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Structural descriptions of ligands in their binding site of integral membrane proteins at near physiological conditions using solid-state NMR

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Abstract Using solid-state NMR approaches, it is now possible to define the structure and dynamics of binding for a small, isotopically (^{2}H, ^{13}C, ^{19}F, ^{15}N) labelled ligand, prosthetic group or solute in its binding site of a membrane-bound protein at near physiological conditions in natural membrane fragments or in reconstituted complexes. Studies of oriented membranes permit the orientational bond vectors of labelled groups to be determined to good precision, as shown for retinal in bacteriorhodopsin and bovine rhodopsin. Using novel magic angle spinning NMR methods on membrane dispersions, high-resolution NMR spectra can be obtained. Dipolar couplings can be reintroduced into the spectrum of labelled ligands in their binding sites of membrane-bound proteins to give interatomic distances to high precision (±0.5 Å). Relaxation and cross-polarization data give estimates for the kinetics for on-off rates for binding. In addition, chemical shifts can be measured directly to help provide details of the binding environment for a bound ligand, as shown for analogues of drugs used in peptic ulcer treatment in the gastric ATPase, and for acetylcholine in the acetylcholine receptor.

Key words Membrane proteins · Solid-state NMR · Receptors · Transporters · Biomembranes

Abbreviations *MAS* Magic angle spinning · *NMR* Nuclear magnetic resonance · *GalP* Galactose transporter of *E. coli* · *TMD* Transmembrane domain · *MI* Meta-I · *TMPIP* 1,2,3-Trimethyl-8-(phenylmethoxy)imidazol[1,2-*a*]pyridinium cation

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Introduction

Small molecules, such as hormones and neurotransmitters, induce conformational changes and then concomitant profound cellular changes through their interactions with membrane-bound proteins. In addition, both nutrient transport into cells and inhibition of important pharmacological targets exploit the biological specificity of the interaction of small ligands with membrane proteins. Despite the importance of defining the structure and dynamics of ligand interactions with integral membrane proteins, atomic details of membrane protein structures and the nature of their interactions with ligands are scare. This scarcity is, in part, a result of the serious technical difficulties in routinely producing two-dimensional or three-dimensional crystals for diffraction studies, and because high-resolution *solution*-state NMR spectra of membrane-embedded proteins are broadened by anisotropic magnetic interactions, resulting in the loss of the spectral resolution required for obtaining structural details.

Solid-state NMR methods have been developed to yield new detailed information about membranes. Two approaches are used; in one, the anisotropic interactions of quadrupolar, ^{13}C and ^{15}N nuclei in oriented membranes are exploited; and in the other, high-resolution-like NMR spectra from randomly dispersed samples are used to give detailed molecular and atomic detail about protein-ligand interactions and protein structures. The approach used depends upon the information to be gained, and the nature of the question being addressed. If orientational information of specific chemical bonds with respect to the membrane axes is required, then nuclei such as ^{2}H and ^{15}N can be incorporated into the protein or peptide backbone, or a prosthetic group, to determine peptide orientation or ligand structure (Seelig 1978; Watts et al. 1995; Smith et al. 1996). For inter-atomic distances or kinetic detail of ligand binding, or information about the binding site environment, then high-resolution solid-state NMR methods are required (Watts et al. 1995; Smith et al. 1996).

The strength of the method is that biological specificity is exploited in ligand binding. Only the constrained ligand is observed (non-bound ligand being invisible) and exhaustive purification of the protein is not required, except when reconstitutions are performed. Usually, therefore, enriched membranes are used and the system retains all its biochemical functionality and integrity.

In this short review, information about several membrane proteins being studied by us using these approaches will be presented, although for more details of the experimental methods the reader is referred to the reviews by Watts et al. (1995) and Smith et al. (1996).

