論文の内容の要旨

NMR study on a reconstituted transmembrane segment of channel protein for its complex model with a lipid soluble natural product

(脂溶性天然物との複合体モデルに向けた チャネルタンパク質膜貫通断片再構成系のNMR研究)

氏名　吉中 藍

Introduction

The interaction between small molecules and membrane proteins is a significant topic from the biological and pharmaceutical point of view. Detailed information on interaction mechanisms can be a guide for clarifying functions and structural features of membrane proteins, modifying small molecules as medicinal drugs, and searching for new drug targets on the protein. However, few successful interaction analyses at the level of molecular structure using full-length membrane proteins have been reported to date because there are still crucial problems. Namely, membrane proteins generally require individually optimized treatment, it is difficult to crystallize membrane proteins with phospholipid, and NMR spectra of membrane proteins usually have low resolution due to low mobility in lipid bilayer. To obtain information on interaction mechanisms while avoiding these problems, I tried interaction analysis using the molecular complex of a transmembrane fragment of a membrane protein and a natural product with NMR techniques.

In this research, domain IV segment 6 (DIVS6) of voltage-gated sodium channel (VGSC) and a neurotoxin veratridine (Figure 1) were chosen for the model system. Voltage-gated sodium channel is responsible for generating action potential in the nervous system. The primary structure of VGSC which consists of α (260 kDa), β1 (36 kDa), and β2 (33 kDa) subunits indicates four homologous domains of six α-helical segments each in α subunit. Veratridine, a lipid soluble alkaloid extracted from lily *sabadilla* seed, is considered to bind segment 6 of each domain and excites VGSC as a partial agonist. DIVS6 has the largest number of interactive residues among four segment 6 as suggested by point mutation experiments of VGSC.

Transmembrane model peptides (TMPs) corresponding to the sequence of DIVS6 (Table 1) were synthesized on solid phase. TMP1 and 2 dissolved in aqueous 2,2,2-trifluoroethanol (TFE) known as a helix-promoting solvent were analyzed firstly by solution NMR to confirm the interaction within the

model system. I started my Ph.D. course study from NMR structure analysis of TMP1 and 2 in the solvent. Secondly, TMP3 reconstructed in dimyristoylphosphatidylcholine (DMPC) liposomes was analyzed by solid state NMR.

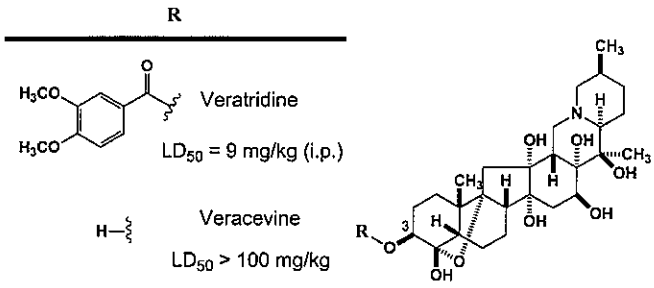

Figure 1. Structures of veratridine and veracevine

Table 1. Amino acid sequences of DIVS6 and TMPs

	1	6	11	16	21	26
DIVS6	GICFF	CSYII	ISFLI	VVNMY	IAIIL	ENF
TMP1		SKII	KSFLK	VVNKY	IAIIL	E
TMP2		KKK SYII	ISFLI	VVNMY	IAIIL	E KKK
TMP3	KKK GICFF	CSYII	ISFLI	VVNMY	IAIIL	ENF KKK

underline : interaction site suggested by point mutation study

Analysis in solution

Peptide synthesis and NMR titration experiments with neurotoxic veratridine (VTD) and its non-toxic analog veracevine (VC) were performed in my master course study. Especially in the titration experiment on TMP1, the observed chemical shift perturbation pattern was same between VTD and VC. VTD and VC share the common steroidal backbone structure but VTD has 3-acyl group which plays an important role for the neurotoxicity. The results suggest that the steroidal backbone of VTD interacts to DIVS6, and neurotoxicity of VTD is caused by interaction to another segment located near DIVS6 such as DIVS4 or DIS6 in the neuronal membrane.

In the next step, structures of TMP1 and 2 were calculated using NMR data and the simulated annealing software package XPLOR-NIH (version 2.21). Distance restraints derived from NOE peak intensities and dihedral-angle restraints derived from coupling constants were used to refine the structure. Refined and accepted structures of TMP1 and 2 were given by calculations repeated 20 times and superimposed for each (Figure 2). Superimposed structures of TMP1 (Figure 2a) show that the center part of the peptide forms α-helix while N-terminus does not converge to a single conformation. Thus N-terminus of TMP1 does not take rigid conformation in the mixture of TFE/H_2O = 1/1. In the titration experiment on TMP1, the amide proton signal of Lys8 disappeared. This disappearance indicates that structure of N-terminus was changed by interaction with VTD. The result from the structure calculation where N-terminus of TMP1 fluctuates supports this consideration. Thus Lys8 and surrounding residues were eliminated from interaction site candidates. The structures of TMP2 (Figure 2b) shows that N-terminal three lysines do not form α-helix and slightly fluctuate as TMP1.

