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Solution structure of neuromedin B by ^{1}H nuclear magnetic resonance spectroscopy

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Abstract The solution structure of neuromedin B (NMB) was investigated using two-dimensional nuclear magnetic resonance (NMR) spectroscopy in membrane-mimicking environments. NMB adopts a relaxed helical conformation from Trp^4 to Met^{10} in 50% aqueous 2,2,2-trifluoroethanol (TFE) solution and in 150 mM SDS micelles. Sidechain atoms of the three residues, Trp^4, His^8 and Phe^9 orient toward the same direction and these residues might play a key role on interacting with hydrophobic acyl chains of the phospholipids in the membrane. NOESY experiments performed on NMB in non-deuterated SDS micelle show that aromatic ring protons of Trp^4 and Phe^9 residues are in close contact with methylene protons of SDS micelles. In addition, proton longitudinal relaxation data proved that the interactions between NMB with SDS micelle are characterized as extrinsic interaction. Trp^4 and Phe^9 seem to be important in interaction with receptor and this agrees with the previous studies of structure-activity relationship (Howell, D.C. et al. (1996) Int. J. Pept. Protein Res. 48, 522–531). These conformational features might be helpful in understanding the molecular mechanism of the function of NMB and developing the efficient drugs.

Key words: Neuromedin B; NMR; Membrane-mimicking solvent; Peptide structure

1. Introduction

Neuromedin B (NMB) [1] having the sequence of GNLWATGHFM-NH_2, was first isolated from porcine spinal cord which belongs to the ranatensin subfamily of bombesin-like peptides [2]. Mammalian bombesin-like peptide, NMB exhibits a wide range of biological responses in the central nervous system and gastrointestinal tracts including thermoregulation [3], stimulation of the secretion of gastrointestinal hormones [4], the regulation of smooth muscle contraction [5], the ability of function as a growth factor in small cell lung cancer cells and murine 3T3 cells [6–8]. Bombesin-like peptides share the similar amino acids in their amidated C-terminal regions and these amino acids are thought to play an important role on the binding to their receptors and thought to be responsible for their related pharmacological effects.

The structure of bombesin has been revealed by NMR spectroscopy as a random coil structure in water or dimethylsulfoxide solution [9] and in aqueous 2,2,2-trifluoroethanol (TFE)-containing solution it is revealed as a relaxed α-helical structure in the C-terminal region [10]. The bombesin receptor agonist and antagonist in dodecylphosphocholine (DPC) micelle have helical structures formed by consecutive β-turns in their C-terminal regions [11]. IR study of bombesin and NMB in phospholipid bilayer also suggested that they adopt α-helical conformation in the C-terminal region [12]. Recent structure-activity relationship (SAR) study of bombesin fragment suggested that Trp^8, His^{12} and Leu^{13} residues, which correspond to Trp^4, His^8 and Phe^9 in NMB are important for the binding to the NMB receptors [13]. Recent circular dichroism, fluorescence and molecular dynamics simulation study also suggests that NMB adopts a α-helical structure [14].

To date, none of the reported data deals with the high-resolution structure of NMB. Binding of NMB to its receptor occurs in the membrane environment. Membrane induces a specific conformation onto the peptide backbone of NMB before interacting with its receptor and this conformational alteration should be an essential step for the recognition by the receptor. Here, we report the three-dimensional structure of NMB in membrane mimetic environments such as TFE/H_2O solution and sodium dodecylsulfate (SDS) micelles studied by NMR spectroscopy. We also discuss about the mechanism of the interaction of NMB with membrane and the structural requirement for the function of NMB.

2. Materials and methods

2.1. Sample preparation

NMB was purchased from Peptides(Japan). TFE-d_3 was purchased from Aldrich, SDS-d_{25} was obtained from Cambridge isotope, and non-deuterated SDS was purchased from Sigma. For NMR experiments in TFE/H_2O solution, 2 mg of peptide was dissolved in 0.40 ml of TFE-d_3/H_2O (1:1, v/v) solution, to make a final concentration of 5.1 mM and pH was adjusted to 4.0 by adding HCl. For the experiments in SDS micelles, 2 mg of peptide were dissolved in 50 mM sodium acetate buffer, pH 4.0, which contains 150 mM SDS-d_{25}. In order to study the interaction between the peptide and SDS micelles, 1 mg of NMB was dissolved in 0.4 ml of 50 mM acetate buffer, pH 4.0, which contains 15 mM of non-deuterated SDS micelles.