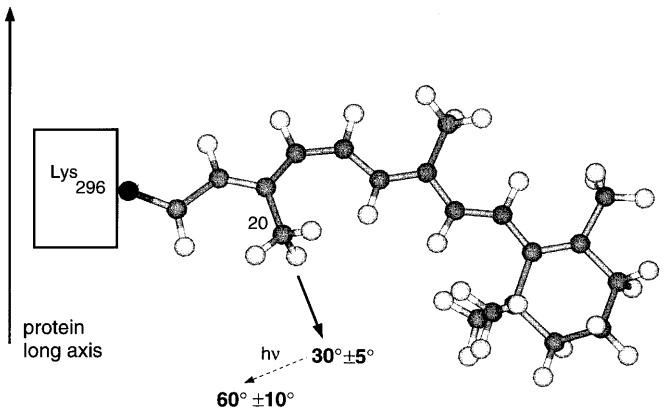

Fig. 1 Orientation and conformation of 11-*cis*-retinal in the binding pocket of bovine rhodopsin at the ground state, constructed from the orientation of the methyl group at the C_{20} position as determined by solid-state ^2H NMR. The relative change of the orientation after photoexcitation at the MI state is also indicated

Results

Static solid-state NMR of retinal in membrane-bound photoreceptor proteins

Retinal is the chromophore of many photoreceptive proteins, including bacteriorhodopsin and retinal rhodopsin (Birge 1990). The mechanisms for photoreception and subsequent biochemical events (as diverse as proton pumping in the bacterial protein and activation of a G-protein cascade in the high life form protein) are still not clear, even though the crystal structure of bacteriorhodopsin has recently been resolved (Pebay-Peyroula et al. 1997).

Since retinal structure and position within the protein are crucial to an understanding of the photoreception mechanisms, we have been using solid-state NMR to define the structure within the protein, using an ab initio approach of oriented membranes in which the chromophore is deuterated at its various C-$(CD_3)_3$ groups in both bacteriorhodopsin and bovine rhodopsin. Although the retinal is not resolved at low (~6 Å) resolution in either of the protein structures (Schertler et al. 1993; Grigorieff et al. 1996; Unger et al. 1997), in the case where the protein structure is known to high (2.5 Å) resolution, precise details of the retinal structure may still not be unambiguously defined (Pebay-Peyroula et al. 1997).

Bacteriorhodopsin

Solid-state ^2H NMR methods have been applied to oriented purple membranes as a non-crystallographic method to determine the detailed local structure of the chromophore in bacteriorhodopsin in the initial state and the M_{412} photointermediate (Ulrich et al. 1995). The spectra from selectively deuterium-labelled methyl groups on the retinal were obtained from oriented patches of the purple membranes on glass plates (about 90 mg on 20 plates), and the chromophore structure obtained from the spectral anisotropy which gave the orientations of the individual methyl groups. The analysis of the orientation dependence of the lineshapes and the quadrupolar splittings of the uniaxially oriented samples with respect to the magnetic field provided angles for the various C-CD_3 bond vectors, defining the retinal conformation precisely.

For the photo-intermediate state trapped at –60 °C, an increase of the local tilt of retinal by ca. 3° upon 13-*cis* isomerization was detected (Ulrich et al. 1995). Recent high-resolution X-ray crystallographic details for bacteriorhodopsin (Pebay-Peyroula et al. 1997) confirm the validity of the ^2H-NMR approach within limits (±10°) for all C-CD_3 bond vectors of retinal except C_{20}, However, it is not clear whether the retinal around the Lys216 residue on helix-G at C_{20} is distorted, and the bond vectors determined by NMR (32°) and crystallography (10°) results are not coincident for this position.

Rhodopsin

For the retinal photoreceptor rhodopsin, a similar deuterium NMR approach has been used to probe the structure of the chromophore in its binding pocket to obtain structural details for the chromophore and hence, for this 7TMD receptor, a closer insight into the molecular mechanism of the activation of the coupling to the G-protein transducin. For this purpose, a preparative procedure was developed enabling the alignment of phospholipid membranes containing rhodopsin (900 µl of a lipid complex containing 20 nM of rhodopsin were oriented onto glass plates and kept at 52% humidity at 4 °C) with 11-*cis*-retinal specifically deuterated at its methyl groups (as C-CD_3) at the C_{20} position in the ground state and photoactivated meta-I (MI) state (Gröbner et al. 1997). To test the orientational integrity of the membranes ^{31}P NMR was used as an independent assessment of the quality of alignment of the membranes by using the phospholipids as internal reporter molecules. Computer analyses of the ^2H NMR lineshapes from the labelled retinal in the protein in aligned membranes at different orientations relative to the magnetic field are providing the orientational constraints for the C-CD_3 bond vector (Fig. 1) for the C_{20} position of retinal

in the dark-adapted ground state of rhodopsin and in its photo-excited MI state, which could be trapped by using a DMPC- matrix at 4 °C (Gibson and Brown 1993). For the ground state an angle of 30±5° was obtained, which increases to about 60±10° for the MI state after photoisomerization (Gröbner et al. 1998). Our results for the conformation and orientation of the chromophore are in good agreement with results obtained from linear dichroism and Fourier transform infrared spectroscopy on mutant forms of rhodopsin (Jäger et al. 1997). To describe the overall orientation and possible distorted structure of the chromophore within the binding pocket of rhodopsin at the various steps of the photocycle in a more comprehensive way, further constraints have to be obtained, in particular for the retinal ring, work which is already in progress using the same ^{2}H NMR approach.

