Towards structure determination of neurotoxin II bound to nicotinic acetylcholine receptor: a solid-state NMR approach

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Abstract Solid-state magic-angle spinning nuclear magnetic resonance (NMR) has sufficient resolving power for full assignment of resonances and structure determination of immobilised biological samples as was recently shown for a small microcrystalline protein. In this work, we show that highly resolved spectra may be obtained from a system composed of a receptor–toxin complex. The NMR sample used for our studies consists of a membrane preparation of the nicotinic acetylcholine receptor from the electric organ of *Torpedo californica* which was incubated with uniformly ^{13}C-,^{15}N-labelled neurotoxin II. Despite the large size of the ligand–receptor complex (> 290 kDa) and the high lipid content of the sample, we were able to detect and identify residues from the ligand. The comparison with solution NMR data of the free toxin indicates that its overall structure is very similar when bound to the receptor, but significant changes were observed for one isoleucine.

Key words: Binding site; Magic-angle spinning nuclear magnetic resonance spectroscopy; α-Neurotoxin; Nicotinic acetylcholine receptor; Solid-state nuclear magnetic resonance spectroscopy

1. Introduction

The nicotinic acetylcholine receptor (nAChR) is found in postsynaptic membranes of central nervous system synapses and the neuromuscular endplate. It is a ligand-gated ion channel which comprises five homologous subunits with the stoichiometry $\alpha_2\beta\gamma\delta$. Each subunit consists of an N-terminal domain of about 200 residues on the extracellular side followed by four membrane-spanning segments with intracellular loops of variable length. The nAChR has a cation-selective pore which opens upon acetylcholine binding. The two acetylcholine binding sites are located mainly on the α-subunits near the subunit interfaces. These two binding sites are competitively blocked by the action of α-neurotoxins (for review, see [1]) (Fig. 1).

There are two classes of postsynaptic neurotoxins according to the length of the polypeptide chain which distinguishes long (66–74 residue) and short (60–62 residue) toxins. With 61 residues, neurotoxin II (NTII), found in the venom of the Asian cobra *Naja oxiana*, belongs to the group of short toxins. The structure of neurotoxins is highly conserved and for a number of them including NTII [2], their three-dimensional structures are known (reviewed in [3]). The interaction between the receptor and the α-neurotoxins has been studied intensively by cross-linking experiments [4], electron paramagnetic resonance (EPR), fluorescence [5], and solution nuclear magnetic resonance (NMR) analysis. In the latter case, analysis of toxins bound to peptide fragments derived from the α-subunit of the nAChR showed changes in the toxin structure [6–8]. The high affinity of the neurotoxins to the nAChR and their specificity have made them an invaluable tool for characterising the structure and function of the receptor.

Solid-state magic-angle spinning (MAS) NMR spectroscopy has emerged as an effective method for structural studies of quasi-immobilised biomolecules. If a sufficient number of structural restraints are collected it is possible to calculate a structure, as was recently determined for the α-spectrin Src-homology 3 domain (SH3) in a microcrystalline state [9]. The two prerequisites for the success of this approach are: (i) availability of isotopically labelled proteins and (ii) a local structural homogeneity within the sample which influences the resolution of the spectra. Recombinant expression of NTII in *Escherichia coli* allows the production of isotopically labelled toxins with ^{13}C-carbon and ^{15}N-nitrogen. The nAChR binding pocket serves as a matrix that provides a structurally defined environment for the ligand. A binding constant in the picomolar range ensures that the equilibrium between the free and bound state of the toxin is strongly shifted towards the receptor-bound state. Excess NTII can be removed by washing without significantly decreasing the number of occupied binding sites in the receptor. The NTII–nAChR complex is ideally suited for structure determination by solid-state MAS NMR spectroscopy, since this system is not easily accessible to high-resolution solution NMR or X-ray crystallography. In this work we show preliminary results which demonstrate the feasibility of solid-state NMR for

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Abbreviations: Bgtx, α-bungarotoxin; MAS, magic-angle spinning; nAChR, nicotinic acetylcholine receptor; NTII, neurotoxin II; PDSD, proton-driven spin diffusion; SH3, α-spectrin Src-homology 3 domain

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structure determination of a receptor-bound ligand in a membrane environment.

