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Neurotoxin II Bound to Acetylcholine Receptors in Native Membranes Studied by Dynamic Nuclear Polarization NMR

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Supporting Information

ABSTRACT: Methods enabling structural studies of membrane-integrated receptor systems without the necessity of purification provide an attractive perspective in membrane protein structural and molecular biology. This has become feasible in principle since the advent of dynamic nuclear polarization (DNP) magic-angle-spinning NMR spectroscopy, which delivers the required sensitivity. In this pilot study, we observed well-resolved solid-state NMR spectra of extensively ^{13}C-labeled neurotoxin II bound to the nicotinic acetylcholine receptor (nAChR) in native membranes. We show that TOTAPOL, a biradical required for DNP, is localized at membrane and protein surfaces. The concentration of active, membrane-attached biradical decreases with time, probably because of reactive components of the membrane preparation. An optimal distribution of active biradical has strong effects on the NMR data. The presence of inactive TOTAPOL in membrane-proximal situations but active biradical in the surrounding water/glycerol "glass" leads to well-resolved spectra, yet a considerable enhancement ($\varepsilon = 12$) is observed. The resulting spectra of a protein ligand bound to its receptor are paving the way for further DNP investigations of proteins embedded in native membrane patches.

Membrane receptors (MRs) are primary targets for pharmaceutical interventions. Nevertheless, many structural details of their interactions with natural or artificial ligands remain unknown, especially as crystallization for X-ray investigations in native membranes is still difficult. Furthermore, a special membrane environment is often required for proper function. Therefore, it is desirable to conduct structural investigations of heterologously expressed MRs in their natural context without the need of any detergent extraction or refolding procedures.

A prominent example of an MR is the nicotinic acetylcholine receptor (nAChR) system,[1−3] which is a ligand-gated ion channel found in postsynaptic membranes of the central nervous system and the neuromuscular junctions. It is involved in many (patho)-physiological processes, such as muscle innervation and epilepsy.[4] Important ligands for the study of nAChRs are toxins such as neurotoxin II (NTII),[5,6] a short α-neurotoxin from an Asian cobra (*Naja naja oxiana*).[7] It binds in the picomolar range[8] to the extracellular site of muscle-type nAChRs. Functional homologues of α-neurotoxins have been found in the central nervous systems of mammals and are thought to be modulators of nAChR function.[9] Structural studies on the nAChR system in native membrane patches by magic-angle-spinning (MAS) solid-state NMR spectroscopy (ssNMR)[8,10] appear to be feasible in principle because the receptor is highly concentrated in the electric organ of the pacific electric ray (*Torpedo californica*).[11] Nevertheless, the concentration is still too low to derive a receptor-bound structure of NTII by conventional ssNMR investigations; weeks of measurement time are needed to acquire even a simple C−C correlation spectrum of membrane patches containing the nAChR/NTII system.[10] In principle, this situation is similar to samples of overexpressed membrane proteins in host organism membranes. For example the binding of D-glucose to the *Escherichia coli* sugar transport protein GalP has been investigated by ssNMR, where experiment times of several days to weeks were also needed.[12]

The use of dynamic nuclear polarization (DNP)[13,14] can enhance the NMR signal by up to 2 orders of magnitude (as indicated by ε, the scaling factor of the intensities with and without DNP) and might help overcome the sensitivity problem. This technique transfers the high polarization in spin systems of unpaired electrons to nuclei of interest using matched microwave irradiation.[15] As an experimental complication, cryogenic temperatures are needed for this transfer, with the danger that different conformations of the analyte are frozen out. The NMR signals then appear inhomogeneously broadened to an extent that depends on the flexibility and water exposure of the corresponding residues.[16] However, resolved DNP signals have been recorded for peptides in nanocrystals,[17] retinal in bacteriorhodopsin,[15,18,19] and a core segment of GNNQQNY fibrils[20] with line widths approaching 1 ppm. All of these systems are highly ordered, but DNP results for proteins in native membrane patches are still lacking.