High-resolution solid-state NMR of ligands bound in membrane proteins

ATPases

The P-type ATPases constitute a small family (four or five members) of pharmacologically important and widespread ion-pumps, which couple nucleotide hydrolysis to cation translocation across cell membranes (Stokes et al. 1994; Carafoli 1992). Ion-pump function typically involves an E1–E2 conformational change during the catalytic cycle, in which binding domains of the protein are exposed alternately to substrates and activating ions on either side of the membrane. There exists a number of structurally diverse and potentially therapeutic ATPase inhibitors that intervene in the catalytic cycle, for example to prevent dephosphorylation of the active enzyme intermediates. Understanding the structural basis for ligand recognition and enzyme inhibition could provide a basis for rational ligand design, and guide the search for novel drugs against peptic ulcer disease, cardiac arrhythmias and congestive heart failure.

Structures of P-type ion-pumps, for example the gastric H^+/K^+-ATPase (Xian and Hebert 1997) and skeletal muscle Ca^{2+}-ATPase (Toyoshima et al. 1993), have been obtained from two-dimensional crystals using electron microscopy and image reconstruction, but are currently of too low resolution (>10 Å) to visualize the structure or location of bound ligands or inhibitors. More detailed structural information on membrane topology and inhibitor binding has been derived indirectly from random mutagenesis of low-level expressed enzyme (Canessa et al. 1992; Palasis et al. 1996; Asano et al. 1997), photoaffinity labelling (Munson et al. 1991) and protein digestion (Goldshleger and Karlish 1997; Heimburg et al. 1997), but these methods are often open to different interpretations.

It has now become possible to obtain direct and highly precise information on inhibitor binding to these enzymes using solid-state NMR distance measurements (Middleton et al. 1996, 1997). Inhibitors of the gastric H^+/K^+-ATPase (M_r<150 k) have potential for the treatment of peptic ulcer disease by ameliorating the highly acidic milieu of gastric glands. The proton pump inhibitors (PPIs) omeprazole, pantoprazole and lanzoprazole are established antisecretory drugs that covalently react with thiol groups (Sachs 1997). More recently, attention has focused on reversible inhibitors as a new generation of drugs (Pope and Sachs 1992). A structurally diagnostic distance in the reversible pump inhibitor 1,2,3-trimethyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridinium (TMPIP) at its site of action in the non-frozen (at 1 °C), fully hydrated protein (~30 mg of total protein in enriched membranes containing ~70% ATPase; 20 nmol binding sites) (Fig. 2) was measured using solid-state (rotational resonance) ^{13}C NMR, and enabled one of the three flexible torsional angles in the molecule to be deduced (Middleton et al. 1997). Work is in progress to measure two further distances, which should solve the entire conformation of TMPIP, place geometric constraints on its functionalities and suggest structures of novel potential inhibitors. The binding location of K^+-competitive inhibitors such as TMPIP is still a matter of debate, and modelling of TMPIP in interaction at its site of action is precluded until this question is resolved. Constructing a model of inhibitors such as TMPIP at their binding sites clearly relies on having some indication of the residues that participate in ligand binding and their proximity to the ligand.

Mutagenic substitutions have helped to pinpoint key residues implicated in ligand binding (Canessa et al. 1992; Asano et al. 1997), but give little or no indication of the spatial arrangement of other residues around the binding site. In proteins where residues have been identified as being important for ligand binding, solid-state NMR distance measurements between isotope labels in the ligand and protein have added valuable constraints to assist in modelling the protein-ligand complex (Klug et al. 1997). Unfortunately, functional P-type ion-pumps have not been expressed to high levels, which poses a problem for introducing isotope labels into the proteins non-invasively. Methods for post-translational, chemical modification of wild-type ATPases are now being investigated as an alternative approach to isotope labelling of specific amino acids (Watts et al. 1995). Points of close contact (<6 Å) of labelled modified residues with labelled inhibitors can be established if the internuclear, intermolecular dipole-dipole interaction between ligand and protein can be re-introduced, for example at rotational resonance.

