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A heteronuclear and homonuclear filtering strategy for studying the structure of membrane peptides in non-deuterated phospholipid vesicles

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RÉSUMÉ

L'étude par RMN des biomolécules membranaires est délicate en raison de leur faible solubilité. Une stratégie d'étude a été élaborée pour obtenir des informations structurales de peptides dans un environnement de vésicules de phospholipides non deutérés. Elle repose sur des filtres isotopiques de type HSQC-NOESY et des filtres homonucléaires avec impulsion sélective, avec une suppression fine de l'eau. Un exemple est donné sur un neuropeptide membranaire de 11 résidus : la substance P.
mots-clés : peptides membranaires, phospholipides non deutérés, filtres RMN.

ABSTRACT

NMR study of membrane biomolecules comes up against a poor solubility in classical solvents. A strategy was elaborated to obtain structural information of peptides in non deuterated phospholipids vesicles. It is based on isotopic (HSQC-NOESY) and homonuclear selective filters, both using a fine water suppression. The method is illustrated with the substance P, a 11-residue membrane neuropeptide.
key words : membrane peptides, non-deuterated phospholipids, NMR filter.

Few studies by high resolution multidimensional NMR have been carried out on membrane compounds due to a poor solubility in standard solvents. The first studies were performed using solid state NMR which supplied information on the environment and the local dynamics of specific enriched atoms. Previous structural studies[1] in organic detergents, micelles, multilayers of phospholipids, or artificial membranes of phospholipid vesicles were carried out using 1D ^{13}C NMR, 2D Dante-Z MAS NMR, transferred NOE method. They supplied some structural information about the internal environment, the structure of peptides in non-deuterated glucosidic micelles[2] and in synthesised perdeuterated phospholipid vesicles.

However the best model for the membrane remains the phospholipid vesicles. In high resolution NMR, using an undeuterated solvent raises numerous problems. The major one is the access to the signals of the peptide or the protein hidden by the large signals of the membrane. The second problem comes from the restricted mobility of the ligand. The lifetime of the vesicles complex is short, inferior to 24 hours due to an irreversible coprecipitation. A low concentration (~1 mM) is also necessary for the stability of the complex. Finally, the water signal must be efficiently suppressed. These problems result in a poor sensitivity of the ligand signals and require a specific strategy.

The present study developed a strategy for obtaining structural NOE information and interactions with the medium, of membrane molecules incorporated into an artificial non-deuterated vesicles membrane. We propose a protocol based on ^{13}C and/or ^{15}N isotopic labelling combined with filtering sequences and on selective homonuclear filtering experiments.

NMR methods

The single and double isotopic Xω filtering sequences[3] incorporated in pseudo-3D-X-filter-type TOCSY/NOESY sequences are suitable for structural studies on ligands in a complex with a macromolecule. Since the heteroatoms were here selectively reduced to ^{15}N or $^{13}C\alpha$ by peptide synthesis, the X atom frequency was set in dimension ω_1 with an $X\omega_1$ type filter to supply a sufficient good resolution. Xω1/ Xω2 filters, HMQC and HSQC, combined with NOESY sequences were assessed to obtain a suitable sensitivity and good water suppression. Simultaneously, water suppression by presaturation or WATERGATE[4] was optimised. The differences of the X filters depend on the sensitivity and water suppression properties. Both HMQC and HSQC are more efficient. HMQC goes through a heteronuclear multiple quantum coherence step which contributes further eliminates the undesirable signals. HSQC contains "two" filters with INEPT and reverse INEPT. In HSQC type sequence, less signal is lost due to relaxation of the single quantum coherences and passive homonuclear couplings do not interfere in

ω_1. Water can also be better suppressed by adding a spin lock during the INEPT part due to T_2 relaxation. A WATERGATE type sequence provides more efficient water suppression when combined with a Z water flip back of the magnetisation introduced before the reading pulse. In order to obtain further information about the interactions with the vesicles, a selective NOESY experiment was run with an off-resonance excitation of the lipids region.