In this study, we investigated the NTII/nAChR complex in native membranes. For this purpose, receptor-containing membrane patches were prepared from the fresh electric organ of *T. californica* through several steps of homogenization followed by a sucrose-gradient centrifugation, yielding receptor-rich membrane patches.[10,21] The neurotoxin was expressed in *E. coli* and isotopically labeled with ^{13}C, either uniformly or following the [1,3-^{13}C]- or [2-^{13}C]glycerol labeling scheme,[22] and always uniformly with ^{15}N. Neurotoxin and membrane patches were incubated and concentrated by centrifugation. A 15 μL sample of the solid pellet was added to 10 μL of solvent (60% glycerol-d_8, 30% D_2O, 10% H_2O) containing the indicated amounts of the biradical TOTAPOL,[23] and the mixture was manually stirred with a small wire and then frozen at 100 K. All spectra were obtained on a Bruker AVANCE III 400 MHz spectrometer with a MAS frequency of 8889 Hz. A Bruker DNP cryoprobe for 3.2 mm rotors tuned to HCN was used. The DNP effect depends upon the

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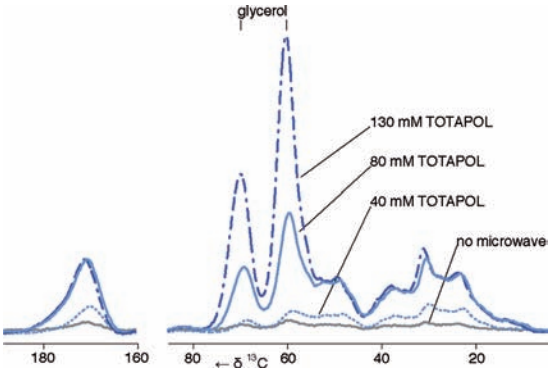

Figure 1. Effect of the TOTAPOL biradical concentration on 1D carbon spectra of uniformly labeled NTII bound to nAChR. The spectrum without microwave irradiation corresponds to a non-DNP spectrum of the same sample at cryogenic temperature. The spectra were recorded with a H−C CP time of 1500 ms, 128 scans, an acquisition time of 25 ms, and a recycle delay of 4.7 s (no microwave, c_T = 40 mM) or 2.4 s (c_T = 80, 130 mM) at 101 K.

concentration of TOTAPOL (c_T; Figure 1): at low c_T (40 mM), the signals of the bound ligand and the lipids were enhanced more than those of the frozen solvent (glycerol ε, 5 ± 0.5; protein/lipid ε, 8 ± 1). Increasing c_T to 80 mM equalized the two measures (glycerol ε, 24 ± 1; protein/lipid ε, 20 ± 1). Finally, at c_T = 130 mM, only the glycerol signals increased, while the protein signals remained unchanged (glycerol ε, 62 ± 2; protein/lipid ε, 21 ± 1). This provides evidence that TOTAPOL was initially localized at membrane and protein surfaces. Hence, at low c_T, the polarization transfer preferentially enhances membrane and protein signals, whereas with increasing c_T, the enhancement of additional molecules such as buffer components also reaches a maximum. In our case, c_T = 60−80 mM appears to be best for optimal enhancement of the protein signals.

In preparation for 2D NMR experiments, we measured the DNP buildup time (T_{DNP}) for ^{13}C by applying direct excitation of ^{13}C in a saturation delay experiment using 50 mM TOTAPOL (see Figure SI A in the Supporting Information). The carbonyl signals had the shortest T_{DNP} of 21.6 s. The overall T_{DNP} of aliphatic signals (28.9 s) was very similar to that of the lipid signals (28.5 s), while the glycerol had the longest T_{DNP} (40.9 s). This is further evidence that the radical is localized at the membrane surface and therefore that more time is needed to transfer the polarization throughout the frozen solvent. However, as most ssNMR spectra are measured via H−C cross-polarization (CP)[24] and $T_{DNP} \approx 1$ s for the protons (see Figure SI B), the practical consequences for measurements are small. Nevertheless, this observation reinforces the idea that an optimal c_T can suppress the solvent signals.

The 2D proton-driven spin diffusion (PDSD) DNP spectra[27,28] of uniformly labeled NTII bound to the nAChR recorded at 100 K reveal many similarities to room temperature solid-state spectra (see Figure SI C). However, the peaks are inhomogeneously broadened[16] with many signals overlapping or covered by broad diagonal peaks. Interestingly, threonine signals that are intense at room temperature are missing in the DNP spectrum (see Figure SI Cc), probably because most of the six threonines are located at the solvent-exposed side of the toxin (see Figure 4). This effect might be explained by two mechanisms: (i) The side chains, especially the methyl groups, undergo microsecond-range dynamics that interferes with proton decoupling, and the slowed dynamics may lead to strong spectral broadening.[16,17]