2. Materials and methods

2.1. Material

Liquid N_2-frozen electric tissue from *Torpedo californica* was supplied by C. Winkler (San Pedro, CA, USA). (u-^{13}C)Glucose was purchased from Spectra Gases (Branchburg, NJ, USA). $^{15}NH_4Cl$ was from Cambridge Isotope Laboratories (Andover, MA, USA). All other reagents and solvents were of the highest purity available.

2.2. Expression of uniformly labelled ^{13}C-, ^{15}N-NTII

E. coli BL21 cells containing the NTII expression vector were grown at 28°C in M9 minimal medium. Cells were collected at $OD_{600} = 0.5$ and resuspended in the same medium containing $^{15}NH_4Cl$ and glycerol-1,2,3-$^{13}C_3$ as sole nitrogen and carbon sources, respectively. Gene expression was induced by increasing the temperature to 37°C. Cells were grown for 24 h during which NTII was secreted into the medium. Cells were removed by centrifugation and the supernatant, acidified to pH 4.5, was heated at 70°C for 20 min, and after centrifugation, extracted onto SP-Sepharose FF (Amersham Pharmacia). NTII was purified further by cation exchange (Mono S, Amersham Pharmacia) and gel filtration chromatography. The yield of isotope-enriched NTII exceeded 15 mg/l.

2.3. Binding assay with [^{125}I]α-bungarotoxin (Bgtx)

Binding of [^{125}I]Bgtx to nAChR-rich membranes was determined using the DE81 filter disc assay [10] with the modification that casein was used instead of bovine serum albumin.

2.4. NMR sample preparation

nAChR-rich membranes were prepared from the electric tissue of *T. californica* as previously described [11]. A receptor-rich membrane suspension with 9.5 mg protein was dissolved in 65 ml ice-cold Ringer solution and incubated with 2 mg of (u-^{13}C,^{15}N)NTII at room temperature for 10 min. The accessibility of the receptor binding sites was increased by addition of Triton X-100 to a final concentration of 0.02% (v/v) [12]. After incubation for 30 min, membranes were collected by centrifugation ($30\,000 \times g$, 20 min) and washed twice with 30 ml of de-ionised water. The sediment was resuspended in 1.6 ml water and centrifuged at $80\,000 \times g$ for 15 h. The supernatant was removed and an additional centrifugation at $10\,000 \times g$ for 20 min was performed. The sediment was transferred into a MAS rotor. Aliquots of nAChR membrane preparation and of NTII were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

2.5. NMR spectroscopy

The solid-state MAS NMR experiments were performed at fields of 9.4 T and 14.1 T on DMX400 and DMX600 wide-bore spectrometers (Bruker, Karlsruhe, Germany). The spectrometers were equipped with a 4 mm MAS probe, operating in double-resonance mode (Bruker). The sample was confined to the centre of the rotor with the use of spacers. The spectra were acquired at MAS frequencies of 8.0 kHz (9.4 T) or 9.5 kHz (14.1 T). Ramped cross-polarisation from ^{1}H to ^{13}C created the initial transverse carbon magnetisation. Carbon magnetisation was exchanged in the 2D experiments by using a proton-driven spin diffusion (PDSD) mixing scheme with a mixing time of 25 ms. Typically, proton RF fields of ~70 kHz were applied for the two-pulse phase modulation decoupling during ^{13}C acquisition and evolution.

3. Results and discussion

The nAChR has two binding sites for NTII located at the interfaces between the α/γ and the α/δ subunits (Fig. 1). In the receptor-rich membrane, however, both binding sites are not fully accessible due to the tight packing of nAChR. Therefore, a procedure to increase access to the binding sites of NTII is helpful to raise the concentration of the labelled bound species in the sample. The addition of Triton X-100 enhances the fluidity of the membranes and makes more binding sites available [13]. We used a small sample of our receptor-rich membrane preparation in a binding assay with [^{125}I]Bgtx, a homologue of NTII, and titrated Triton X-100 into the membrane preparation. The number of binding sites increased by about