The membrane peptide used was the Substance P (RPKPQQFFGLM). It was synthesised, $^{13}C\alpha$ or ^{15}N selectively labelled and introduced to a concentration of 1.3 mM. The phospholipids were PC and PS 4:1 introduced at 6.66 mg/ml in stable small unilamellar vesicles (SUV). The vesicles were prepared less than 12 hours before use and NMR experiments were run as soon as the complex was formed. The spectra were acquired at 300K on a DMX 500 MHz Bruker spectrometer equipped with a TBI $^1H/^{13}C/^{15}N$ 8mm Z gradient probe. The Z flip back of the water magnetisation was obtained by a E-burp pulse. The final pseudo 3D HSQC-NOESY spectra were recorded with τ_m=300ms, water Z flipback, a 1.5ms spin-lock during the INEPT part, 10 (^1H) and 20ppm (^{13}C) spectral widths leading to an experimental time of 10 hours.

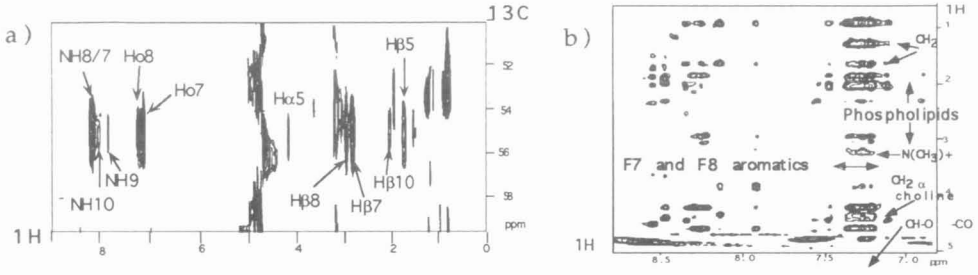
RESULTS

$^{13}C\alpha$-F8/^{15}N-G9 Substance P in perdeuterated micelles.

The NMR experiments were optimised in 80mM perdeuterated micelles. The vesicles cause a 2.3 fold experimental loss in signal-to-noise ratio and the experimental time must be less than about 15 hours. In the micelles it had to be optimised to less than $15/2.3^2$~3 hours. The previously described X filters sequences were compared for their sensitivity. The expected NOE crosspeaks were monitored from ^{15}N G9 and $^{13}C\alpha$ F8 and integrated. The HSQC sequence suppressed water magnetisation 10-fold more efficiently than the Xω-presaturation type experiments. The WATERGATE sequence also suppressed the huge water signal and avoided the initial bleaching of the ^1H α- resonances close to the water peak that occurs when presaturation was applied. It also prevents the loss of exchangeable amide proton.

The 8 mm probe allowed the S/N ratio to be doubled while maintaining a necessary low concentration. The final experimental time was thus reduced to 3 hours. HMQC type sequence supplied a similar result with a 10% inferior signal to noise ratio.

^{13}Cα-F7,F8/^{15}N-G9 Substance P in non-deuterated phospholipid vesicles. The above sequence was applied to a 1.3 mM ^{13}Cα-F8, F7 and ^{15}N-G9 Substance P in non-deuterated vesicles. ^{15}N- and ^{13}C-filtered spectra showed NOEs expected for a helical or U-turn structure : NN(i,i+1), HαHβ(i, i+3). The Hα chemical shifts moved to +1 Wishart index supporting the evidence for a helical type structure.



Figures a) HSQC-NOESY spectrum of ^{13}CαF7 SP b) Selective NOESY spectrum of SP

This is in agreement with recent dynamic calculations[5] on Substance P in DMPC vesicles which suggested a helical conformation. Moreover selective ^{1}H excitation of the off-resonance region of the lipids signals provided NOEs interactions between the aromatic side chains of SP with the surface phospholipids.

To conclude, we have shown that the 2D HSQC(t_1)-NOESY WATERGATE sequence is a sensitive sequence with a good water suppression to filter out signals from ^{13}Cα or ^{15}N-labelled membrane peptide placed in non-deuterated phospholipids vesicles. The method showed the helical/turn structural motifs of the Substance P and illustrated the feasibility of NMR studies on peptides in interaction with artificial non deuterated membranes[6]. Homonuclear selective filtering showed the interactions with the vesicles and provided some information about the surface position of the peptide in the membrane.

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