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Conformation of Substance P in Neutral Phospholipid Micelles

Seonggeum Kim, Eunjung Bang[‡] , and Yangmee Kim*

Department of Chemistry, Konkuk University, Seoul 143-701, Korea
[‡] Korea Basic Science Institute, Seoul 136-701, Korea
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Abstract: A linear undecapeptide, Substance P (SP) is involved in a wide variety of physiological processes such as pain, inflammation, salivation, and hypertension. Tertiary structure of SP in dodecylphosphocholine (DPC) micelles has been investigated by CD, NMR spectroscopy, and DGII calculation. CD spectrum of SP in the presence of 7.5 mM DPC micelles does not show any favorable secondary structure. The tertiary structure determined by NMR spectroscopy and DGII calculation shows that the Phe[7]-Phr[8]-Gly[9]-Leu[10] region adopts a turn structure, while the N-terminal region is quite flexible. Both prolines in SP exist preferentially as the trans isoforms and the aromatic ring of Phe7 protrudes outward. Conformation of SP may be restrained by the contact of the Phe7 aromatic ring with the hydrophobic side chains of the DPC micelles and this interaction induces a turn structure. Structure of SP in aqueous solution in the presence of DPC micelles can represent a good model to study the conformation recognized by the receptor near neutral membrane.

INTRODUCTION

SP, an undecapeptide with a sequence of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2 is present in most parts of the central nervous systems of all mammals, in the periphery, in the primary sensory neurons, and in the neurons of the gastrointestinal tract.[1-3] In view of its wide spectrum of physiological actions, SP is of great pharmacological interest as a neurotransmitter. C-terminal penta peptide, Phe-Xaa-Gly-Leu-Met-NH2 are shared in most of the tachykinins.[4,5] All of them have similar activities including hypotension, vasodilatation, salivation, and contraction of various smooth muscles. The C-terminal part is known to be more important as a messenger sequence for most SP-influenced processes than N-terminal part.[1-7]

The conformation of SP and its analogues have been extensively studied by various

spectroscopic techniques such as UV, CD, IR, and NMR spectroscopy and they have shown that the conformations of SP are dependent on its local chemical environments.[8-16] It has been reported that SP adopts a random-coil structure in water.[12] According to the NMR studies, addition of sodium dodecylsulfate (SDS) or TFE to the aqueous solution induces a well defined α-helical structure of SP.[10,13,14] It has been suggested that a biological membrane serves two major functions in the binding of a neuropeptide to a membrane-embedded receptor.[17] The first function is to increase the concentration of the neuropeptide near the receptor. The second is to induce a specific conformation onto the polypeptide backbone of the neuropeptide prior to its interacting with its receptor. Binding with phospholipids induces a change in the three-dimensional structure of many peptides such as gramicidin and mellitin.[18-20] It has been further postulated that these conformational alterations should be an essential step for the recognition by the receptors. SP has been found to interact with phospholipid membranes.[11] In this study dodecylphosphocholine (DPC) model membrane system was selected because it can be used to mimic the amphiphilic local chemical environment of membrane embedded receptor and DPC micelle is closer to biological membrane system than SDS micelle. We determined the tertiary structure of SP in 7.5 mM DPC micelles by NMR spectroscopy and DGII calculation.

EXPERIMENTALS

NMR Experiments

SP was purchased from Sigma Chemical Co. Inc.. One milligram of SP was dissolved in 0.4ml of DMSO-d_6 solvent for NMR experiment. For the determination of the tertiary structure of SP in DPC micelles, perdeuterated DPC was purchased from Cambridge Isotope Co. and SP in 7.5mM DPC micelles was prepared in 0.4ml of 90% H_2O and 10% D_2O buffered to pH of 4.0 with 50mM sodium acetate.