This approach is being applied to the Na^+/K^+-ATPase, the pharmacological receptor for the cardiac glycosides (CGs). Ouabain, a well-known CG, was derivatized to incorporate ^{13}C labels into the inhibitor with only a minor loss of potency. Labels were introduced into the protein by reductive methylation of primary amines (Watts et al. 1995 and refs. therein), predominantly from lysines, to produce a multiple N,N-$(^{13}CH_3)_2$ derivative. Cross-polarization magic angle spinning (CP-MAS) NMR (Spooner and Watts 1992) was able to detect and resolve in the same spectrum ^{13}C signals from the bound inhibitor and a broad signal envelope from labelled protein. Preliminary experiments showed that, at rotational resonance, magnetization was exchanged between the protein and Ouabain ^{13}C spin-systems. This ob-

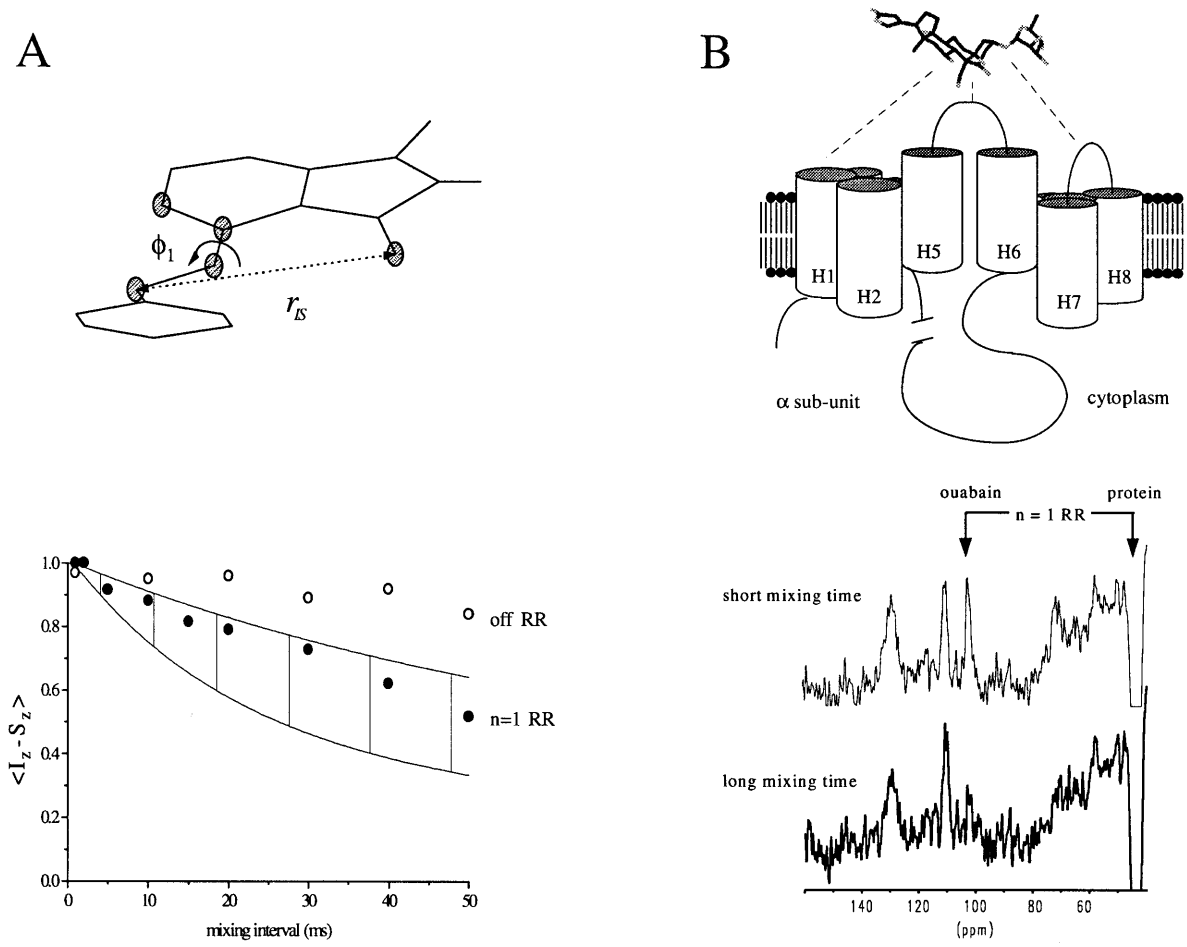

Fig. 2 A, B CP-MAS methods for probing the interactions of non-covalent inhibitors with P-type ATPases. **A** The H^+/K^+-ATPase inhibitor TMPIP was ^{13}C labelled at two sites (*top*) and a rotational resonance magnetization exchange curve was obtained from ^{13}C spectra of the inhibitor at its high affinity site (*bottom*). Numerical simulations of the data indicated that bound TMPIP adopts a '*syn*-type' configuration with respect to angle θ_1 (Middleton et al. 1997). **B** The Na^+/K^+-ATPase inhibitor ouabain has been shown to interact with a number of residues in the alpha subunit (*top*). Ouabain was ^{13}C labelled and protein NH_2 groups were methylated with $^{13}CH_3$. At rotational resonance, the decay of the ouabain resonance in the ^{13}C spectrum with increasing mixing time (*bottom*) suggested a close contact (<6 Å) between the labelled inhibitor and at least one labelled residue

servation suggests that at least one of the labels within the sodium pump is in close proximity (<6 Å) to the highly specific ligand in its binding site. Further biochemical and NMR experiments will help to clarify which of the labelled residues falls within a close radius of the ligand.

