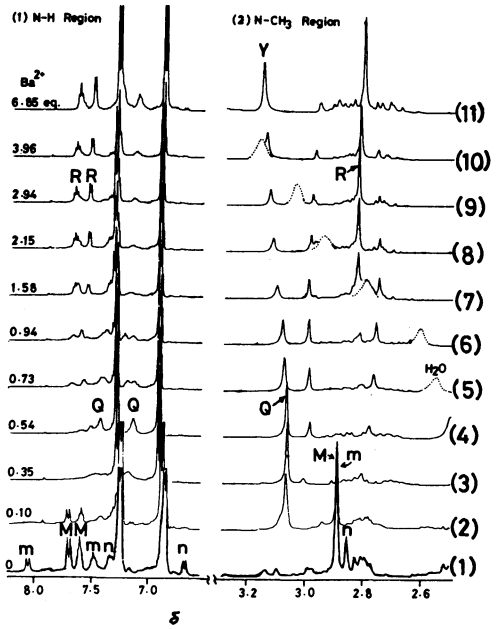

Fig. 8. ^{1}H-NMR spectra of NH and N–CH_3 regions of CGCSP in CD_3CN as $Ba(ClO_4)_2$ is added.
Molar ratio of $Ba(ClO_4)_2$ to CGCSP: (1) 0, (2) 0.10, (3) 0.35, (4) 0.54, (5) 0.73, (6) 0.94, (7) 1.58, (8) 2.15, (9) 2.94, (10) 3.96, (11) 6.85.

^{1}H-NMR and ^{13}C-NMR. In order to elucidate the conformational properties of the cyclic tetrapeptide on complexing with a metal cation, the amide proton and the N–CH_3 proton regions of ^{1}H-NMR were examined in acetonitrile-d_3, titrating with $Ba(ClO_4)_2$. The results are presented in Fig. 8. At least three different types of complex formations can be deduced from the coalescence phenomena of the NH and N–CH_3 proton signals. That is, when $Ba(ClO_4)_2$ was added to the solution, a new signal of N–CH_3 resonance appeared at 3.07 ppm in addition to the signal already present. The signal became pronounced until 0.5 equiv. of salt has been added. The resulting single major resonance at 3.07 ppm for N–CH_3 protons and the two resonances at 7.15 and 7.44 ppm for NH protons are associated with the first complexed species, **Q**. The second complexed species, **R**, which is deduced from the major signal at 2.83 ppm for N–CH_3 protons and the signal at 7.67 and 7.54 ppm for NH protons, appears on the addition of *ca.* 3 equiv. of salt. In the presence of an additional, larger amount of salt, a new resonance, different from that of the second species, appears and grows. This third species, **Y**, is characterized by the signal at 3.18 ppm for N–CH_3 protons. The above results of titration experiments using CD and ^{1}H-NMR leads us to the conclusion that at least three types of complex formations between the cyclotetrapeptide and Ba^{2+} occur in the range of salt concentration of 0—6.85 equiv.

The step-by-step addition of $Ba(ClO_4)_2$ to CGCSP in acetonitrile-d_3 yields ^{13}C-NMR spectra which suggest a convergence to a single conformer. With the increase in the salt concentration, the Pro C_β and the Pro C_γ resonances positioned at the chemical shifts typical of the cis Sar–Pro peptide bond diminished in intensity. The ^{13}C-NMR spectrum in Fig. 4B when 1.0 equiv. of Ba^{2+} is added provides a single resonance for each carbon present in cyclic tetrapeptide. The chemical shifts of the Pro C_β and Pro C_γ resonances (30.5 ppm and 25.4 ppm) are indicative of the presence of the trans Sar–Pro peptide bond (>90%).