2.2. NMR spectroscopy

All the NMR experiments for the sample in TFE/H_2O (1:1, v/v) solution were performed at 277 K and experiments for the samples in SDS micelles were conducted at 298 K. All the phase sensitive two-dimensional experiments such as DQF-COSY [15], TOCSY [16] and NOESY [17] experiments were performed using time-proportional phase incrementation (TPPI) method [18]. For these experiments, 400–512 transients with 2 Kb complex data points were collected for each of the increments with a relaxation delay of 1.2–1.5 s between the successive transients and the data along the t_1 dimension were zerofilled to 1 Kb before two-dimensional-Fourier transformation.

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Abbreviations: NMB, neuromedin B; DPC, dodecylphosphocholine; SDS, sodium dodecyl sulfate; TFE, 2,2,2-trifluoroethanol; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; SAR, structure-activity relationship

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TOCSY experiment was performed using 80–100 ms, MLEV-17 spin-lock mixing pulse. NOESY experiments were performed using mixing time of 150 and 250 ms. The suppression of water signal was achieved by the field-gradient method with WATERGATE sequence [19] for the sample in deuterated SDS micelles.

NMR spectroscopy can give useful information about the interaction between the neuropeptides and micelles. As reported for the substance P and bradykinin, proton longitudinal relaxation data and the nuclear Overhauser effect can be used to characterize these interactions as intrinsic or extrinsic [20]. NOESY experiments for the sample in non-deuterated SDS micelles was executed at 298 K with a mixing time of 250 and 650 ms. All proton spectra to determine the T_1 relaxation time were acquired using the Inversion-Recovery method with a relaxation delay of 10 s between the scans. All NMR spectra were recorded on Bruker AMX-500 spectrometers in the Inter-University Center for Natural Science Research Facilities in Seoul National University, Bruker DPX-400 spectrometer in Konkuk University and Bruker DMX-600 spectrometer in the Korea Basic Science Institute. All NMR spectra were processed off-line using the FELIX software package (Molecular Simulations, San Diego, CA, USA) on a SGI workstation in our laboratory.

Chemical shifts of the samples were measured relative to the methyl resonance of internal 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) at 0 ppm. $^3J_{HN\alpha}$ coupling constants were either measured in a one-dimensional spectrum or calculated by the formula derived by Kim and Prestegard [21] from the separation of absorptive peaks and dispersive peaks in the DQF-COSY spectrum. DQF-COSY spectrum was processed to the 4 Kb×2 Kb matrix and used to measure peak-to-peak separations. P.E.COSY [22] experiment was executed to obtain $^3J_{\alpha\beta}$ coupling constants. To identify slowly exchanging amide protons, a series of one-dimensional spectra were acquired after deuterium oxide was added to the sample.

2.3. Structure calculation

Structure calculations were carried out using X-PLOR version 3.851 [23]. All the NOE intensities are divided into three classes, i.e. strong, medium and weak with the distance ranges of 1.8–2.7, 1.8–3.5, and 1.8–5.0Å, respectively. Standard pseudoatom corrections [24] were applied to the non-stereospecifically assigned restraints, and the additional 0.5 Å was added to the upper bonds for NOEs involving methyl protons [25]. Standard distance geometry-dynamical simulated annealing hybrid protocol [26,27] was employed to generate the structures. Center averaging was used to correct distances involving methyl

Table 1
Chemical shifts of NMB in (a) TFE/H_2O (1:1, v/v) at pH 4.0, 277 K and (b) 150 mM SDS micelles at pH 4.0, 298 K

(a)				
	NH	CαH	CβH	Others
Gly^1	7.97	3.68		
Asn^2	8.45	4.65	2.58	$C\delta_{21}H$ 7.42$C\delta_{22}H$ 6.58
Leu^3	8.28	4.00	1.31	$C\gamma H$ 1.41$C\delta_1H$ 0.72$C\delta_2H$ 0.65
Trp^4	7.85	4.37	3.16, 3.23	$C\delta_1H$ 7.07$C\varepsilon_1H$ 9.72$C\varepsilon_3H$ 7.37 $C\zeta_2H$ 7.20$C\zeta_3H$ 6.94$C\eta_2H$ 7.00
Ala^5	7.86	4.03	1.24	
Thr^6	7.82	4.01	4.14	$C\gamma_2H$ 1.10
Gly^7	8.06	3.71		
His^8	7.76	4.37	2.69, 2.95	$C\delta_2H$ 6.82$C\varepsilon_1H$ 7.94
Phe^9	8.02	4.29	2.84, 2.92	CδH 7.02CεH 7.18CζH 7.13
Met^{10}	7.83	4.22	1.74, 1.93	$C\gamma_1H$ 2.27$C\gamma_2H$ 2.36
NH_2	6.60, 6.44			
(b)				
Gly^1		3.88, 3.95		
Asn^2	8.55	4.75	2.65, 2.75	$C\delta_{21}H$ 6.80$C\delta_{22}H$ 7.50
Leu^3	8.24	4.12	1.46, 1.70	$C\gamma H$ 1.60$C\delta_1H$ 0.82$C\delta_2H$ 0.90
Trp^4	7.76	4.56	3.24, 3.30	$C\delta_1H$ 7.25$C\varepsilon_1H$ 9.91$C\varepsilon_3H$ 7.49 $C\zeta_2H$ 7.40$C\zeta_3H$ 7.01$C\eta_2H$ 0.90
Ala^5	7.77	4.21	1.29	
Thr^6	7.85	4.15	4.23	$C\gamma_2H$ 1.26
Gly^7	8.17	3.80, 3.87		
His^8	7.98	4.34	2.95	$C\delta_2H$ 6.63$C\varepsilon_1H$ 8.41
Phe^9	8.14	4.48	3.06, 3.18	CδH 7.34CεH 7.31CζH 7.21
Met^{10}	7.89	4.33	1.98, 2.10	$C\gamma_1H$ 2.44$C\gamma_2H$ 2.52
NH_2	6.93, 7.05			