Sugar transporters

CP-MAS ^{13}C NMR techniques have been used to detect substrate, selectively labelled with ^{13}C nuclei, associated with sugar symport proteins which have been over-expressed in their native fluid membranes and retained in their functional state. Despite the typically weak association of substrate with these transport systems (k_d~mM), bound substrate could be discriminated at high resolution and with good sensitivity (~22 mg of total membrane protein of which 55–60% is GalP; 250 nmol–1 μmol of binding sites; 4 °C) (Spooner et al. 1994). New NMR methods (dephasing delayed CP) have been developed to show that although substrates are weakly bound, their overall exchange with the protein is slow (>10^{-1} s). We attribute the good sensitivity from CP in these fluid membrane systems to two factors: a relative immobilization of protein as a result of its amplified expression in the native membranes and an immobilization of the substrate during its slow translocation in the protein. Substrate binding has been characterized in terms of its specificity, anomeric selectivity and the kinetics of the translocation process, for GalP (Spooner et al. 1994) and FucP (Spooner P, O'Reilly J, Rutherford N, Henderson P, Watts A, unpublished data), the galactose-H^+ and fucose-H^+ symport proteins, respectively, from *E. coli*, and, more recently, LacS, the lactose symporter from *S. thermophilis* (Spooner P, Veerhoff L, Poolman B, Watts A, unpublished data). All these measurements show a high degree of substrate selectivity for the target proteins and, importantly, this is allowing studies on the native systems to proceed without any special requirements for purification and reconstitution.

As shown in Fig. 3, initial measurements showed that selective substrate binding could be readily detected