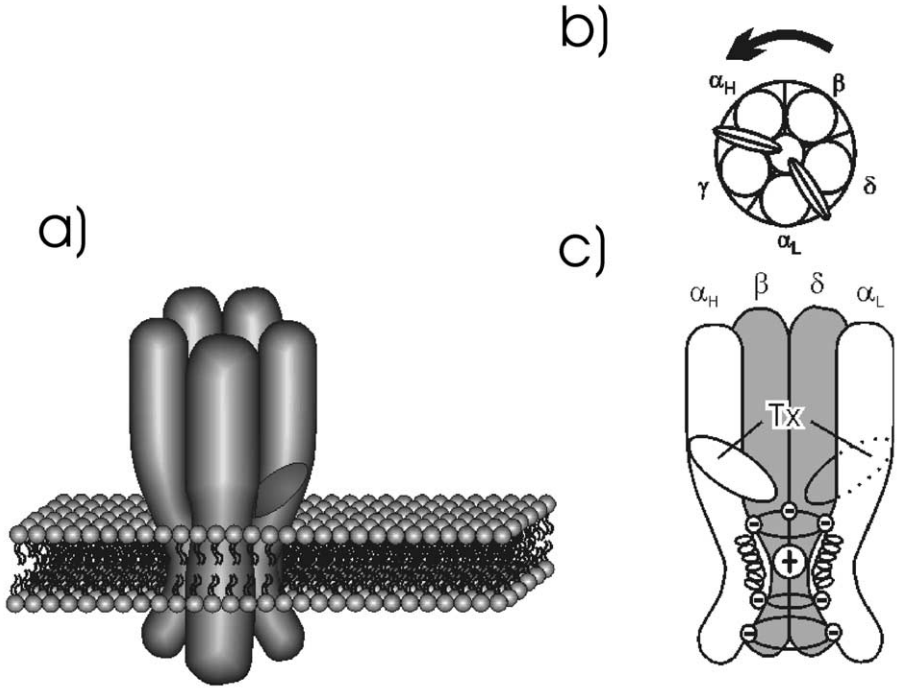

Fig. 1. a: Model of the nAChR–NTII complex in lipid bilayer including bound neurotoxin. b: Top view of nAChR from the extracellular side with the toxin binding sites, subunits are indicated. c: Cut through the α-subunits of nAChR perpendicular to the membrane normal presenting the toxin binding sites and pore region of the receptor.

50% when Triton X-100 was added to a percentage of 0.02%. Although still higher concentrations of Triton X-100 led to a slight increase in toxin binding, significant amounts of the receptor became solubilised at that concentration as well (data not shown). Since the structure and the binding mode of NTII to nAChR are highly comparable to those of Bgtx [3], the preparation of the [u-^{13}C,^{15}N]NTII–nAChR complex for the MAS NMR measurements was performed in the same way. We estimate that the rotor used for the NMR measurements contained approximately 7.5 mg of nAChR and 0.3 mg of NTII, with an average binding stoichiometry of 1.5 toxin molecules to each receptor.

3.1. 1D NMR experiments

Fig. 2a shows a 1D ^{13}C solid-state MAS NMR spectrum of [u-^{13}C,^{15}N]NTII–nAChR complex in lipid membranes recorded at 263 K. For comparison, a 1D spectrum of a micro-crystalline preparation of SH3 recorded at 280 K is shown in Fig. 2b. Both spectra were recorded at the temperature which provided the highest resolution for each sample. For the toxin–receptor preparation, we estimate that the amount of unlabelled membrane protein and lipid comprises about 98–99% of the total mass. The ^{13}C natural-abundance background of 1% of the receptor-rich membranes contributes about 50% of the total signal intensity. Hence, the 1D spectrum is a superposition of labelled NTII and background signals of unlabelled proteins and lipids. The narrow lines observed in Fig. 2a show that the toxin was in a defined environment, which we assume were the binding pockets. A random distribution of the toxin in the sample would give rise to line broadening due to variations in the surroundings of each toxin molecule and would result in less resolved spectra as observed for lyophilised proteins [14,15]. The appearance of the 1D spectrum of the toxin remained unchanged over a period of several months, indicating that the overall structure within the sample was stable. Spectra recorded at different temperatures in the non-frozen state of the sample, from 260 K to 290 K, showed no significant changes. Small changes were observed if spectra were recorded from a frozen sample at temperatures below 260 K. These were fully reversible and might be due to ice formation close to the toxin binding sites or rearrangements of the receptor in the membrane (data not shown).

