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An NMR Study on the Conformation of Substance P in Acidic Bicelles

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The conformation of a neuropeptide, substance P (SP), in isotropic (q = 0.5) acidic bicelles was investigated using two-dimensional NMR techniques. By the nuclear Overhauser effect (NOE) cross peaks between SP and long-chain lipid molecules SP was probed to bind on the flat surface of the disc-like bicelles. Structural analysis of NMR data indicated that the helical conformation of SP extended to the C-terminal region of Leu10 as well as in the mid-region from Pro4 to Phe8. As compared with the conformations of SP bound on the sodium dodecylsulfate (SDS) or the dodecylphosphocholine (DPC) micelles with curved surfaces, the surface curvature of the membrane mimics was found to be one of the major factors inducing the biologically relevant conformation of SP. The negative surface charge of the membrane is also a key factor inducing both the binding of SP on the membrane and its biologically active structure.

Key Words : Substance P, Bicelle, NMR, NOESY, Surface curvature, Conformation

Introduction

Substance P (SP) is a neuropeptide of the tachykinin family and composed of 11 amino acid residues (Arg1-Pro2-Lys3-Pro4-Gln5-Gln6-Phe7-Phe8-Gly9-Leu10-Met11-NH_2), which is thought to be involved in many important physiological processes including pain transmission, inflammation, blood flow, salivation and various muscle contractions.[1,2] Such wide range of physiological activities of SP has been ascribed to the lack of selectivity for a specific receptor type. Thus, SP is known to activate three membrane-embedded receptor subunits with more or less extents of potencies, and this can be accounted to the conformational flexibility of SP peptide.

To investigate the effects of conformation on the activity of SP, extensive conformational studies have been conducted for the past several decades using diverse techniques such as circular dichroism,[3,4] infrared spectroscopy,[5] nuclear magnetic resonance (NMR),[6-13] and molecular dynamics.[12,14] The results indicate that SP has a random structure in aqueous solutions,[7-11] whereas it exhibits helix-like turns or partial helical structures in various membrane mimetic systems such as micelles and phospholipids bilayers.[6,8-10,13] In addition, SP is known to experience at least three types of lipid environments. such as the storage vesicles, the presynaptic and the postsynaptic membranes,[9] during the physiological processes. The lipid membrane has been expected to play an important role in inducing and stabilizing the physiologically active conformation of SP. The detailed studies on the conformations of SP and its interaction with the lipid membranes could provide basic information explaining the peptide-receptor binding mechanism and its activity.

NMR spectroscopy has emerged as a major tool to study both the conformation of peptides and the interaction of peptides with model biomembranes. For high resolution NMR, however, the model membranes have to reorient fast and isotropically in the solution with a correlation time of nanosecond scale, and consequently micelles or small unilamellar vesicles (SUV) have been widely used as a model membrane due to such requirements.[11,15] Recently, the bicelle that consist of both long- and short-chain lipids has drawn much attention as a membrane mimic because the disk-like bicelle has a flat surface made of long-chain phospholipids along with a curved surface made of short-chain lipids on its rim.[16,17] The geometrical size of bicelle can be altered by controlling the long-to-short-chain lipid molar ratio (q). In particular, the small isotropically tumbling bicelle (q < 1) has a fast reorientional motion in the solution enough to provide highly resolved NMR spectra for bicelle-bound peptides,[18,19] and such conformational studies have been reported in many cases.[19-23]

In the present study, the conformation of SP in an solution containing an isotropic (q = 0.5) acidic bicelle including 25% anionic phospholipids out of the long-chain lipid bilayers, which is the most common lipid composition for biological membranes, was investigated using two-dimensional NMR spectroscopy and the structural analysis. The results show that SP adopts a helical conformation in the region from Pro4 to Met11.