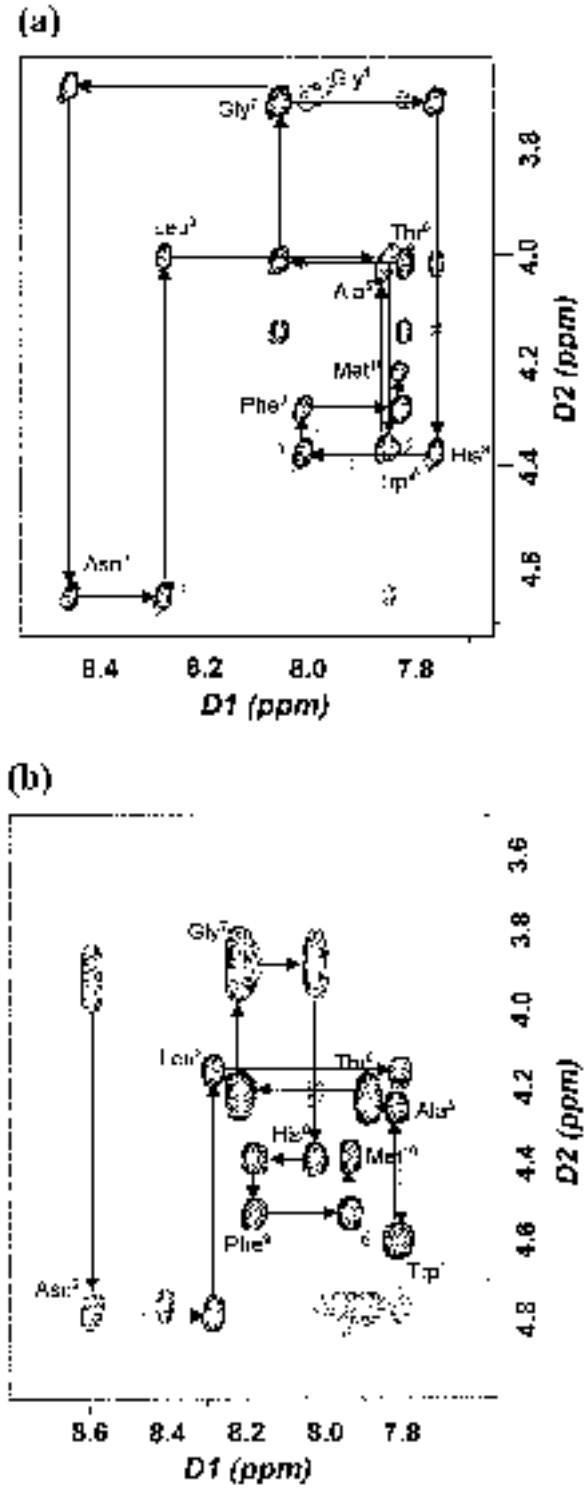

Fig. 1. Fingerprint region of NOESY spectrum of NMB a: TFE/H_2O (1:1, v/v) b: 150 mM SDS micelles recorded with a mixing time of 250 ms. Assigned amino acid residues and sequential connectivities are indicated.

groups and non-stereospecifically assigned methylene protons. The target function that is minimized during simulated annealing comprises only quadratic harmonic potential terms for covalent geometry, square-well quadratic potentials for the experimental distance and torsion angle restraints and a quartic van der Waals repulsion term for the non-bonded contacts. A total of 36 sequential, 27 medium-range (*i*-*i*+2, *i*-*i*+3, *i*-*i*+4), 33 intraresidual distance restraints, 8 ϕ torsion angle restraints, and 1 χ torsion angle restraints were used as experimental restraints for the structure in TFE/H_2O solution. For the structure of NMB in SDS micelles, 55 sequential, 31 medium-range(*i*-*i*+2, *i*-*i*+3, *i*-*i*+4), 44 intraresidual distance restraints, and 8 ϕ torsion angle restraints were used as experimental restraints.