3.2. 2D NMR experiments

Two-dimensional solid-state MAS NMR ^{13}C-^{13}C correlation experiments were recorded using the PDSD sequence with a mixing time of 25 ms and at temperatures of 240–250 K (frozen) and 260–290 K (non-frozen). All spectra showed comparable cross-peak patterns, but the overall resolution was better in spectra recorded from the non-frozen state of the sample. Fig. 3a shows a 2D spectrum recorded at a temperature just above the freezing point of the sample. The strong diagonal signals arise from ^{13}C carbon atoms of the fully labelled NTII and from natural-abundance ^{13}C background atoms. The resonances due to the ^{13}C background nuclei, however, do not yield off-diagonal signals due to their low concentration and random distribution. Hence, cross-peaks corresponding to correlations between ^{13}C carbon atoms all arise from NTII, and form amino acid-specific correlation patterns [14]. This is illustrated in Fig. 3a for the correlation pattern of one threonine of NTII.

We have compared the chemical shifts of the solid-state resonances with the assignment obtained on free NTII from solution NMR [16]. As an example, the ^{13}C chemical shifts of the threonine in solution are indicated in the solid-state spectrum, shown in Fig. 3b. Most of the threonine solution chemical-shift assignments coincide with cross-peaks of the 2D MAS NMR spectrum. A similar close correspondence between solution and solid-state data was also found for correlation patterns from other residues (data not shown). Since chemical shifts are highly conformation-dependent, the high

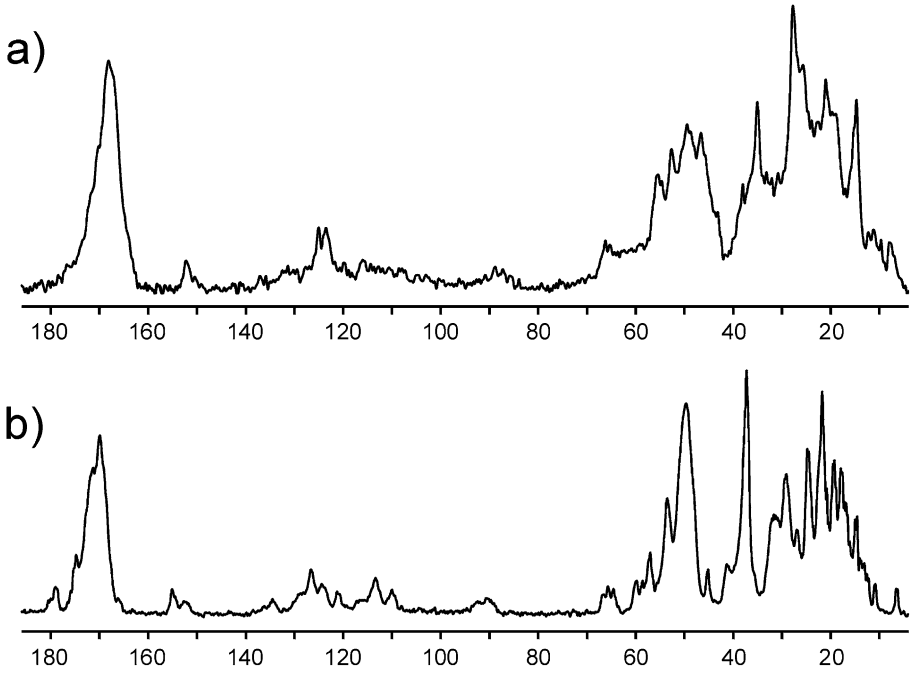

Fig. 2. 1D ^{13}C solid-state MAS spectrum of (a) [u-^{13}C,^{15}N]NTII–nAChR complex in membranes and (b) microcrystalline SH3. Experimental set-up: 263 K (a) or 280 K (b), 2048 scans (a) or 32 scans (b), spinning speed 8 kHz, field 9.4 T.