All the phase sensitive two dimensional experiments such as DQF-COSY[21], TOCSY[22], NOESY[23], and ROESY[24] experiments were performed using TPPI method.[25] For these experiments, 512 transients with 2K complex data points were collected for each of the increments with a relaxation delay of 1.5 s between successive transients and the data along the t_1 dimension were zero-filled to 1K before 2D-Fourier transformation. TOCSY experiment was performed using a 95 ms, MLEV-17 spin-lock mixing pulse. Mixing times of 150, 200, and 250 ms were used for NOESY and ROESY experiments. PE-COSY experiment was performed using a 39° second pulse to measure the accurate passive coupling constants.[26] A 1D spectrum was acquired with a digital resolution of 0.1 Hz/point for the measurement of accurate $J_{HN\alpha}$ coupling constants. Chemical shifts are expressed relative to TMS signal or DSS signal at 0 ppm. All spectra were recorded at 303K on Bruker AMX-500 and DMX-600 spectrometers in Inter-University Center for Natural Science Research

Facilities at Seoul National University and Korea Basic Science Institute. All the NMR spectra were processed off-line using the FELIX software package on SGI workstation in our laboratory.[28]

CD Experiments

All CD experiments were done on Jasco model J-500 spectrometer using a 5 mm cell at 25°C. Spectra are expressed in units of mean residue ellipticity [θ]r (deg cm^2 $dmol^{-1}$). All the samples for the CD experiments were prepared with 100uM SP in the appropriate solvents in the acetate buffer from pH 4 to pH 6.

Structure Calculation

Distance constraints were extracted from the NOESY and ROESY spectra with a mixing time of 150 ms. The volumes of the NOEs between the two NH protons in the side chain of Gln or the two methylene protons in Met are used as references. All other volumes were converted to distances by assuming a simple $1/r^6$ distance dependence. All the NOE intensities are divided by three classes (strong, medium, and weak) with the distance ranges of 1.8-2.7, 1.8-3.3, and 1.8-5.0 Å, respectively.[29,30]

Tertiary structures were calculated by using DGII programs.[31] Distance bounds were computed for all atoms using triangle inequalities and subsequently improved using tetra-angle inequalities for quadruples of atoms in sequential residues. Initial random distances were chosen by prospective metrization, and the bounds were embedded. Twenty structures were generated and the coordinates are optimized using simulated annealing for 20000 steps of 0.2 ps and analyzed.

RESULTS AND DISCUSSION

Fig. 1 shows the CD spectrum of SP in water, 7.5 mM DPC micelles, and 15mM SDS micelles. SP has a random coil structure in water. Addition of SDS and DPC micelles induced changes in the CD spectrum as shown in Fig. 1. CD spectrum of SP in the presence of SDS micelles exhibited characteristics of the α-helical structure. DPC micelles induced structural changes, but SP in DPC micelles did not show any distinguished secondary structure in CD spectrum. In order to understand the structural changes induced by the DPC micelles, we determined the tertiary structure of SP in DPC micelles using NMR spectroscopy.

We performed NMR experiments on SP in DMSO to investigate the flexibility in the structure of SP. DMSO is known to be more hydrophobic than aqueous solution and the solubility of SP in DMSO is much better than that in water. TOCSY spectrum of SP in DMSO as shown in Fig. 2-A-a shows that there are sets of resonances corresponding to the

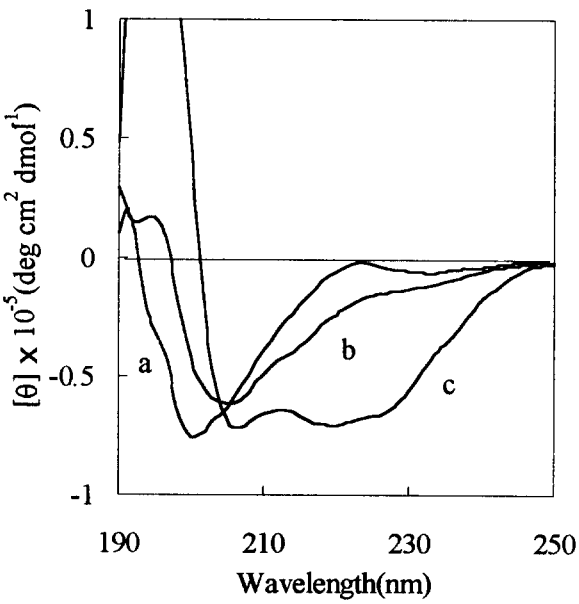


Fig. 1. CD spectra of SP (50uM) a) in water, b) in 7.5mM DPC micelles, and c) in 15mM SDS micelles.