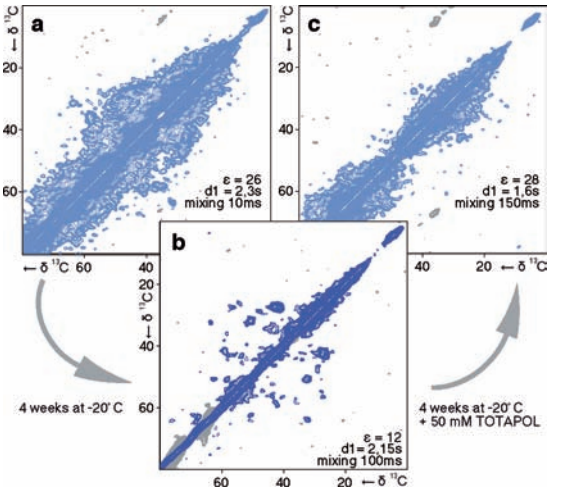

Figure 2. 2D ^{13}C−^{13}C spectra of [2-^{13}C]glycerol-labeled NTII bound to nAChR as a function of the concentration of active biradical. The first spectrum (a) was recorded with 50 mM TOTAPOL (0.625 mmol). After 4 weeks at −20 °C, the enhancement (ε) was reduced by 50%, but the resolution improved remarkably (b). After another 4 weeks, the enhancement was only 6 (spectrum not shown), and another 0.625 mmol of TOTAPOL was added (c). All changed parameters are shown in the figure. All spectra used H−C CP with a CP time of 1000 μs, 32 scans, and acquisition times of 30/8.5 ms (ω_2/ω_1) at 100 K. All spectra were processed with a Gaussian window function in the direct dimension (line broadening of 30 Hz, Gaussian offset of 0.06) and with a mixed sin^2/cos^2 window function in the indirect dimension (see the SI) and scaled to 5 times the calculated noise of 2000 points, as calculated with Sparky.[25]

(ii) Also, the close proximity to the biradicals located at the membrane surface leads to paramagnetic relaxation enhancement (PRE),[15,29,30] which increases the line width greatly.

To ease the analysis of cross-peak patterns in the spectra of uniformly labeled NTII, we recorded DNP spectra of a [2-^{13}C]glycerol-labeled sample (Figures 2 and 3). The following aliphatic carbons were ^{13}C-labeled in such samples with greater than 50% efficiency: K ($\alpha, \gamma, \varepsilon$); L ($\beta, \gamma$); V ($\alpha, \beta$); I ($\beta, \delta$); and P ($\beta, \delta$).

In contrast to samples of proline and SH3 with similar TOTAPOL concentrations, whose enhancements remain unchanged over months,[31] the enhancement of our NTII samples decreased by a factor of 2 after handling upon measurement and storage for 4 weeks at −20 °C (Figure 2). We interpret this loss in enhancement as a result of chemical reduction of the radicals located close to the membrane and protein surfaces, most probably through reactions with thiol groups of cysteines.[32] This would explain the lack of the observed reduction in samples of proline and SH3, which do not contain cysteines. This might also explain the relatively high optimal c_T relative to samples of proline or SH3 (data not shown).

The spectrum recorded directly after sample preparation (Figure 2a) is a typical DNP protein spectrum with broad diagonal peaks. After storage, ε decreased from 26 to 12, but remarkably well-resolved spectra were recorded (Figure 2b). After another four weeks, the enhancement was only 6, so another 0.625 mmol of TOTAPOL was added, including thawing of the sample. The spectrum was again poorly resolved and had a broad diagonal (Figure 2c). We could exclude the long mixing time of Figure 2b as main source of the narrow diagonal signals, because a shorter mixing time of 50 ms in the same sample state showed a comparable diagonal. Furthermore, the spectrum in Figure 2c was recorded with an even longer mixing time of

