Magnetic resonance of lipids and proteins in membranes

Derek Marsh

Magnetic resonance spectroscopy (NMR and electron spin resonance [ESR]) yields uniquely both dynamic and structural information on lipids and proteins in biological and bilayer membranes. Recent advances have been made in elucidating the three-dimensional structure of membrane-embedded proteins by solid state NMR techniques and by site-directed ESR spin labelling.

Addresses
Abt. Spektroskopie, Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-37077 Göttingen, Germany;
e-mail: DMARSH@gwdg.de

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Abbreviations

ESR	electron spin resonance
MAS NMR	magic angle spinning NMR
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect

Introduction

Magnetic resonance methods have a long and successful history in studying lipids in membranes because of the unique sensitivity of both spin label electron spin resonance (ESR) and solid state NMR to membrane dynamics. Applications to peptides and proteins in membranes, although potentially extremely powerful, are still undergoing rapid development. The reason for this is, on the one hand, that ESR methods require specific labelling and, on the other hand, that the multi-dimensional methods of solution state NMR may only be applied to micellar systems [1] and not to intact membranes. Considerable advances are being made, however, both in the area of site-directed ESR spin labelling [2] and in various forms of solid state NMR spectroscopy [3,4,5•], that have already allowed determination of high resolution protein or peptide structures in membranes. Whilst summarizing some of the recent results on lipids, this review will concentrate more on the advances in determining membrane protein structure. Equally of interest are methods for studying the overall features of membrane assembly and of protein insertion, for which spin label ESR methods are well suited. Magnetic resonance methods applied to channel proteins have recently been described within the wider context of complementary spectroscopic studies [6]. Results obtained with peptides as models for the transmembrane segments of channel proteins have also been reviewed recently [7].

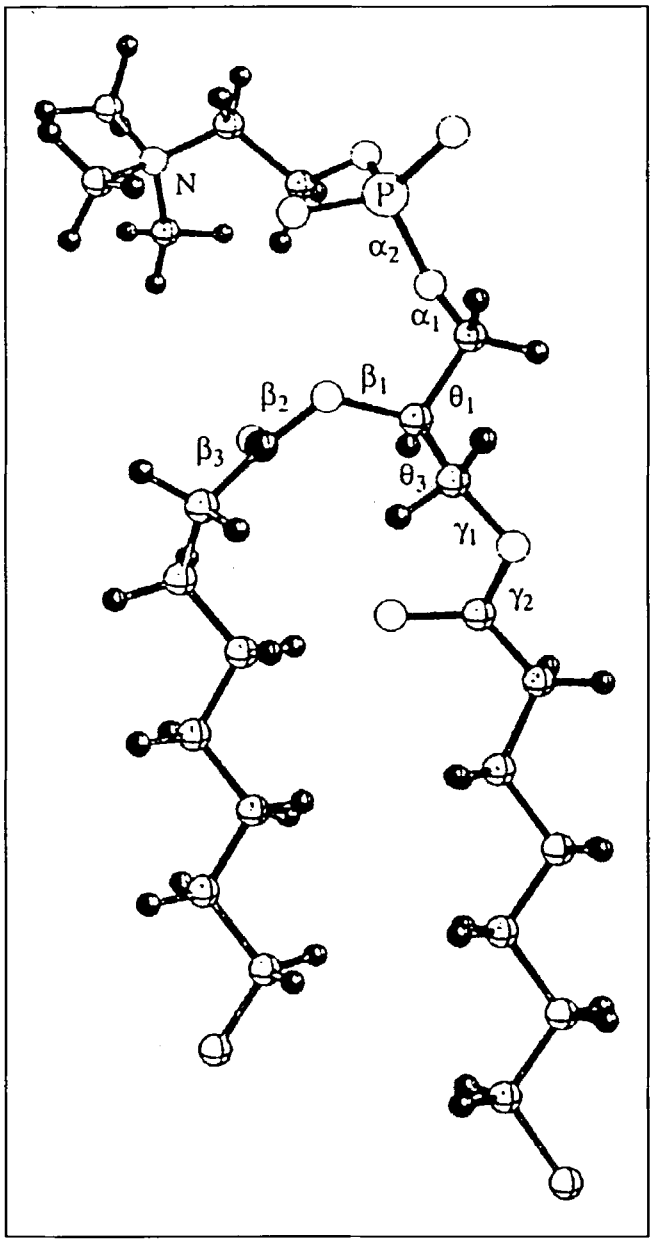
Lipids in membranes

The solid state (i.e. broad-line) ^{2}H-NMR spectra of deuterated lipids may be used to determine the angular amplitudes of motion or ordering of the chain segments in phospholipid bilayers. These in turn are related to the equilibrium area per lipid molecule, which is determined by the balance of lateral stresses within the membrane [8••]. This approach has been used to study the effects of phospholipid headgroup, chainlength, chain unsaturation and temperature on the mean cross-sectional areas of the lipids [9–11]. Particular attention has also been paid to the influence on chain order of additives that promote the formation of nonlamellar lipid phases, because these are likely to increase the curvature stress in bilayer membranes [8••,12]. Such studies are important not only for the elastic and other properties of the lipid membranes, but also because these internal membrane stresses are likely to have a direct effect on the activity of embedded proteins.