Experimental

Sample Preparation. Substance P was purchased from Sigma Chemical Co., and used without further purification. 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylserine (DMPS), and 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC) were obtained from Avanti Polar Lipids Inc. The acidic bicelle solution was prepared by dissolving DMPC, DMPS,

and DHPC in 0.5 mL of sodium acetate buffer solution (pH 6.0, 0.1 M NaCl, 90% H_2O and 10% D_2O) with the ratio of q = ([DMPC] + [DMPS])/[DHPC] = 0.5 under the inert nitrogen gas environment. The molar ratio of anionic lipid DMPS to neutral lipid DMPC was 1:3 (25%). The lipid suspension was allowed to be hydrated under thermal cycling between 38 ºC and 4 ºC in order to make bicelle phase out of lipids. Each cycle was then followed by vigorous vortexing for 30 minutes at room temperature to ensure complete mixing of the lipids. This procedure was carefully repeated several times until the bicelle solution was cleared and then the solution was centrifuged to remove the undissolved ingredients. The SP buffer solution was added drop-by-drop to the bicelle solution with constant agitation until the molar ratio of peptide to DMPC reached 1:40. The final concentration of SP in the bicelle solution was 2.8 mM, and the total amount of lipids was 15% (wt/v).

NMR Spectroscopy. All NMR experiments were carried out on a Bruker DRX500 spectrometer (500.13 MHz for ^{1}H frequency) equipped with a broad-band inverse probe and pulsed field z-gradient capability. Two-dimensional total correlation spectroscopy (TOCSY)[24] and nuclear Overhauser effect spectroscopy (NOESY)[25] were applied to obtain the spectral and sequential assignments of the peptide. Phase-sensitive TOCSY and NOESY were collected with both 256 scans, a relaxation delay of 2 s, and a spectral width of 5000 Hz, as well as 4096 and 512 data points in t_2 and t_1 dimension, respectively. The NOESY spectrum was recorded with a mixing time of 200 ms. In all 2D experiments, water suppression was achieved by selective irradiation of water resonance during the relaxation delay.[26] NMR data were processed with Bruker XWIN NMR program, and the assignments of proton resonances were carried out using SPARKY software.[27]

Results and Discussion

Binding of SP in Isotropic Acidic Bicelles. Figure 1 shows the regional NOESY spectrum revealing the spatial distances between aliphatic and amide protons of the peptide. In addition to the cross peaks representing the intramolecular structure information, the marked cross peaks between methylene protons of DMPC acyl chain and aromatic protons of Phe7 and Phe8 in SP were also found, which indicates that SP molecule binds on the flat region composed of DMPC molecules in a bicelle. The strong intermolecular NOE cross peaks exhibit the deep insertion of SP into the hydrophobic region of the lipid bilayer in an isotropic acidic bicelle and also indicate the very low mobility of SP in the lipid bilayer. As seen in Figure 1, no cross peaks between methylene protons of DHPC and aromatic protons of SP appeared. This indicates that SP did not bind to the rim region made out of DHPC molecules. These facts imply that SP exclusively binds to the flat surface in the disk-like bicelle made out of long- and short-chain lipid molecules and our experimental condition for the study on the conformation of SP bound to the flat membrane surface was well adjusted.

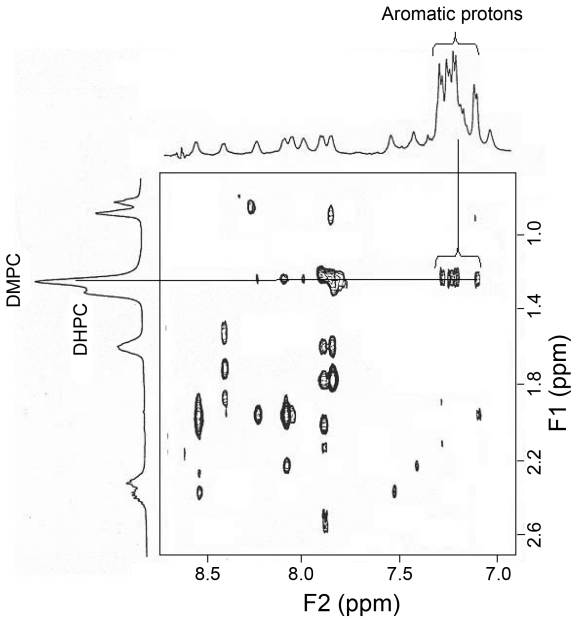

Figure 1. 2D NOESY spectrum of SP and lipids in DMPC: DMPS:DHPC (3:1:8) bicelles at pH = 6.0 and 300 K. The NOE cross peaks between the aromatic protons of SP and the methylene protons of DMPC are marked.