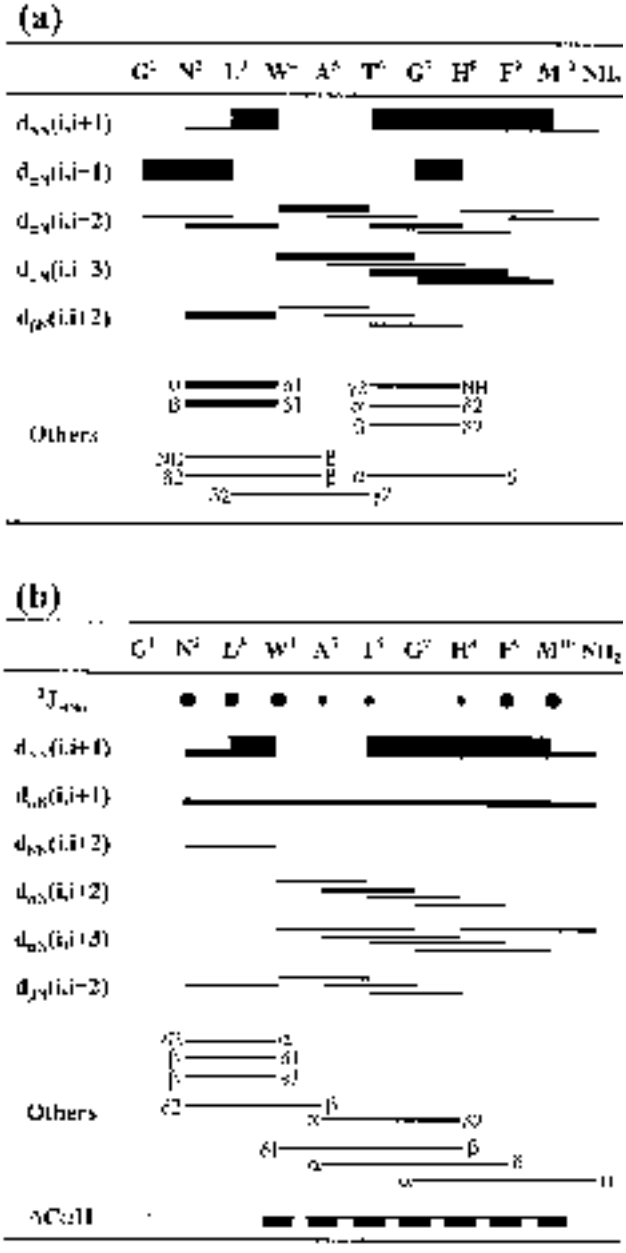

Fig. 2. A summary of interresidual NOE connectivities found in NOESY spectrum of NMB a: TFE/H_2O (1:1, v/v) B: 150mM SDS micelles. The thicknesses of the bars are proportional to the NOE intensity. b: $^3J_{HN\alpha}$ coupling constants and chemical shift index values of Cα protons were also indicated. Filled circles represent the approximate sizes of coupling constants.