Interacting residues suggested by titration experiments with VTD are shown on calculated structures in Figure 3. In TMP1, chemical shift perturbation was observed at the hydrophobic side of the helix, which is consistent with point mutation experiments. Therefore VTD is expected to interact with TMP1 in a similar manner to interaction with VGSC. Combined with the results in titration of VC, it is possible that this hydrophobic side faces other segment which is related to activation by VTD. Further interaction analysis on other segments and VTD is expected to give information on tertiary structure of VGSCs (Figure 4).

As for TMP2, VTD could interact to N-terminus region of the peptide. A noteworthy point is the chemical shift perturbation at the amide proton of Leu14 of both TMP1 and 2. Leu14 is thus suggested to be the key residue for interaction between VTD and TMPs. Leu14 of TMPs is L1580 in the sequence of a rat skeletal muscle VGSC, Nav1.4 (μ1). In point mutation experiments, μ1-L1580 was not included in required residues for binding. NMR experiments in this study indicated μ1-L1580 as a new possible VTD binding site.

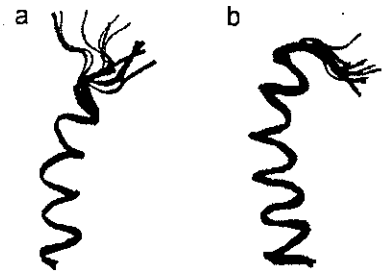

Figure 2. Superimposed structures of TMP1 (a) and TMP2 (b)

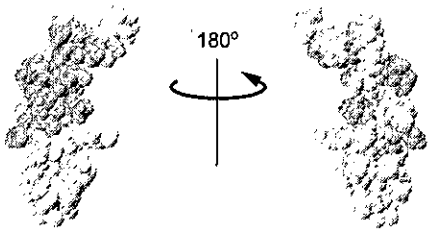

Figure 3. Suggested interaction site (colored) on the calculated structure of TMP1

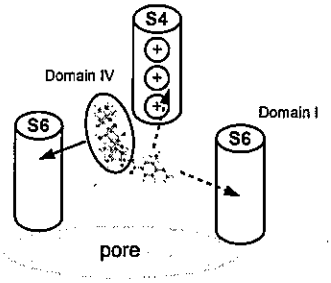

Figure 4. Suggested interaction model

Analysis in lipid bilayer

Peptide synthesis

Since native sequence of DIVS6 is too hydrophobic for normal conditions of peptide synthesis and purification, three lysines as hydrophilic amino acids are added on each terminus of the DIVS6 sequence at the design of TMP3 also to direct the helix perpendicular to lipid bilayer. While unlabeled TMP3 was synthesized on solid phase peptide synthesizer for CD measurement, ^{13}C, ^{15}N-labeled TMP3 for solid state NMR measurements was synthesized manually on solid phase to achieve high yield (approximately 10%, total 34 coupling cycles including a few repetitions).

Optimization of reconstitution conditions

The amino acid sequence of DIVS6 predicts that DIVS6 forms α-helix in lipid bilayer. To reconstitute TMP3 in lipid bilayer with α-helical conformation, conditions frequently used to reconstitute membrane associated peptides and membrane proteins were screened and evaluated with CD and solid state NMR analysis. The conditions can be separated into two methods, namely f dialysis and thin-film hydration. Conditions of dialysis resulted in the mixture of α-helix and β-strand in one sample. The sample where TMP3 forms α-helix uniformly in DMPC liposomes was obtained with one of the conditions of thin-film hydration as follows: TMP3 dissolved in hexafluoroisopropanol and DMPC dissolved in chloroform (molar ratio of peptide : lipid = 1 : 25) were mixed at the same volume, dried on vacuum line after solvent evaporation, and then hydrated with 5 mM phosphate buffer (pH 7.4) by vortex mixing.

Solid-state NMR measurement

First, cross polarization (CP) experiments at various temperatures were performed to confirm that TMP3 is reconstituted properly in lipid bilayer. CP which is one of the important techniques in solid state NMR enhances signals of dilute spins such as ^{13}C or ^{15}N by transferring polarization from abundant spins such as ^{1}H. This cross polarization produces the effect only to molecules with low mobility. So CP spectra can indicate the mobility of peptides in lipid bilayer.

1D CP spectra at three temperatures are shown in Figure 5. In the CP spectra, signals of TMP3 were observed at –60°C where sample is completely frozen, and at 10°C where DMPC is in gel phase and only molecules outside of lipid bilayer have high mobility. On the other hand, signals disappeared at 25°C where DMPC is liquid crystal phase. These CP experiments showed that mobility of TMP3 in the sample correlated with phase transition of lipid. To obtain more detailed information of TMP3 with lipid bilayer, ^{13}C (peptide)-observed ^{2}H (lipid)-selective ^{1}H (peptide)-demagnetization (CODSHD) experiment and water-edited CP experiment were performed. Signal intensities of a CODSHD spectrum reflects the distance between deuteriums of DMPC and protons of TMP3. The spectrum showed that most of TMP3 is located within lipid bilayer. A water-edited CP spectrum shows the distance between water and protons of TMP3. This experiment indicated that center part of TMP3 is located at the center of lipid bilayers. Thus it is confirmed that TMP 3 was reconstituted in lipid bilayer. Subsequently, 2D ^{13}C-^{13}C (DARR) experiments were performed to assign carbon signals and to obtain constraint data for structure calculation. The 2D DARR spectrum with reasonable resolution for signal assignment were obtained at –60 °C with magic angle spinning (MAS) at 12.5 kHz and resonance at 175 MHz (Figure 6).