Chemical Shifts and Conformation. ^{1}H NMR chemical shift assignments of SP in isotropic (q = 0.5) acidic bicelles were accomplished by using the well-established procedure developed by Wüthrich.[28] TOCSY (Fig. S1) was primarily used to identify the spin systems of the residues. The peptide was dissolved in a non-deuterated lipid bicelle solution and resulted in severe overlaps between the peptide resonance peaks and lipid resonance peaks in the aliphatic region of ^{1}H spectrum. Thus, the absence of amide protons in Pro2 and Pro4 makes it difficult to assign the aliphatic ^{1}H resonances belonging to the two Pro residues. 2D NOESY facilitated the ^{1}H resonance assignments by providing the sequential connectivity information. Figure 2 shows the fingerprint regions of ^{1}H NOESY spectrum for SP in acidic bicelles and displays the sequential connectivities between the cross-peaks. All the assigned ^{1}H chemical shifts are given in Table 1.

Since the deviations in chemical shift values from the random coil conformation reveal different types of secondary structures the chemical shift index analysis provides highly reliable predictions of the peptide secondary structures.[29,30] Figure 3 shows the $C_\alpha H$ chemical shift deviations ($\Delta\delta_{C\alpha H}$) of SP in the acidic bicelle, where the successive negative values from Pro4 to Gly9 are strong indication of a helical structure for the given residues. The upfield shifts of NH resonances from the random coil values can also be used as a diagnostic probe for any changes in the local environments such as the existence of intramolecular hydrogen bonding and/or burial of the given residues into hydrophobic atmosphere. In the acidic bicelle the NH chemical shifts from Gln6 to Met11 residues were upfield-shifted by greater than 0.2 ppm except for Phe7. In particular, Gly9 shows a

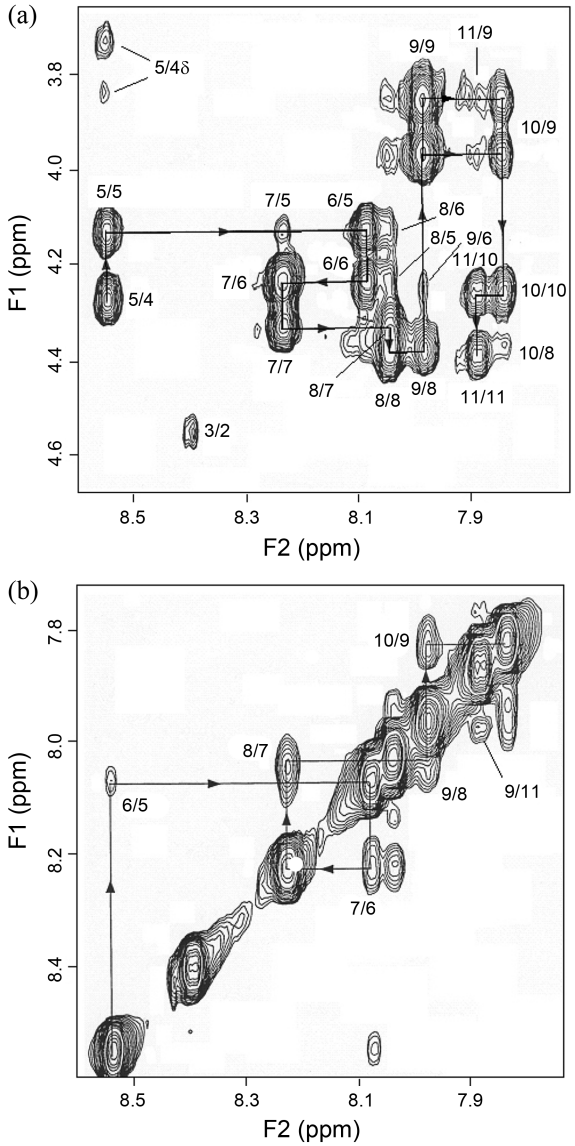

Figure 2. The NOESY spectra of the (NH-C_αH) region (A) and the (NH-NH) region (B) for 2.8 mM SP in acidic bicelles obtained with the mixing time of 200 ms at 300 K. Sequential connectivities between NH and C_α protons are indicated by solid lines.

large upfield shift of 0.41 ppm as compared to those of 0.27 and 0.17 ppm measured in SDS[8,10] and DPC micelles,[8] respectively, implying that the conformation and/or the hydrophobicity around Gly9 in bicelles are very different from those in micelles. The higher upfield shift of NH resonances might be caused by the deep burial of the hydrophobic residues such as the Phe7 and Phe8 into the hydrophobic acyl chain region of DMPC lipid. These chemical shift data are consistent with the NOESY spectrum showing the strong intermolecular cross peaks between SP and DMPC as an evidence for the deep insertion of the peptide SP into the bilayer.