Discussion

Free Peptide. If one considers the cis-trans rotational isomers around the Cys$\overset{a}{—}$Sar and Sar$\overset{b}{—}$Pro peptide bond, four distinct conformers are possible; (a-cis, b-cis), (a-cis, b-trans), (a-trans, b-cis), and (a-trans, b-trans). From the results of ^{1}H-NMR, it is deduced that **M** and **n** contain the cis Cys–Sar peptide bond (a-cis). Therefore, the (a-cis, b-cis) and (a-cis, b-trans) sequence of the Cys$\overset{a}{—}$Sar$\overset{b}{—}$Pro peptide linkages must certainly be found in **M** and **n**. If one considers the peptide bond sequence of the Cys–Sar–Pro bond in **m** as (a-trans, b-trans), this is inconsistent with the result from the ^{13}C-NMR data, for it reveals that the cis Sar–Pro peptide bond is superior to the trans bond in population. These considerations enable us to deduce that **m** takes a skeletal conformation containing the (a-trans, b-cis) Cys$\overset{a}{—}$Sar$\overset{b}{—}$Pro peptide linkages. Moreover, the predominance of the cis Cys–Sar bond over the trans bond suggests that the skeletal conformation of the predominant species **M** contains the (a-cis, b-cis) sequence of the Cys–Sar–Pro linkages. The remaining species, **n**, is considered to take a conformation which contains the (a-cis, b-trans) sequence. From a Ramachandran relationship[11] using the vicinal coupling constants (J_{AX}, J_{BX}, and J_{MX}) in Table 1, the conformational angles of the Cys and the Gly residues are calculated:

	M	**m**	**n**
Cys ϕ/°	−120	−120	−139
Gly ϕ/°	128	103	130

Utilizing these conformational parameters, the CPK model construction to be seen in Fig. 9 is sterically allowed. It should be noted that the Cys NH proton is nonlinearly hydrogen-bonded to the Pro C=O oxygen. This finding is supported by the independence of the Cys NH resonance of the temperature. By the same manner as has been described above, **m** is assumed to have one intramolecularly hydrogen-bonded (Pro C=O···Cys NH). This hydrogen bond has the same features as that in **M**. The conformational properties of the several synthetic cyclic tetrapeptide have been studied by the measurement of NMR[12] and by X-ray analysis.[13,14] However, most of these peptides have the simple ^{1}H-NMR spectrum of one conformer in solution. On the contrary, it is surprising that the NMR studies of the present peptide revealed the coexistence of three species with potentially permitted conformational states. This result is closely related to the conformational properties of peptide linkage. Since typical values of an energy barrier to rotation about the peptide bond are within 20 ± 1 kcal mol^{-1},[15] the presence of cis and trans N-substituted peptide bonds can generate different cyclic conformations. In addition, the rotational cis-trans isomerism about two N-substituted peptide bonds in CGCSP is slow on the NMR time scale. Thus, it permits us to observe separate resonances for each proton of the different cyclic conformers. This cis-trans isomerism can often be observed about X-imino acid (X; any amino acids) peptide bonds in cyclic hexa-[2,16] or cyclic octapeptide.[2,17] The above results indicate that, even in a cyclic tetrapeptide, the implantation of some N-substituted amino acids in the peptide yields significant multiple conformations. It should also be noted that these three conformational species are almost entirely consistent with those present in dimethyl-d_6 sulfoxide, as reported in the preceding paper.[1]

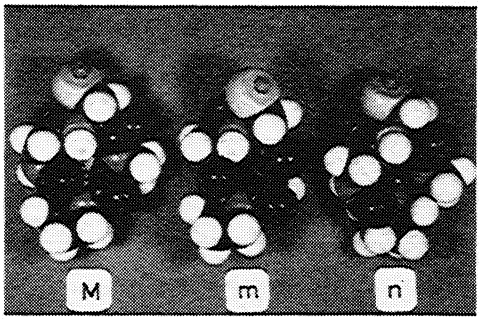

Fig. 9. Photograph of CPK model of proposed three conformations of free CGCSP in CD_3CN. Note the non-linear γ-turn hydrogen bond from the Cys to the Pro in **M** and **m**.

Ion Binding. Since chiroptical properties, such as CD and ORD, depend uniquely upon the molecular geometry, the CD spectral change observed for CGCSP on the addition of salt reflects the change in skeletal conformation. That is, it can be said that this peptide changes its conformation depending on the size and charge of the cation, upon complexation. This conformational change is accompanied by the rotational isomerism about N-substituted peptide bonds. The ^{1}H- and ^{13}C-NMR spectral changes of CGCSP in Fig. 4 and Fig. 8, resulting from the addition of salt, give us some information about the cis-trans isomerism of the two N-substituted peptide bonds (Cys–Sar and Sar–Pro bonds). The chemical shifts of N–CH_3 proton resonances usually serve to differentiate the trans peptide bond containing the sarcosyl N atom from the cis bond. It has been reported that a resonance for N–CH_3 protons whose geometry is anti to carbonyl oxygen occurred in a lower field region than a resonance for N–CH_3 protons which are involved in the cis peptide bond.[18,19] This, together with the results from ^{13}C-NMR, leads us to the confirmation that the first 2 : 1 complex (**Q**) contains trans Cys–Sar and trans Sar–Pro bonds, namely, all trans peptide bonds. This all-trans species, **Q**, is distinct from the three different ones, **M**, **m**, and **n**,