intraresidual $P^{\alpha} - P^{\delta}$ connectivities for Pro^2 and Pro^4. Fig. 2-A-b which is the NOESY spectrum of SP in DMSO shows that there are strong $X^{i,\alpha} - P^{i+1,\delta}$ correlations for Pro^4 which correspond to the trans forms, while Pro^2 does not show any $X^{i,\alpha} - P^{i+1,\delta}$ correlations in this spectrum. All of the NMR data reveals that there are cis/trans two isoforms for Pro^2. N-terminal region of SP in DMSO is affected by *cis/trans* isomerization of Pro^2 and this results in the two sets of resonances also for the Pro^4. When Pro^2 has *cis* form, $Pro^{4',\delta}$ shows NOEs to $Lys^{3,\alpha}$ and $Arg^{1,\alpha}$. When Pro^2 has *trans* form, $Pro^{4,\delta}$ shows NOE to $Lys^{3,\alpha}$. However, as shown in Fig. 2-B-a, TOCSY spectrum of SP in DPC micelles shows that there is only one set of resonances for both of prolines. In the Fig. 2-B-b which is the NOESY spectrum of SP in DPC micelles, there are strong $X^{\alpha,i} - P^{\delta,i+1}$ correlations for both of the proline residues. This indicates both of Pro^2 and Pro^4 exist as *trans* isoforms in DPC micelles. Therefore, conformation of SP in DPC micelles is stabilized by the interaction with the phospholipid and both of prolines exist preferentially as the trans isoforms.

Sequence specific resonance assignment were performed using mainly DQF-COSY, TOCSY, and NOESY data.[32] Table. 1 shows the resonance assignments of SP in the presence of DPC micelles at 298K and pH 4.0, referenced to DSS and the $J_{HN\alpha}$ coupling constants. These $J_{HN\alpha}$ coupling constants are much larger than those values found in α-helical structure.

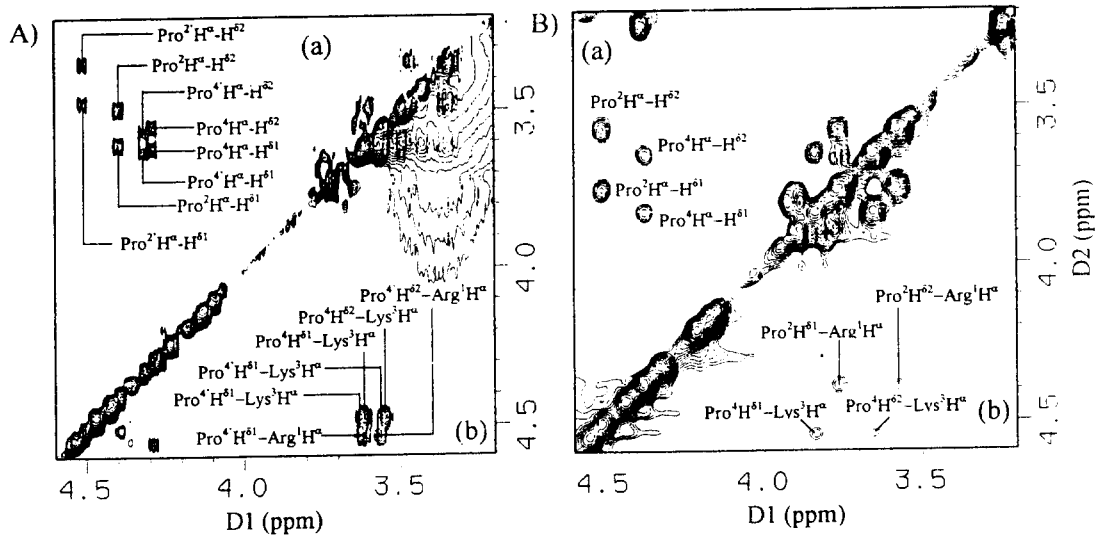


Fig. 2. 500 MHz ^{1}H NMR spectrum of SP A) in DMSO-d_6 (a) TOCSY spectrum showing the scalar connectivities of $Pro^{\alpha,i} - Pro^{\delta,i}$ in the upper left of the diagonal. (b) NOESY spectrum showing the correlations between $X^{\alpha} - Pro^{\delta}$ in the lower right of the diagonal. B) in DPC micelles (a) TOCSY spectrum showing the scalar connectivities of $Pro^{\alpha,i} - Pro^{\delta,i}$. (b) NOESY spectrum showing the correlation between $X^{\alpha,i} - Pro^{\delta,i+1}$.