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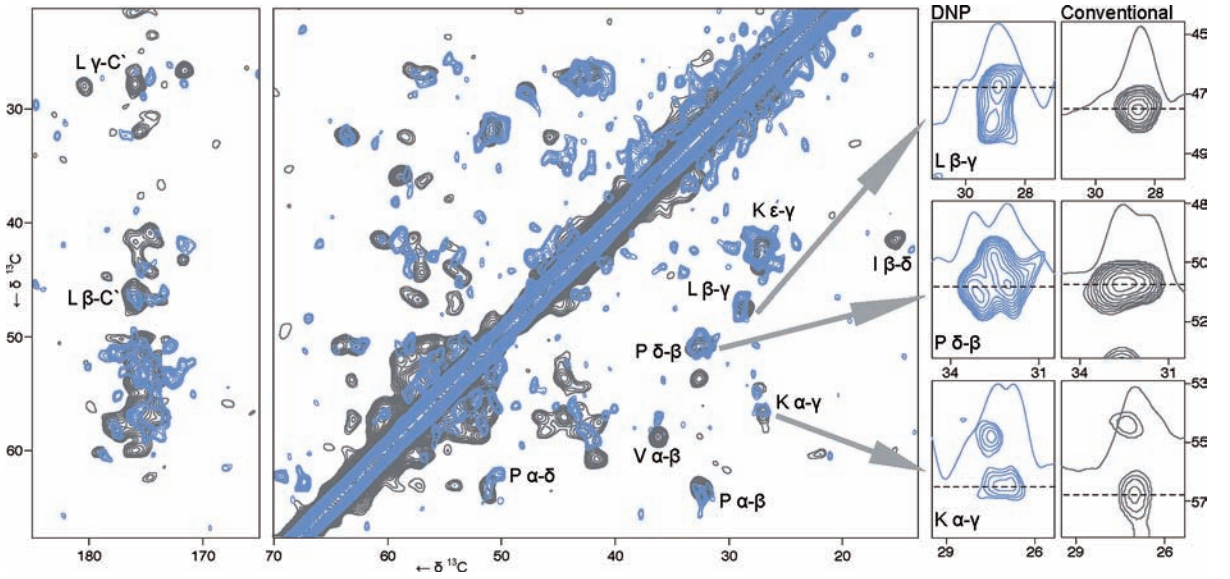

Figure 3. 2D PDSD $^{13}C-^{13}C$ spectra of [2-^{13}C]glycerol-labeled NTII bound to nAChR, recorded with DNP at 100 K in 14 h (blue) and with conventional ssNMR at 265 K in 9 days (gray), both at a proton frequency of 400 MHz. The DNP spectrum was recorded after 4 weeks of storage at −20°, corresponding to Figure 2b. At the right are magnifications of several cross-peaks with DNP (blue) and conventional ssNMR (gray). The 1D spectra represent the slices at the dotted lines. The DNP spectrum was recorded using HC CP with a CP time of 1000 μs, a PDSD mixing of 100 ms, 32 scans per row, and acquisition times of 30/8.5 ms (ω_2/ω_1). The delay time was 2.15 s and the spinning frequency 8889 Hz. The direct dimension was processed with a Gaussian window function (line broadening of 15 Hz, Gaussian offset of 0.1) and the indirect dimension with a mixed $\sin^2/\cos^2$ window function (see the SI). The parameters of the conventional spectrum were as follows: CP time, 1000 μs; PDSD mixing time, 300 ms; 512 scans; acquisition times, 15 × 9.4 ms; delay time, 2.55 s; spinning frequency, 9500 Hz. It was processed similarly to the DNP spectrum but with a line broadening of 40 Hz (offset of 0.07) and a $\cos^2$ window function; otherwise cross-peaks were unobserved.

150 ms but also showed a broad diagonal; when this spectrum was deliberately measured with a short delay time (data not shown), it did not show any differences relative to the spectrum recorded with the optimal delay time. Therefore, the changed concentration of active biradical appears to be the main factor in the observed change in resolution.

The spectrum in Figure 2b is remarkably well resolved, and an assignment of spin systems to individual residue types is possible using the known room-temperature and solution chemical shifts (Figure 3 and Figure SI D). In comparison with the conventional C−C correlation spectrum recorded in 9 days, some peaks appear to be better resolved in the DNP spectrum recorded in just 14 h. For example, all four proline Cβ−Cδ cross-peaks of NTII appear in the DNP spectrum, while only two proline cross-peaks were detected in the conventional spectrum (Figure 3 right, second row). More importantly, the number of observed cross-peaks does not exceed the number of residues of the respective kind. When the conventional spectrum was processed in the same way as the DNP spectrum, the line widths were smaller (e.g., for the leucine Cβ−Cγ peak, 0.85 ppm vs 1.32 ppm with DNP), but almost no cross-peaks were detectable. On the other hand, line widths of 1 and 1.2 ppm (observed for carbons of bacteriorhodopsin at a similar field) have been reported as the narrowest resonances of a membrane protein in DNP/MAS experiments to date.[15]

The observed signals result mostly from residues in the binding site of NTII (see Figure 4). This might explain their relatively narrow and not inhomogeneously broadened lines, as they are hindered in motion. We associate the overall good resolution of the spectrum shown in Figure 2b with the following hypothesis (Figure 5): At the storage temperature of −20 °C, chemical reactions in the hydration shell around the membrane and the protein surface are still possible. Thus, the biradical is reduced over