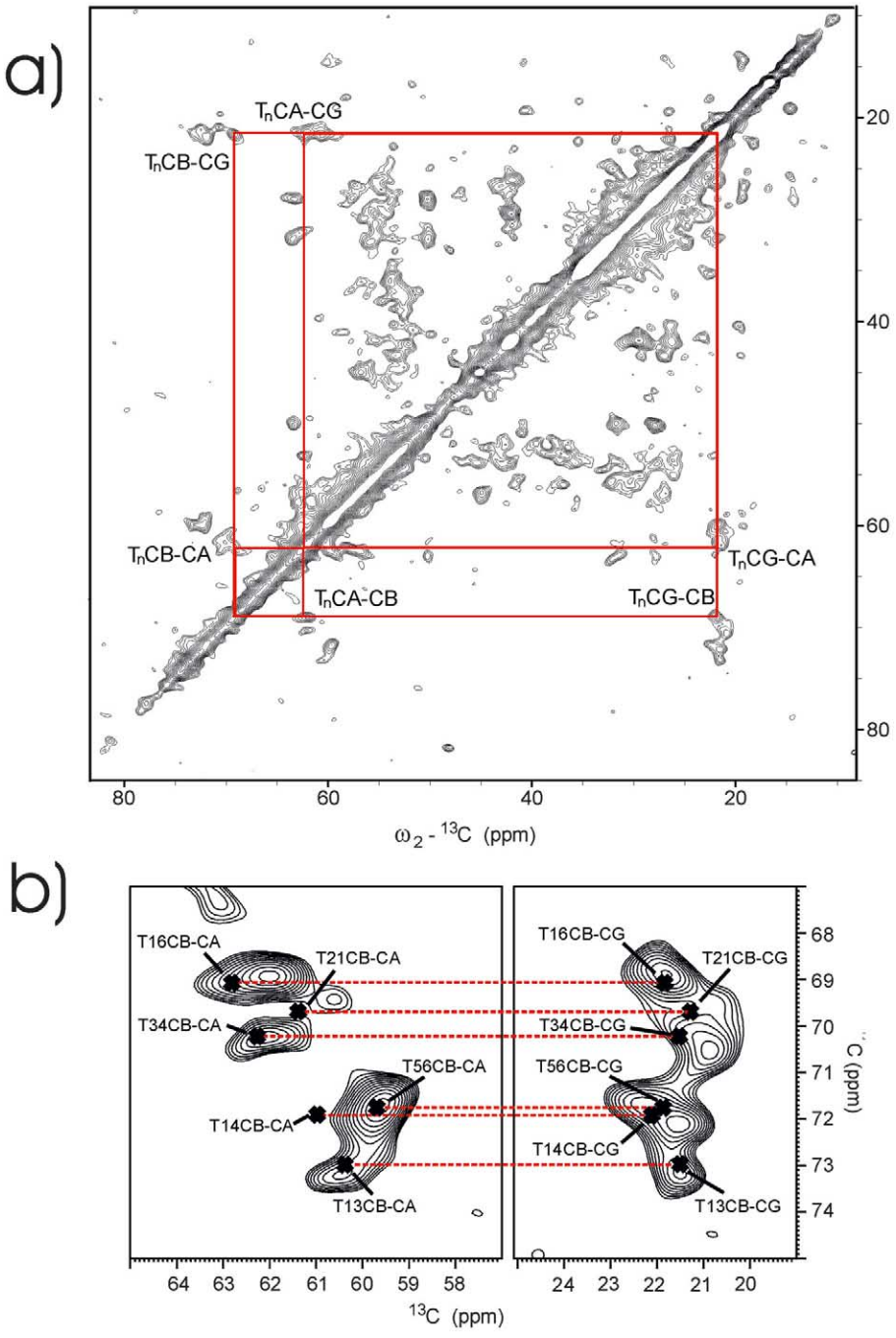

Fig. 3. a: ^{13}C-^{13}C correlated 2D PDSD MAS NMR spectrum of [u-^{13}C,^{15}N]NTII–nAChR complex in membranes. Experimental set-up: 263 K, 9.4 T, 8 kHz spinning speed, 512 scans per slice, 512 slices, CP 1 ms, mixing time 25 ms. Cross-peak signal pattern attributed to one of the six threonines of NTII. b: Region of the 2D spectrum of panel a containing the signal patterns attributed to threonines. The positions of the threonine resonances of free NTII determined by solution NMR are indicated.

degree of identity for most chemical shifts of NTII in solution and in the receptor-bound state strongly suggests that the overall fold of the toxin is the same in free and bound state. In the 2D solid-state NMR spectrum, two neighbouring correlation patterns were detected and identified as isoleucine networks (Fig. 4). The correlation patterns of the two isoleucines in the solution 2D NMR spectra are well separated. In particular, whilst the solution chemical shifts of the Ile36 signals nearly coincide with a set of cross-peaks in the solid-state spectrum, the signals of Ile35 were shifted upfield in solution and did not match with signals in the solid-state spectrum. This observation can be interpreted in two ways: the first interpretation is that Ile35 does not show signals in the solid-state experiment. Hence, the two correlation patterns are both assigned to Ile36. The doubling would arise from different conformations or environments of the ligand in the two binding pockets. The second, more likely, explanation is that Ile35 has very different chemical shifts in the solid state. According to the solution structure of NTII, Ile35 is in close vicinity and above the aromatic ring of Trp28 (Fig. 5). The ring current of