NOE Measurements and Conformation. Figure 4 shows the NOE peaks that are important to characterize the secondary structure of SP in acidic bicelles. The observations of consecutive strong NH_i-NH_{i+1}, $C_\alpha H_i$-NH_{i+1} and a number of

Table 1. Summary of ^{1}H chemical shift assignments (in ppm) of 2.8 mM substance P in isotropic (q = 0.5) acidic bicelles

Residue	NH	C_αH	C_βH	C_γH	Others
Arg1	n/a	n/a	n/a	n/a	n/a
Pro2	-	4.56	n/a	n/a	n/a
Lys3	8.39	n/a	1.85	1.53	C_δH 1.71, C_εH 1.49
Pro4	-	4.28	n/a	n/a	C_δH 3.72, 3.83
Gln5	8.54	4.13	2.00	2.36	$N_\delta H_2$ 6.82, 7.53
Gln6	8.08	4.24	1.95	2.22	$N_\delta H_2$ 6.79, 7.42
Phe7	8.23	4.33	2.94, 3.00	-	2,6H 7.08, 3,5H 7.19
Phe8	8.04	4.39	3.01, 3.27	-	2,6H 7.22, 3,5H n/a
Gly9	7.98	3.85, 3.97	-	-	-
Leu10	7.83	4.26	1.77	1.60	C_δH 0.90
Met11	7.88	4.40	2.00, 2.12	2.49, 2.54	NH_2 7.02, 7.27

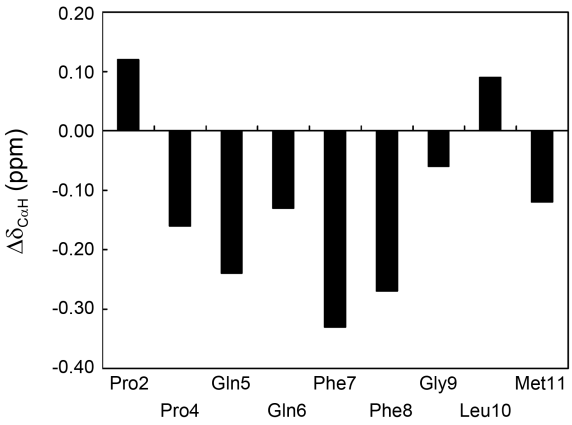

Figure 3. Plot of the C_αH chemical shift deviations (in ppm) from the random coil values for SP in acidic bicelles.

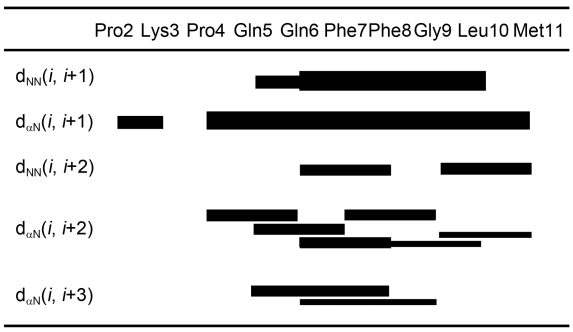

Figure 4. Summary of sequential and medium-range NOE data in acidic bicelles at 300 K. Solid lines represent the NOE connectivities between residues, and the line thickness indicates the intensity of the NOE correlations as strong, medium, and weak.

medium-range connectivities such as $C_\alpha H_i$-NH_{i+2} and $C_\alpha H_i$-NH_{i+3} reveal that SP adopts a helical structure in the mid-region from Pro4 to Phe8 when dispersed in the bicellar solution. In particular, the presence of $C_\alpha H_i$-NH_{i+2} peaks and the absence of $C_\alpha H_i$-NH_{i+4} peaks would point to a 3_{10}-helix conformation, while ruling out a standard α-helical conformation, in good agreement with those values given in Table 7.1 from the Wüthrich's book.[28]