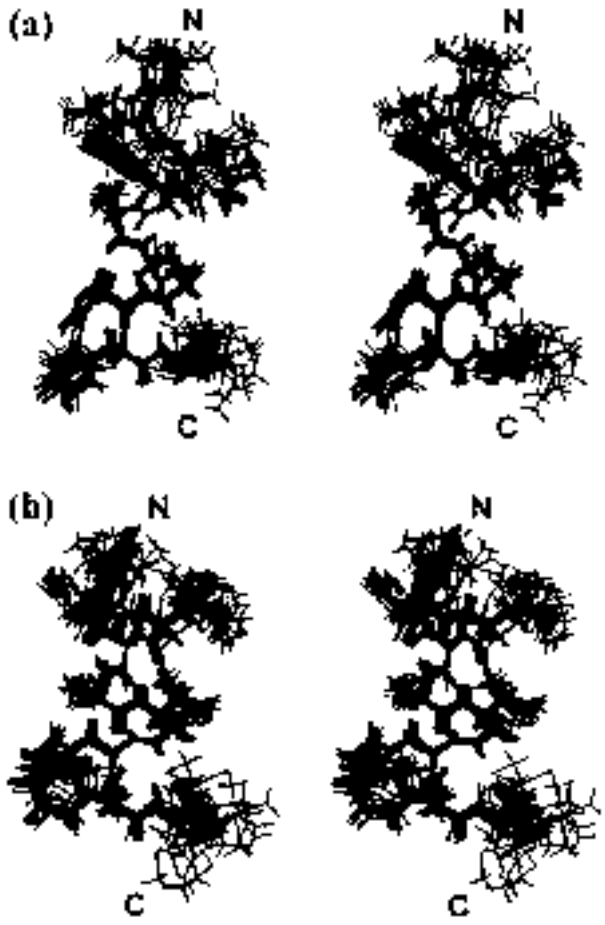

Fig. 3. Stereoview of the structure of NMB a: TFE/H_2O(1:1, v/v) b: 150 mM SDS micelles. All heavy atoms of the Trp^4–Phe^9 region of 20 structures were superimposed with respect to the restrained-minimized average structure.

3. Results and discussion

3.1. Resonance assignments and secondary structures

Using the standard sequential assignment strategy [28], all the proton resonances were assigned. TOCSY and DQF-COSY spectra were used to assign spin systems of most of the amino acid residues and the spin systems of three aromatic residues were identified using the NOE connectivities between Cβ protons and aromatic ring protons. By direct comparison of TOCSY and NOESY spectra, sequence-specific resonance assignments were completed. Table 1(a) and (b) lists the complete assignments of the proton chemical shifts of NMB in TFE/H_2O (1:1, v/v) solution and in SDS micelles. The sequential NOE connectivities in the fingerprint region of NOESY spectra of NMB in TFE/H_2O (1:1, v/v) solution and in 150 mM SDS micelles are illustrated in Fig. 1a and b, respectively.

3.2. The structure of NMB in TFE/H_2O (1:1 v/v) solution

Fig. 2a illustrates the summary of the NOE connectivities of NMB in TFE/H_2O (1:1, v/v) solution, which were extracted directly from a NOESY spectrum recorded with a mixing time of 250 ms. Many medium-range, i.e. $i-i$+2, $i-i$+3 NOE connectivities are observed. $d_{\alpha N}$(i,i+3) connectivities observed from Trp^4 to Met^{10} strongly suggest the presence of helical conformation in NMB. A total of 50 structures were gener-

Table 2
Structural statistics for the 20 final structures of NMB in (a) TFE/H_2O (1:1, v/v) solution and (b) 150 mM SDS micelles

	(a)		(b)	
	$\langle SA \rangle^a$	$\langle \overline{SA} \rangle_r^a$	$\langle SA \rangle$	$\langle \overline{SA} \rangle_r$
rms deviations from experimental restraints				
NOE (Å)	0.0888 ± 0.0024	0.0928	0.0677 ± 0.0003	0.0698
Dihedral angles (deg)	0.597 ± 0.260	1.074	1.020 ± 0.026	0.946
rms deviations from covalent geometry				
Bonds (Å)	0.0064 ± 0.0001	0.0067	0.0083 ± 0.0001	0.0084
Angles (deg)	0.696 ± 0.024	0.785	0.985 ± 0.005	0.976
Impropers (deg)	0.361 ± 0.016	0.356	0.679 ± 0.005	0.771
Energies (kcal mol^{-1})[b]				
E_{tot}	75.81 ± 3.27	83.03	91.25 ± 0.38	95.30
E_{NOE}	37.90 ± 2.02	41.37	29.81 ± 0.24	31.63
E_{cdih}	0.23 ± 0.19	0.63	0.51 ± 0.03	0.44
E_{repel}	8.94 ± 0.75	6.10	2.29 ± 0.05	3.10
E_{L-J}	−25.49 ± 1.12	−25.62	−28.40 ± 1.03	−29.26

[a]$\langle SA \rangle$ represents the 20 final simulated annealing structures $\langle \overline{SA} \rangle_r$ is the restrained minimized mean structure obtained by restrained minimization of the mean structure.
[b]E_{NOE}, E_{tor} and E_{repel} are the energies related to the NOE violations, the torsion angle violations and the van der Waals repulsion term, respectively. E_{L-J} is the Lennard-Jones potential calculated using the CHARMm empirical energy function.

ated by hybrid distance geometry-dynamical simulated annealing algorithm and 20 structures having lowest energies were selected for further analysis. Superposition of the 20 lowest energy structures of NMB in aqueous TFE solution over the all heavy atoms of residue Trp^4–Phe^9 of the restrained-minimized average structure is shown in Fig. 3a. As shown in this figure, most of the atoms including sidechain atoms are well-defined. None of the structures have violations over 0.5Å from the NOE distance restraints and three degrees from dihedral angle restraints and all the structures exhibit good covalent geometry. Table 2(a) lists the structural statistics for the 20 final structures having the lowest energies and the atomic rms differences to the restrained-minimized average structures are summarized in Table 3(a). When we superimpose the final structures to the restrained-minimized average structure on Trp^4-Phe^9 region, backbone atomic rms differences were 0.19Å. All heavy atoms were also converged well and the superimposition of the structures over the residue Trp^4–Phe^9 of the restrained-minimized average structure with respect to all heavy atoms gave the rms differences of 0.54Å.