the tryptophan produces upfield shifts of the Cγ and Cδ resonances of Ile35. In the solid-state NMR data we observed downfield shifts for Ile35 relative to the solution NMR data. These shift differences could arise if the aromatic ring of Trp28 moves away from Ile35 when the toxin binds to the receptor. This hypothesis is very well in line with results from solution NMR experiments on a complex of Bgtx and a small receptor-derived peptide [17,18], and with EPR and fluorescence studies [19]. In the NMR studies it was found that the loop II of Bgtx shows a prominent bend at the homologous position of Ile35 when the peptide is bound. The EPR/fluorescence work analysed the surface area between NTII and nAChR and identified Lys26, Ile35 and Trp28 as in contact with the receptor. If we assume a similar binding mechanism for NTII to the native receptor and consider the different chemical environment upon binding, this could explain the changes in chemical shifts of Ile35.

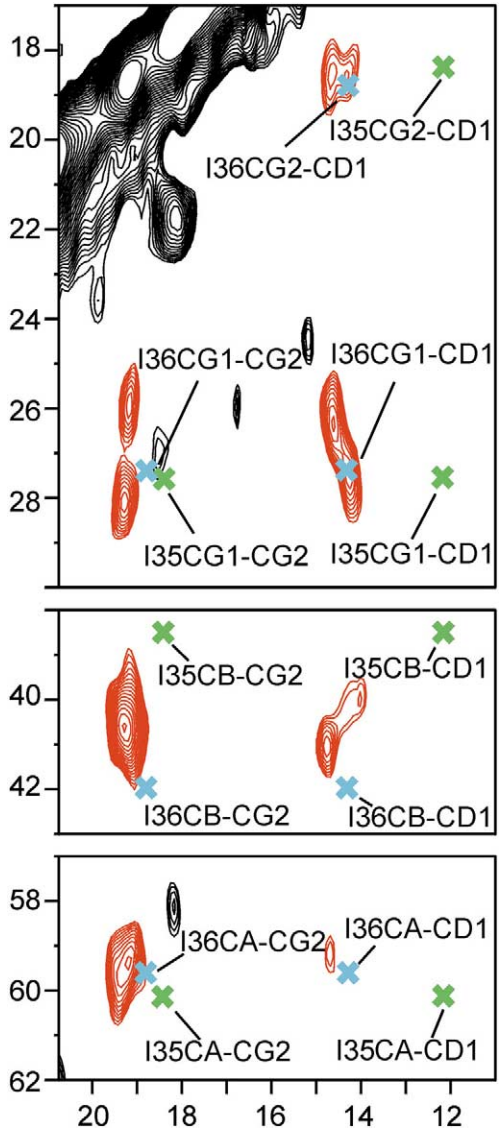

Fig. 4. Regions of a 2D correlation experiment showing the isoleucine signal patterns (red contours). The positions of resonances for Ile35 (green) and Ile36 (blue) of NTII determined by solution NMR of free NTII are indicated. Experimental conditions: field 14.1 T, 9.5 kHz spinning speed.

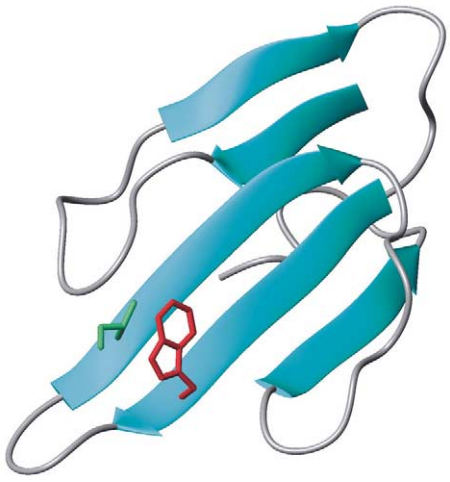

Fig. 5. Structure of NTII in solution determined by solution NMR [2]. The side chains of Ile35 and Trp28 are shown.