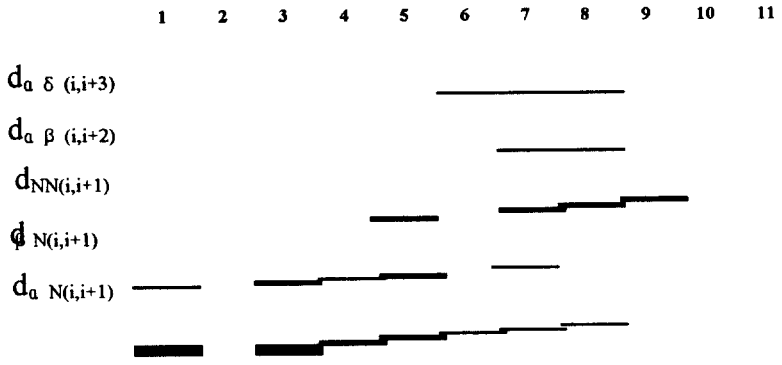


Fig. 3. Schematic representation of NOE connectivities of SP in 7.5 mM DPC,

90%H_2O/10%D_2O at 298K. The relative thickness of the lines represents the intensities of the NOEσ belonging to three classes (strong, medium, and weak NOEs).

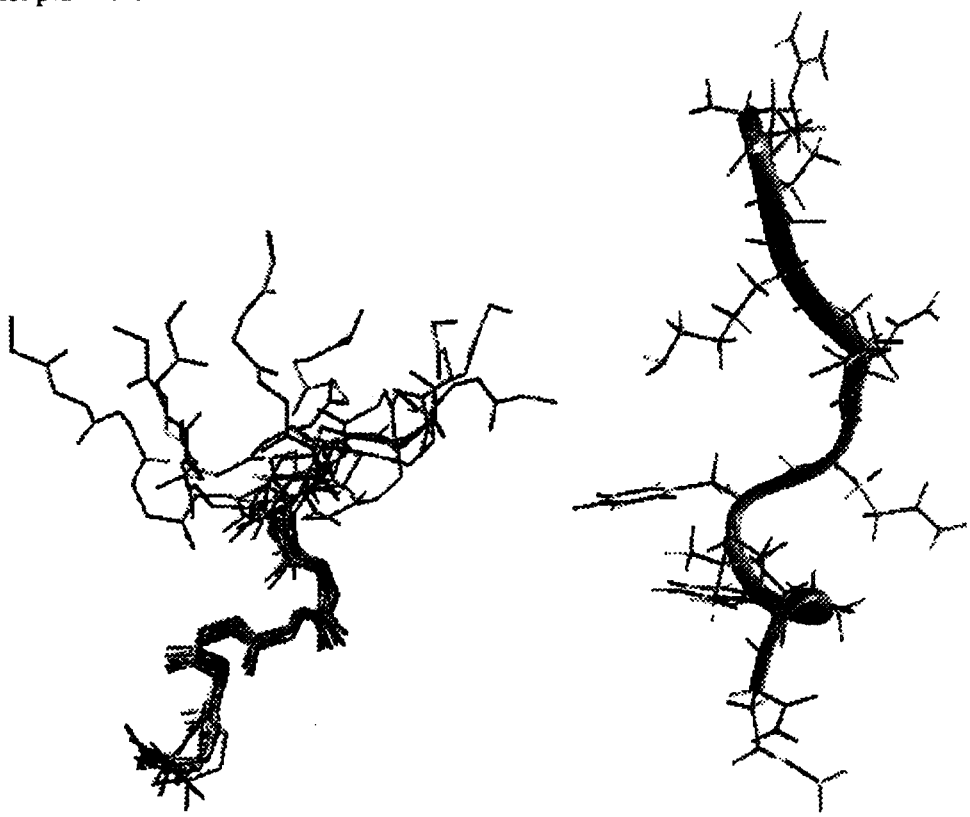
Fig. 3 shows the summary of the observed inter-residual NOE connectivities of SP in DPC micelles. NOE patterns do not agree with the characteristics of α-helix or β-sheet conformations. $J_{HN\alpha}$ of Phe8 is 7Hz and $J_{HN\alpha}$ of Gly9 is 5Hz. As shown in Fig. 3 there are weak $d_{\alpha N(i,i+1)}$ NOE and medium $d_{NN(i,i+1)}$ NOEs between Phe8 and Gly9, and between Gly9 and Leu10. Also there are NOEs between Phe7 and Leu10, and NOEs between Phe8 and Leu10. All of these NOE distance constraints and $J_{HN\alpha}$ agree with the turn I′ structure in Phe[7]-Phr[8]-Gly[9]-Leu[10] region.