3.3. The structure of NMB in SDS micelles

NOE connectivities of NMB in 150 mM SDS micelles are presented in Fig. 2b. Many medium-range NOE connectivities such as $d_{\alpha N}(i,i+2)$, $d_{\alpha N}(i,i+3)$ and $d_{\beta N}(i,i+3)$ connectivities are observed from Trp^4 to Met^{10} and these data indicates that NMB forms a helical conformation from Trp^4 to Met^{10}. Small $^3J_{HN\alpha}$ coupling constants and negative chemical shift index (CSI) values [29] also suggested a helical structure from Trp^4 to Met^{10}. Superposition of the 20 lowest energy structures of NMB in 150 mM SDS micelles over the all heavy atoms of residue Trp^4–Phe^9 of the restrained-minimized average structure is shown in Fig. 3b. The restrained-minimized average structure is shown in Fig. 4b. Summarized in Table 2(b) and Table 3(b), respectively, are the structural statistics and atomic rms differences of 20 structures calculated. Similar to the structures in aqueous TFE solution, most of the residues were well-defined in SDS micelles. When we superimpose the Trp^4–Phe^9 region over the restrained-minimized average structure, rms differences were 0.17Å for backbone atoms and 0.67Å for all heavy atoms.

3.4. Interactions between NMB and SDS micelles

More detailed interactions between NMB and micelles were investigated by NOESY experiments in non-deuterated SDS micelles as shown in Fig. 5. As shown in this figure, aromatic ring protons of Trp^4 and Phe^9 are in contact with methylene protons of SDS micelles. Since these aromatic residues orient toward the same direction both in TFE/H_2O solution and in SDS micelles, it can be concluded that the sidechain atoms of these aromatic residues have close contacts with the hydrophobic acyl chains of micelle. Since resonances of methylene protons of SDS are the average of the 18 methylene protons of SDS, the accurate position of the contacts can not be estimated from NOEs. NOEs involving methyl or headgroup protons of SDS micelles were not observed. Also, T_1 longitudinal relaxation times were obtained to investigate the interactions between the SDS and NMB [20]. The correlation time of SDS micelles interacting with NMB should be longer than the correlation time of SDS micelles by itself and T_1 relaxation time should be reduced in the presence of NMB. However, T_1 relaxation time of all the side chain protons in SDS is reduced only by 5%. If we consider the experimental error, 5% is not significant at all. Therefore, it can be con-

Table 3
Atomic rms deviations[a] for the 20 final structures of NMB in (a) TFE/H_2O (1:1, v/v) solution and (b) 150 mM SDS micelles

Superimposing	(a)		(b)	
	Backbone (Å)	Heavy (Å)	Backbone (Å)	Heavy (Å)
Whole peptide	0.55 ± 0.23	0.93 ± 0.28	0.37 ± 0.11	0.83 ± 0.18
Residues 4–9	0.19 ± 0.06	0.54 ± 0.20	0.17 ± 0.05	0.67 ± 0.18

[a]The rmsd values were obtained by best fitting the coordinates of restrained minimized structure to the 20 converged structures for corresponding residues. The numbers given for the backbone and all heavy atoms represent the mean values ± standard deviations.