Figure 5 shows the superimposed image of 20 SP confor-

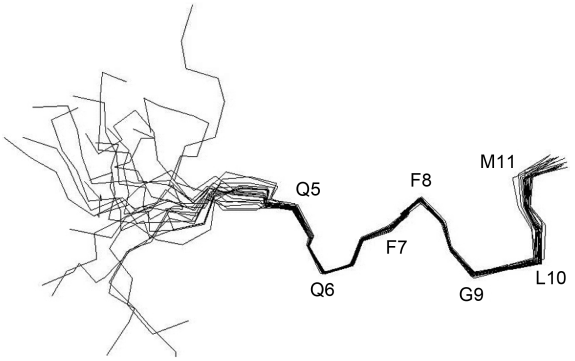

Figure 5. Line representations of ensembles of 20 SP structures in DMPC:DMPS:DHPC (3:1:8) bicelles at pH = 6.0 and 300 K.

mations simulated by XPLOR using the NOE data. Residues of Gln5-Leu10 are found to form a 3_{10}-helical structure and residues of Lys3, Pro4, and Met11 may be involved in the helical structure as well. The side chain groups have some variations in their positions but the backbone structures are well defined. For instance, the side chain groups of Gln5 and Gln6 and the phenyl ring of Phe7 residue are well defined but the phenyl ring of Phe8 residue is not, as shown in Figure S2. While the C-terminal region is characterized as a helical structure, the N-terminal region is not well structured which might be results from the residence of N-terminal region in aqueous phase. Both the NOE connectivities and the simulated structures reveal that the conformation of SP in acidic bicelle solution is rather close to 3_{10}-helix, which is in good agreement with those observed in micelles.[10,13]

Along with above analysis, the chemical shift of NH resonance in Gly9 suggests the possibility of the extension of a helical structure to the C-terminal. The large upfield-shift of NH resonance in Gly9 from the random coil value means that there could be a formation of the intramolecular hydrogen bond around Gly9, which is also supported by the observation of medium range NOE between Gln6 $C_\alpha H$ and Gly9 NH. This hydrogen bonding may induce the extended helical structure. For SP in SDS and DPC micelles, no such NOE connectivity was observed.[8-10] Thus, it appears that the conformation of SP depends not only on the solution condition, but also on the type of model membranes, of which the bicelle with flat bilayer surface might provide a better environment for the extended helix formation than the micelle with highly curved surface. The well-aligned hydrophobic acyl chain in the flat surface can interact more effectively with the terminal region of the straight helical structure of SP. This is one of the plausible explanations for the extension of the SP helical structure in the bicellar bilayer.

In the article published recently, SP in isotropic bicelles composed of DMPC/CHAPS was reported to have a helical structure spanning residues Phe8-Met11 in the presence of Ganglioside Monosialo 1 (GM1), which is consistent with our result of the extended helical structure to the Met11 residue. The NMR conformation of SP in GM1-containing bicelles when docked on NK_1R exhibited the highest binding energy and best fit to the active site of NK_1R.[31] As one of the major effects of GM1 is to make the lipid bilayer surface negatively charged, the similar structure of SP bound in the GM1-containing bicelles and in our anionic bicelles can be also attributed to the strong interaction between SP and the negatively charged surface of the lipid bilayers along with the surface curvature effect.

Conclusions

The conformation of SP has been reported to strongly depend on the solution conditions.[8-10] Many conformational studies indicate that SP shows no preference in conformation when dispersed in aqueous solutions,[7-11] while it favors relatively well-defined conformations in membrane mimics and organic solvents.[6,8-11,13] In the present study, we have investigated the conformational features of SP in isotropic (q = 0.5) acidic bicelle with a flat bilayer surface using two-dimensional NMR techniques. Structural analysis of NMR data suggests that SP forms a 3_{10}-helix as in micelles but the helical conformation extends from Pro4 to Met11 contrary to the micellar one having a helical conformation only in the mid-region. As our results are combined with others, the longer 3_{10}-helical structure of SP is thought as the more biologically active conformation. Furthermore, both the negatively-charged surface and the flat membrane curvature are of importance to induce the above conformation.

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Supplementary Materials. Supplementary figures S1 and S2 for TOCSY spectrum and line/ribbon representations of ensembles of 20 SP structures, respectively, in DMPC:DMPS :DHPC (3:1:8) bicelles are available at the bkcs website.

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