Twenty structures are generated from the DGII and SA calculation with 58 NOE

Table 1. Complete Assignment of ^1H Resonances, Coupling Constants of 5mM SP Dissolved in 7.5mM DPC Micelles Buffered with Sodium Acetate at pH 4.0 in H_2O/D_2O(90:10 v/v %) at 500MHz and 293K, Referenced to DSS.

	Proton chemical shifts						
Residue	**NH**	**$C^{\alpha}H$**	**$C^{\beta}H$**	**$C^{\gamma}H$**	**$C^{\delta}H$**	**Others**	**$^3J_{NH\alpha}$**
ARG[1]	7.29	4.39	1.95	1.74	3.24		
PRO[2]		4.51	1.87,2.34	2.01	3.58, 3.77		
LYS[3]	8.59	4.55	1.69,1.83	1.50	1.72	CεH 2.99	6
PRO[4]		4.37	2.30,1.89	2.04	3.66,3.84		
GLN[5]	8.55	4.18	1.96	2.30		NH_2 6.91,7.58	6
GLN[6]	8.32	4.22	1.85	2.14		NH_2 6.90,7.49	6
PHE[7]	8.27	4.54	2.93,3.03			7.15(o),7.25(m),7.27(p)	
PHE[8]	8.29	4.56	2.97,3.18			7.25(o),7.31(m),7.31(p)	7
GLY[9]	7.96	3.80				3.89(α2)	5
LEU[10]	8.14	4.32	1.62,1.68	1.64	0.89, 0.94		7
MET[11]	8.31	4.44	2.02,2.11	2.50,2.60		$CONH_2$ 7.13,7.46	4

restraints and 6 dihedral angle constraints of SP in DPC micelles. RMSDs for 20 structures range from 0.1 to 0.9. All of structures satisfies the experimental NOEs well within 0.02 Å atoms of residue 5-11 and they converge well to a turn-like structure in the Phe^{7}-Phr^{8}- RMSD. Fig. 4a shows the superposition of 10 low energy structures on the backbone atoms of residue 5-11 and they converge well to a turn-like structure in the Phe^{7}-Phr^{8}-Gly^{9}-Leu^{10} region. Fig. 4b shows the ribbon structure of the lowest energy structure with all atoms. The aromatic ring of Phe7 protrudes outward and this aromatic ring is expected to contact with the phospholipid membrane. There is a stable turn I′ structure at the C-terminal region, while N-terminal region is very flexible. Conformational changes induced by DPC micelles is primarily due to the hydrophobic interaction between SP and the hydrophobic acyl chains of phospholipid in the micelles.



a) b)

Fig. 4. a) The superposition of the 10 structures of SP in DPC micelles calculated by DGII and SA calculations. Backbone atoms of the residues 5-11 are superimposed. Only the backbone atoms are shown here except Phe7. Bottom, C-terminus; top, N-terminus. b) The ribbon structure of the lowest energy conformation of SP in DPC micelles showing all atoms.

Other conformational studies of SP in the presence of SDS micelles have shown that SP can fold to form an α-helical structure when it interacts with negatively charged SDS micelles.[13,14] We suggest that interactions between SP and the neutral phospholipid in DPC micelles are largely weak hydrophobic interactions and the protruded aromatic ring of Phe7 has hydrophobic interaction with DPC micelles. According to our NOE and T_1 relaxation data, the aromatic ring of Phe7 does not penetrate deeply into the hydrophobic core of DPC micelles but has close contacts with the side chains of DPC micelles (data not published yet). Hydrophobic interactions between SP and DPC micelles are much weaker than the strong electrostatic interactions between the positively charged SP and the negatively charged SDS micelles. According to our CD and NMR data including the CSI data, SP in the presence of 7.5 mM neutral DPC micelles does not adopt an α-helical structure but a turn structure.

The aqueous solution in the presence of DPC micelles may represent an attractive model for defining the conformation recognized by the receptor near neutral membrane. Tertiary structure determined here will be used to study the interactions between NK-1 receptor and SP. NMR studies on the structures of various neuropeptides in neutral phospholipid bilayer as well as anionic phospholipid bilayer are going on in our laboratory and the structure of SP in DPC micelles will be helpful to understand the underlying mechanisms of the binding of neuropeptides with lipid bilayer.

Acknowledgments

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