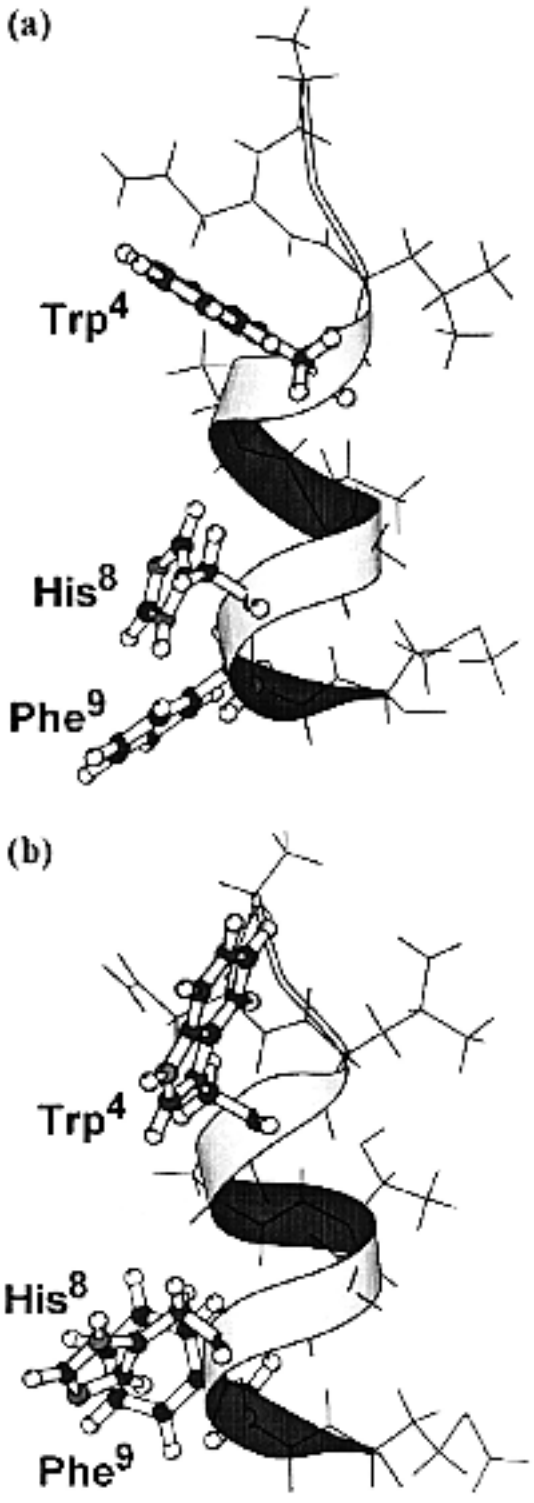

Fig. 4. Ribbon representation of the restrained-minimized average structure of NMB a: TFE/H_2O (1:1, v/v) b: 150 mM SDS micelles. These figures were generated by MOLSCRIPT [34].

cluded that the interactions between NMB and the SDS micelles are mainly weak hydrophobic interactions and the aromatic rings of NMB are not deeply inserted into the hydrophobic core of SDS micelles. There is only close contact between the methylene protons of SDS micelles and the sidechain atoms of Trp^4 and Phe^9 residues of NMB. Since residues in the sequence of NMB are very hydrophobic, it can be expected that there are no strong electrostatic interactions between the charged residues in NMB and the negatively charged SDS micelles. T_1 relaxation data and NOE data observed here represents the averaged values of the free NMB and NMB bound to the micelles and they reveal that the free form of NMB is dominant in SDS micelles.

3.5. Three-dimensional structure of NMB

Restrained-minimized average structures in TFE/H_2O solution and in SDS micelles are presented in Fig. 4. The overall structure of 7NMB in aqueous TFE solution is a 3_{10}-helix from Trp^4 to Met^{10}. In SDS micelles, as shown in Fig. 4b, NMB adopts a 3_{10}-helical structure as expected from the NOE connectivities, $^3J_{HN\alpha}$ coupling constants, and CSI values. In both solutions, the sidechain atoms of Trp^4, His^8, and Phe^9 orient toward the same direction.

The previous studies on bombesin suggested that it adopts a relaxed α-helical conformation in aqueous TFE solution [10], dodecylphosphocholine micelle [11] and lipid bilayer [12]. Recent structural study using circular dichroism, fluorescence and molecular dynamics simulation have suggested that NMB has an α-helical conformation and fluorophore of the Trp^4 residue is exposed to the solvent [14], which is also in accord with our results. Erne and Schwyzer [12] have suggested from IR studies on NMB that in aqueous TFE solution, two hydrogen bonds between the sidechain atoms of Trp^4 and His^8 and between the sidechain atoms of Thr^6 and Met^{10} might be formed, but these hydrogen bonds do not appear in our present structure.

In case of bombesin, the amino acid sequence includes Leu residue instead of Phe residue in NMB and this residue is known to contribute significantly in receptor recognition or binding affinity. The recent results from SAR studies of Ac-bombesin (7–14) using alanine scan [13] has revealed that Trp^8, His^{12} and Leu^{13} residues of bombesin which corresponds to Trp^4, His^8 and Phe^9 in NMB, respectively, are of primary importance in binding to NMB receptor. And, a recent site-directed mutagenesis study on NMB receptors suggest that four amino acids of NMB receptor, which are located in the extracellular loops and transmembrane helices near the membrane solution interface are critical for binding of NMB to NMB receptor [30]. Therefore, it is likely that the residues of NMB involved in binding to NMB receptor, i.e. Trp^4 and Phe^9 must be located in the same side or in close positions and that NMB approaches to its receptor in parallel orientation. Results from the present study indicate that the C-terminal part of NMB has an ordered helical structure in membrane-mimicking environments, while the N-terminal part of NMB has a rather random structure, and that sidechain atoms of Trp^4, His^8 and Phe^9 orient toward the same direction, rendering some amphipathic characters. These structural features which seem to be essential for the biological activity will probably be maintained until NMB approaches its receptor. Several reports suggested that there might be a conformational change to a β-turn type structure upon binding to the receptor [31–33] and the structure in the present study suggests that NMB, interacting with membrane surfaces, has a helical conformation in membranous solutions. It is certain that the development of potent receptor agonist or antagonist would be significantly improved with the aid of our results.