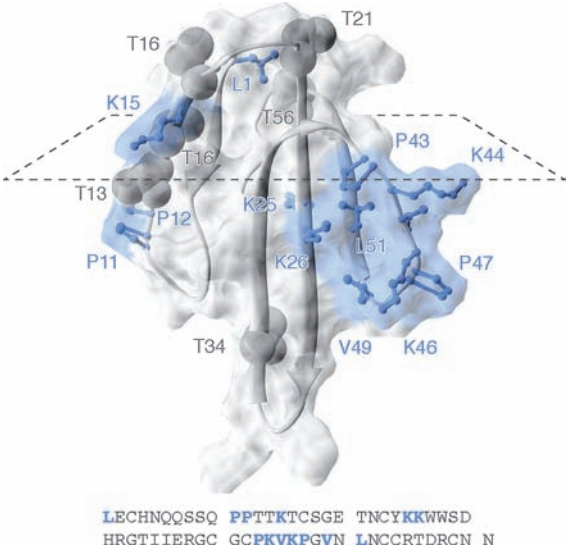

Figure 4. Crystal structure and sequence of neurotoxin II (PDB entry 1NOR[26]). The residues with extensive ^{13}C-labeled carbons in the [2-^{13}C]glycerol-labeled sample (leucines, lysines, valines, and prolines) are colored blue, and the threonines are colored dark-gray. The top part above the dotted line is solvent-exposed, and the three "fingerlike" structures to the bottom bind to nAChR.

time. However, exchange with the active biradical in the solid glassy solvent is less efficient. Therefore, inactive forms of TOTAPOL remain around the sites of interest, and unwanted effects such as PRE do not broaden the protein NMR signals. Directly subsequent to the measurement of the spectrum shown in Figure 2b, we recorded an equally well resolved 2D DNP spectrum with a PDSD

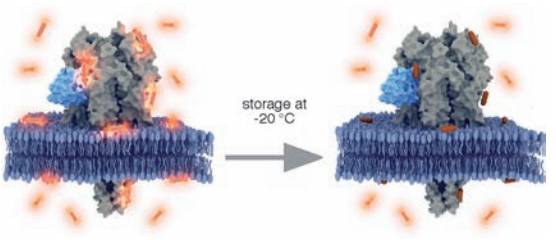

Figure 5. Hypothesis about the well-resolved data. After the biradical and analyte are mixed, the active TOTAPOL (orange lights) is localized at membrane and protein surfaces and broadens the NMR signals (left). Upon handling and storage, the radical reacts with membrane components, and only the active biradical in the bulk solvent enhances the resulting narrow NMR signals (right).

mixing time of 50 ms (see Figure SI E). The cross-peak pattern is mostly the same, but the intensities are changed, as expected.

To support our hypothesis of reduced radicals, we measured a uniformly labeled NTII sample without active TOTAPOL at 100 K (see Figure SI F). Even though no cross-peaks were detectable because of the missing DNP enhancement and the longer H T_1, the measured diagonal peaks were narrow. This is further evidence that proximal TOTAPOL is the main source of the observed broadening in Figure 2a. To reproduce the observed effect, we applied this method to a sample of [1,3-^{13}C]glycerol-labeled NTII on the nAChR in membranes (see Figure SI G). We observed the similarly well resolved spectra as shown in Figure 2: the enhancement dropped after 2 weeks of handling and storage, and the proton T_1 time increased. Again, we were then able to record well-resolved data in just 10.5 h.

In summary, we have demonstrated the possibility of observing narrow signals of membrane proteins with DNP−MAS NMR spectroscopy. This enables the study of extensively labeled membrane proteins in their native membranes. While weeks of measurement time were needed to obtain the conventional MAS NMR data, we were now able to record resolved spectra in just 6% of that time. The factors leading to the well-resolved spectra in Figure 2b and with the [1,3-^{13}C]glycerol-labeled sample will be investigated further. For the moment, we assume that during handling and storage, those TOTAPOL molecules that were located on the membrane surface were reduced and that the DNP effect resulted then from molecules in the bulk. As a result, the line width was favorable.

■ ASSOCIATED CONTENT

Supporting Information. ^{13}C and ^{1}H DNP buildup data, 2D PDSD spectra of uniformly labeled NTII, a spectrum with indicated cross-peaks as expected for a [2-^{13}C]glycerol-labeled NTII from the solution-NMR chemical shifts, a 2D PDSD spectrum with a mixing time of 50 ms for the [2-^{13}C]glycerol-labeled sample, and 2D PDSD spectra of [1,3-^{13}C]-labeled NTII after preparation and after storage. This material is available free of charge via the Internet at http://pubs.acs.org.

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