3.3. Conclusion

Fully ^{13}C-, ^{15}N-labelled NTII was analysed by solid-state MAS NMR spectroscopy while the toxin was bound to nAChR. The toxin–receptor complex was studied in its native membrane environment. The 1D and 2D solid-state NMR spectra of the sample clearly showed that NTII was in a defined environment, most likely provided by the binding pockets of the receptor. The resolution of the 1D ^{13}C MAS spectrum of the NTII–nAChR complex was highly comparable to that of the SH3 preparation. This result is quite remarkable considering the different nature of the two samples: the SH3 sample was composed of about 10 mg pure, fully labelled hydrated protein in a microcrystalline state, whereas the receptor–toxin complex sample contained only 0.3 mg labelled NTII, unlabelled protein and lipid.

Our results suggest the calculation of the backbone conformation of NTII should be feasible in the near future, in analogy to the solid-state NMR structure determination of SH3 [9]. Additional solid-state experiments are first needed to sequentially assign the amino acid correlation patterns. In combination with further toxin–receptor preparations, which contain multiple labelled NTII [9], the solid-state-derived NMR data should provide the basis for structure calculation of the receptor-bound toxin. This work demonstrates the power of solid-state NMR for analysing receptor–ligand complexes.

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References

[1] Hucho, F. and Weise, C. (2001) Angew. Chem. Int. Ed. Eng. 40, 3100–3116.
[2] Golovanov, A.P., Lomize, A.L., Arseniev, A.S., Utkin, Y.N. and Tsetlin, V.I. (1993) Eur. J. Biochem. 213, 1213–1223.
[3] Tsetlin, V. and Hucho, F. (2004) FEBS Lett. 557, 9–13.
[4] Hucho, F., Tsetlin, V.I. and Machold, J. (1996) Eur. J. Biochem. 239, 539–557.
[5] Tsetlin, V.I., Karlsson, E., Arseniev, A.S., Utkin, Y.N., Surin, A.M., Pashkov, V.S., Pluzhnikov, K.A., Ivanov, V.T., Bystrov, V.F. and Ovchinnikov, Y.A. (1979) FEBS Lett. 106, 47–52.

[6] Moise, L., Piserchio, A., Basus, V.J. and Hawrot, E. (2002) J. Biol. Chem. 277, 12406–12417.
[7] Zeng, H. and Hawrot, E. (2002) J. Biol. Chem. 277, 37439–37445.
[8] Samson, A.O., Chill, J.H., Rodriguez, E., Scherf, T. and Anglister, J. (2001) Biochemistry 40, 5464–5473.
[9] Castellani, F., van Rossum, B., Diehl, A., Schubert, M., Rehbein, K. and Oschkinat, H. (2002) Nature 420, 98–102.
[10] Hartig, P.R. and Raftery, M.A. (1979) Biochemistry 18, 1146–1150.
[11] Schiebler, W. and Hucho, F. (1978) Eur. J. Biochem. 85, 55–63.
[12] Saez-Briones, P., Krauss, M., Dreger, M., Herrmann, A., Tsetlin, V.I. and Hucho, F. (1999) Eur. J. Biochem. 265, 902–910.
[13] Saez-Briones, P., Krauss, M., Dreger, M., Herrmann, A., Tsetlin, V.I. and Hucho, F. (1999) Eur. J. Biochem. 265, 902–910.
[14] Pauli, J., van Rossum, B., Forster, H., de Groot, H.J.M. and Oschkinat, H. (2000) J. Magn. Reson. 143, 411–416.
[15] Martin, R.W. and Zilm, K.W. (2003) J. Magn. Reson. 165, 162–174.
[16] Bocharov, E.V., Lyukmanova, E.N., Ermolyuk, Y.S., Shulga, A.A., Pluzhnikov, K.A., Dolgikh, D.A., Kirpichnikov, M.P. and Arseniev, A.S. (2003) Appl. Magn. Reson. 24, 247–254.
[17] Zeng, H., Moise, L., Grant, M.A. and Hawrot, E. (2001) J. Biol. Chem. 276, 22930–22940.
[18] Moise, L., Piserchio, A., Basus, V.J. and Hawrot, E. (2002) J. Biol. Chem. 277, 12406–12417.
[19] Tsetlin, V.I., Karlsson, E., Arseniev, A.S., Utkin, Y.N., Surin, A.M., Pashkov, V.S., Pluzhnikov, K.A., Ivanov, V.T., Bystrov, V.F. and Ovchinnikov, Y.A. (1979) FEBS Lett. 106, 47–52.