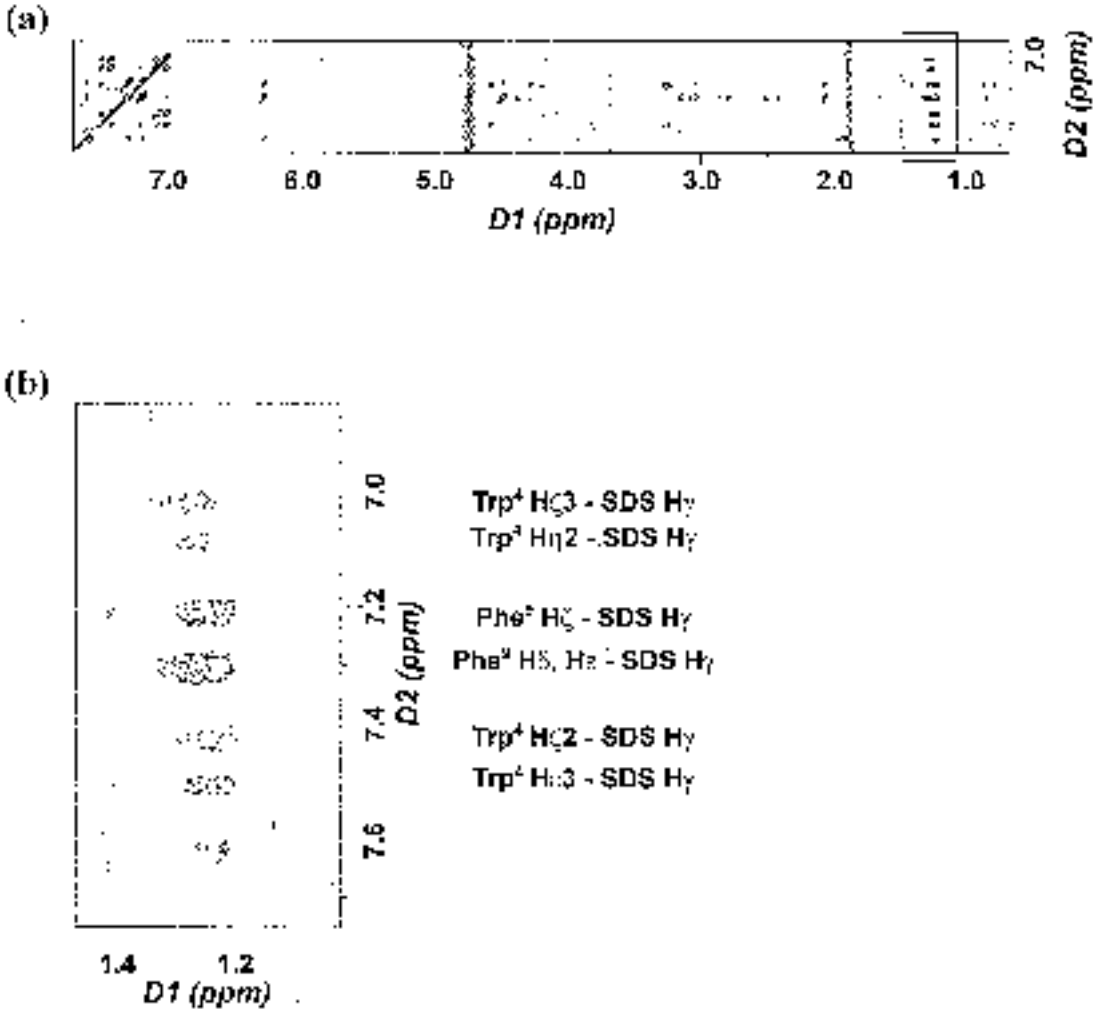

Fig. 5. a: NOESY spectrum of NMB in 15 mM SDS micelles recorded with a mixing time of 650 ms. b: Magnification of the region of NOESY spectrum in which the intermolecular NOEs are observed. NOE contacts between the aromatic ring proton of Trp^4, Phe^9 and methylene proton of SDS are indicated.

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