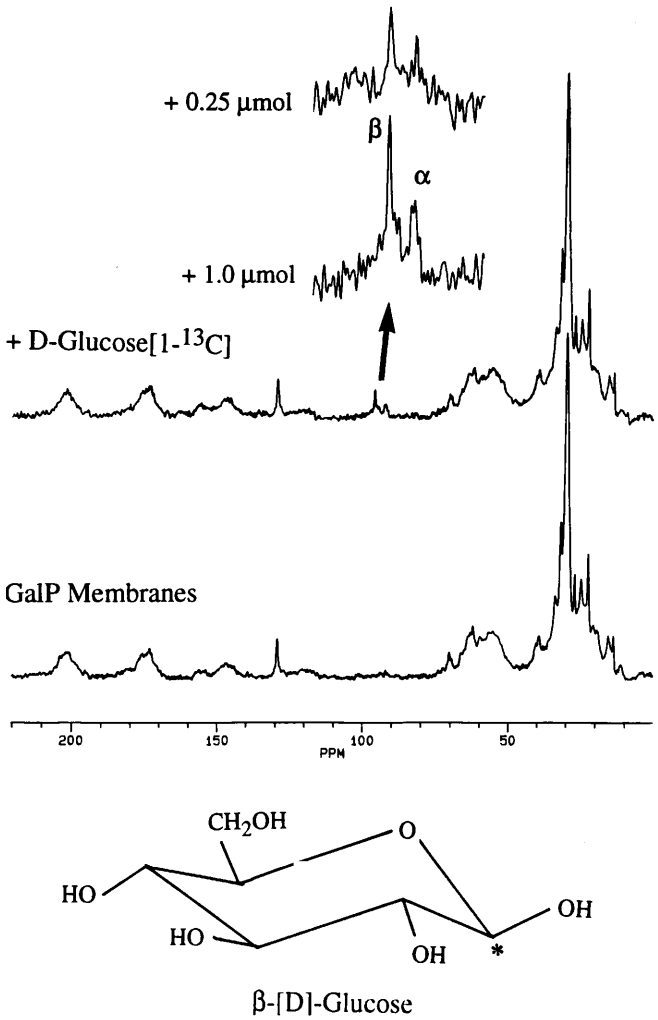

Fig. 3 The MAS NMR spectrum from inner membranes containing 0.54 mol (~26 mg) of GalP which was ~55% of the total membrane protein. The lower spectrum shows natural abundance ^{13}C (1.1% of the total carbon) NMR resonances which are due to the lipids and proteins in the membranes and would not normally be resolved without the MAS technique. In the *upper spectrum*, the resonances due to added D-[1-^{13}C]glucose are resolved, with the uppermost insets showing the expanded part of the spectra for two different amounts (1 µmol and 0.25 µmol) of added labelled glucose, where both the α- and β-anomers are resolved. In each case the specific label is resolved in a part of the spectrum (90–100 ppm) not occupied by other resonances from the membranes and very similar to those observed for the solid, amorphous labelled glucose by the same method. CP (1-ms contact time) conditions, with a 3-s recycle time and 8000 acquisitions per spectrum, were used to resolve only constrained molecules, including bound glucose, with any free, isotropic glucose in the solution and not bound being invisible in this type of experiment (adapted from Spooner et al. 1994)

from adding as little as 250 nmol of substrate to the membranes with around 50% of total protein as GalP. In more recent work, with transport systems achieving more modest levels of over-expression and in membranes of greater chemical complexity, as with LacS-containing membranes, we have detected and characterized substrate binding to as little as 60 nmol of the transporter.

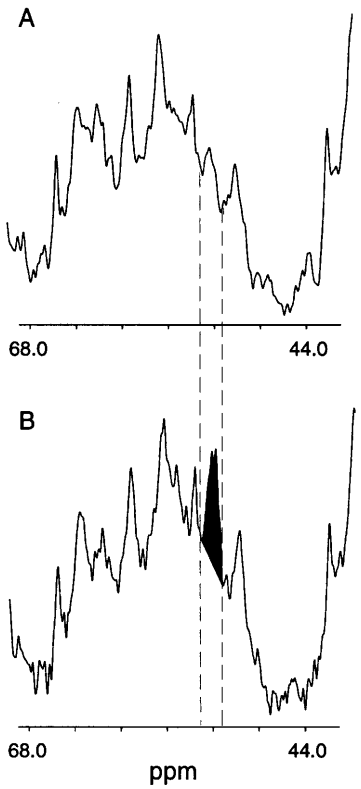

Fig. 4 A, B CP-MAS NMR ^{13}C spectra (64–48 ppm) of nicotinic acetylcholine receptor enriched membranes containing 40 nmol of acetylcholine binding site recorded with 1 ms CP and averaged over 8600 acquisitions at 283 K. Data processed with 20 Hz line broadening. Membranes in the absence of acetylcholine (**A**); membranes with 40 nmol of $N^+(^{13}CH_3)_3$-acetylcholine added (**B**). The resonance assigned to bound acetylcholine is *shaded* in **B**

Nicotinic acetylcholine receptor

The nicotinic acetylcholine receptor, a ligand gated ion channel, is a member of the four transmembrane helix superfamily of receptors which include the GABA, glycine and 5-HT3 receptors which play a key role in the transmission and modulation of nervous impulses (Galzi et al. 1991). These receptors are recognized targets for a variety of pharmaceutical agents for a range of neurological diseases (Cooper et al. 1996). Using high-resolution MAS NMR, we have been able to obtain high-resolution-like spectra of NMR-labelled agonist and of nicotinic acetylcholine-enriched membranes.