which are initially present in free peptide. An inspection of the CPK model indicates that the conformation can be allowed in which three carbonyl groups direct to the same side, preferentially for ion binding. The binding sites in **Q** appear to be the Sar C=O, the Cys C=O, and the C=O of Gly. Therefore, the intramolecular hydrogen bond can not be found. Actually, when a 1.0 equiv. of salt is added to peptide, the diminution of the negative $n\rightarrow\pi^*$ CD transition band and the downfield shift of the Pro C_β resonance about 0.9 ppm can be observed. This finding strongly supports the disruption[20] of the 1←3 hydrogen bond of γ-turn present in the free cyclic peptide. The second species, **R**, is characterized by the peptide linkages containing the cis Cys–Sar and trans Sar–Pro peptide bond. Judging from the large difference of CD pattern and the difference of the chemical shift for the Gly and Cys NH protons, **R** is likely to be different in backbone conformation from **n**, particularly in Pro ϕ angle. An inspection of the CPK model for **R** reveals that the Cys, Sar, and Pro carbonyls are oriented favorably for binding to a metal cation. The ^{1}H-NMR spectrum of CGCSP on the addition of 6.85 equiv. of salt demonstrates that the third species, **Y**, starts to spring up in addition to **R**. This **Y** is considered to contain the trans Cys–Sar and trans Sar–Pro peptide bonds. This cation-sandwich species, **Y**, appears to be the same as **Q**, judging from the similarities in the chemical shifts of the NH and N–CH_3 signals of the peptide. However, one can not exclude an alternate species which has a different conformation from that of **Q**, for there is only one available carbonyl group in **Y**, which should form a secondary binding site in a cation-sandwich complex. In order to account for the above observations in NMR and CD studies, the following scheme for the Ba^{2+} binding by CGCSP may be proposed;

$$\mathbf{M}_{\text{cis-cis}} \rightleftarrows \mathbf{m}_{\text{trans-cis}} \rightleftarrows \mathbf{n}_{\text{cis-trans}}$$

$$\downarrow K_{1/2}, +Ba^{2+}$$

$$\mathbf{Q}_{\text{trans-trans}}\ Ba^{2+}\mathbf{Q}_{\text{trans-trans}}\ (2:1\ \text{complex})$$

$$\downarrow K_1, +Ba^{2+}$$

$$\mathbf{R}_{\text{cis-trans}}\ Ba^{2+}\ (1:1\ \text{comprex})$$

$$\downarrow +Ba^{2+}$$

$$\mathbf{R}_{\text{cis-trans}}Ba^{2+} \rightleftarrows Ba^{2+}\mathbf{Y}_{\text{trans-trans}}Ba^{2+},$$

(1 : 1 complex) (1 : 2 complex)

where the subscript of each species denotes the peptide bond sequence a—b of Cys—Sar—Pro.

Previous studies have established the conformational fitting of cyclic penta-[20] and cyclic hexapeptide[21] on binding with alkali and alkaline earth cations. In these bindings, from two to four carbonyl groups in peptide were essential for coordination to occur. The present study of CGCSP provides the first evidence for conformational fitting on ion binding by a homodetic cyclic tetrapeptide. These facts demonstrate that even a cyclic tetrapeptide can have a potential for binding if two or three carbonyls favoring the cation interaction are available to form a coordination site. In conclusion, it is reasonable to think that conformational flexibility resulting from the cis-trans isomersim around the two N-substituted peptide bonds is responsible for the conformational fitting of CGCSP to a cation.

The authors are grateful to Dr. Yukio Imanishi of Kyoto University for his valuable suggestions and discussions. We are also grateful to Dr. Shoei Fujishige of our Institute for his useful comments.

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