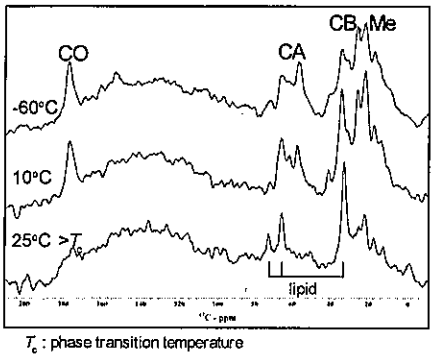

Figure 5. 1D CP spectra of TMP3

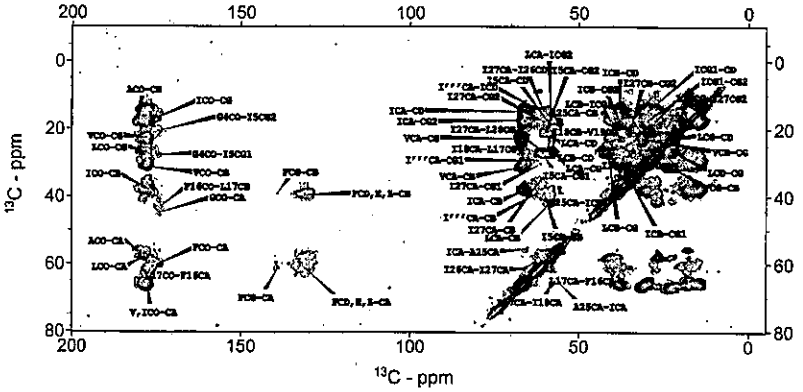

Figure 6. 2D DARR spectrum of TMP3 with signal assignments

Structure calculation

Solid state NMR spectra of uniformly isotope labeled peptides or proteins have usually too low resolution to obtain structural constraints such as distances and dihedral angles. While site specific isotope labeling can simplify the spectra, a small number of structure constraints limits the calculative determination of whole molecular structure. Thus, combination of NMR experiments and computer simulation is needed. In this research, combination of NMR and replica exchange molecular dynamics (REMD) simulation with an implicit membrane model was applied to structure determination of TMP3 in DMPC liposomes.

REMD simulation generated transmembrane α-helical structure as major conformational state at 300 K. The generated structures were subjected to ^{13}C chemical shift prediction by SHIFTX. Backbone of NMR structures of TMP3 was selected from major conformational state by comparison of predicted and observed chemical shifts. Subsequently, orientation of the selected backbone structures with lipid bilayer was evaluated with spectral calculation of CODSHD and water-edited CP. The structure whose 1D simulated spectra was consistent with observed spectra was selected as the NMR structure. Finally structure of TMP3 in lipid bilayer was obtained from REMD simulation (Figure 7). The obtained structure and the crystal structure of pore segments of ion channels showed some similarities.

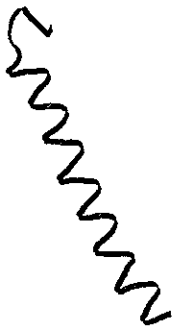

Figure 7.
An NMR structure of TMP3 calculated by REMD

Conclusion

Analysis in solution

NMR titration experiments using TMP1 with VTD and VC indicated that the steroidal backbone of VTD can interact to DIVS6 and 3-acyl group of VTD can cause neurotoxicity by probably interaction to another segment. Structures of TMP1 and 2 in a helix-promoting solvent were obtained by simulated annealing method. Combination of the calculated structure and results of titration experiments indicated the perspective of interaction: a slight structure change can occur around Lys8 by interaction with VTD due to fluctuation at N-terminus of TMP1; the possible interaction site of TMP1 shown on the calculated structure is located at hydrophobic side of α-helix; Leu14 (μ1-L1580) in VGSC was newly suggested as VTD binding site. The interaction manner of VTD suggested by titration experiments agreed with the expected manner by competitive binding study and point mutation experiments reported before. Obtaining the interaction information by NMR analysis in solution phase using model system was achieved.

Analysis in lipid bilayer

Reconstitution of TMP3 to lipid bilayer was achieved by screening a broad array of conditions. CP, CODSHD and water-edited CP experiments on solid state NMR indicated that TMP3 was reconstituted within lipid bilayer. The reconstituted TMP3 gave 2D DARR spectra with assignable resolution. The NMR structure of TMP3 was obtained by using combination of NMR experiments and REMD simulation with implicit membrane model. Reconstituted TMP3 gave the structure information necessary for interaction analysis using model system.