Synthetic introduction of carbon-13 labels into the receptor agonist acetylcholine, at the *N*-methyl position [$N(^{13}CH_3)_3$-acetylcholine], permits direct observation of the bound agonist in the receptor. Making use of the favourable CP behaviour of membrane systems, in contrast to those in free solution, we have been able to observe the appearance of a resonance at 52.34 ppm upon the addition of 40 nmol of $N(^{13}CH_3)_3$-acetylcholine to 50 mg of protein at 4 °C in excess buffer (Fig. 4), assignable to the $N(^{13}CH_3)_3$-acetylcholine motionally restricted within a receptor binding site. Upon the addition of the same amount

of agonist to membranes pretreated with the specific inhibitor α-bungarotoxin (Galzi et al. 1991), which both displaces and prevents binding to the acetylcholine binding site, no such resonance appears (Williamson et al. 1998). This demonstrates that the resonance observed at 52.34 ppm arises from the $N(^{13}CH_3)_3$-acetylcholine specifically bound to its binding site in the nicotinic acetylcholine receptor (Williamson et al. 1998).

The resonance at 52.34 ppm from bound agonist is shifted by –1.5 ppm from that observed for $N(^{13}CH_3)_3$-acetylcholine in free solution or as a crystalline solid, as measured in solution-state and solid-state NMR, respectively (Williamson et al. 1996). Such information provides information relating to the local electrostatic environment experienced by the bound ligand. The upfield perturbation in chemical shift may arise from the presence of aromatic residues in the binding pocket, which generates ring currents causing local field effects (Günther 1992). The nicotinic acetylcholine receptor is proposed to have a binding pocket lined by aromatic residues similar to that found in acetylcholinesterase (Raves et al. 1997) in which the aromatic rings in the binding pocket ligate the quaternary ammonium group of acetylcholine (Raves et al. 1997; Dougherty 1996). Simulation of chemical shifts (Williamson 1993; Asakura et al. 1995) based on the crystal structure of acetylcholine bound to acetylcholinesterase (Raves et al. 1997) predict a similar perturbation in chemical shift upon the binding of acetylcholine to acetylcholinesterase.

Through the exploitation of differing relaxation properties between ligands free in solution and those bound to the receptor, we have been able to carry out dipolar dephasing experiments which demonstrate that acetylcholine is resident in the receptor binding site for a period exceeding 100 ms. Such information provides additional mechanisms for obtaining kinetically valuable information about ligand binding events which are critical to our understanding of receptor activation.

Our ability to observe selectively ligands bound to this class of receptor provides us with the opportunity to use fully isotopically enriched ligands and recent advances in dipolar recoupling NMR techniques (Lee et al. 1995; Baldus et al. 1997) to provide multiple restraints critical to the elucidation of the structure of the bound ligand.

Conclusions

Observation at high resolution and sensitivity for nuclei in the binding site of membrane proteins now provides opportunities for characterizing the structure within these functionally important regions of the transporter, receptor and ion-translocation proteins. Work is now proceeding with uniformly labelled proteins (uniformyl ^{15}N, labelled sugar transporters, for example) and using substrate binding to probe the vicinity of the binding sites with strongly interacting nuclei (e. g. ^{19}F). Then, connectivities resolved through magnetic interaction between ligand and local (7 – 15 Å) residues will help to build up a picture of the ligand at its site of action. It is expected that the unique application of these methods for correlating ligand-polypeptide distance measurements will yield detailed structural information within the binding sites of proteins, from which we can begin to deduce functional mechanisms and details of the specificity of the binding process. One major hold-up in these approaches is in the molecular biology required for expression of a large variety of active membrane proteins at sufficient levels (>30% of the total membrane protein; 5 – 20 mg per NMR experiment) for such approaches to be used, but this is thought not to be a hurdle which cannot be overcome at some time in the future.

The solid-state methodology can also be adapted for elucidating the dynamic features of the functional events in transport proteins, as already demonstrated with GalP (Spooner et al. 1994). These dynamic processes are likely to play a significant role in understanding the mechanisms of translocation, and therefore solid-state NMR will hold the unique position of being capable of yielding both structural and dynamic information in a direct and non-crystallographic way.

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