The connection between lipid chain order, determined by ^{2}H-NMR, and the curvature in nonlamellar lipid phases is of related interest [13]. The chain order of diacylglycerol and of phosphatidylcholine in the lamellar and nonlamellar phases of their mixtures has been studied by ^{2}H-NMR [14•]. Diacylglycerol is the endogenous lipid activator of the regulatory peripheral membrane enzyme protein kinase C, and its location and chain dynamics in the same systems have also been studied by spin label ESR [15•,16]. Additionally, spin label ESR and ^{31}P-NMR have been used to study the phase diagram of polymorphic binary mixtures of the avidin-binding lipid, biotin-phosphatidylethanolamine, with phosphatidylcholine [17].

In a different area, considerable progress has been made recently in analyzing the conformation of phosphatidylcholine in fluid lipid bilayer membranes by using dipolar couplings obtained from solid state NMR [18••]. Two-dimensional ^{13}C–^{1}H chemical shift correlation spectroscopy indicated that the phosphatidylcholine headgroup is bent back towards the beginning of the *sn*-2 chain, excluding one of the two established crystal conformations for the liquid-crystalline bilayer. Combining 20 known anisotropic NMR couplings, mostly dipolar but also chemical shift anisotropies, yielded a unique conformation for the core of the molecule that is consistent with all the NMR data (Fig. 1). The glycerol G1–G2 torsion angle is non-*trans*, which is significantly different from the crystal conformer with bent-back headgroup.

Figure 1



Structure proposed for the glycerol backbone and adjacent segments of phosphatidylcholine in fluid lipid bilayers. It is consistent with 20 measured NMR dipolar couplings (^{31}P–^{13}C, ^{13}C–^{1}H, ^{1}H–^{1}H and ^{13}C–^{13}C) and chemical shift anisotropies (^{13}CO and ^{31}P). The torsion angles ($\alpha,\beta,\gamma,\theta$) that are fixed are indicated. Most of the rest of the lipid molecule is mobile and has interconverting conformations. Reproduced with permission from [18••].

Another significant aspect of lipid chains in membranes is their attachment to acylated proteins, which has also been studied by ^{2}H-NMR. The acyl chain conformation of palmitoylated gramicidin has been determined for the peptide incorporated in lipid bilayers [19••,20,21]. The otherwise mobile C-terminal ethanolamine attachment site is immobilized in a fixed conformation, and segments in the C1–C5 region of the palmitoyl chain are bent over, probably in a fixed conformation, such that the rest of the chain is immersed in the lipid bilayer and is mobile. This requires only displacements of the immediate C-terminal side chains of the gramicidin helix to accommodate the acyl chain.

The lateral rearrangement of lipids within the membrane plane, induced by surface association of cationic proteins, can be studied by solid state NMR of the lipid headgroup resonances. Segregation of phosphatidylglycerol by binding cardiotoxin II to mixtures with phosphatidylcholine has been found by ^{31}P- and ^{2}H-NMR [22]. Preferential interactions of cytochrome *c* with diphosphatidylglycerol in phospholipid mixtures have also been resolved by magic-angle spinning ^{31}P-NMR [23]. These studies are relevant both to lipid interactions with peripheral proteins and lipid domain formation in membranes.

Solid state protein NMR

Structural determinations, by NMR, of proteins and peptides in lipid membranes (as opposed to in detergent micelles) require the use of solid state techniques. There are currently two major approaches to determining the structure of proteins and peptides by solid state NMR [4,5•]. One is the use of orientational constraints obtained from the angular anisotropy of the dipolar and quadrupolar couplings and of the chemical shift. This is best done with oriented membranes. The other is from direct distance determinations by using the dipolar couplings either between nuclei of the same type, or between different isotopic labels. Line narrowing is performed by magic-angle spinning and the dipolar interaction is recoupled by rotational-resonance methods [24,25] in the homonuclear case, or by rotational-echo double-resonance (REDOR) [26] in the heteronuclear case. Additionally, chemical shifts obtained by magic-angle spinning NMR may be used in structural (and functional) determinations [27••].

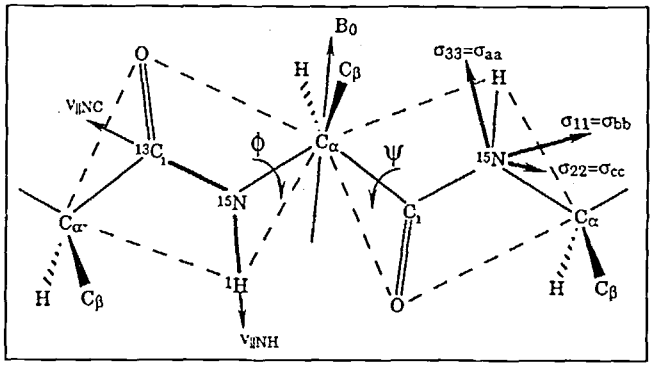
Gramicidin A

The full, atomic-level structure of the ion-channel forming peptide gramicidin in oriented dimyristoyl phosphatidylcholine bilayers has been determined solely by using orientational constraints derived from solid state NMR [28••]. This is the first complete structure of a polypeptide in a phospholipid membrane to be determined by this method and one of relatively few structures determined for integral membrane polypeptides overall. The backbone conformation was obtained from the ^{15}N–^{1}H and ^{15}N–$^{13}C_1$ dipolar splittings and the ^{15}N chemical shift anisotropy of the peptide amide group, together with C^{α}–^{2}H quadrupole splittings, for gramicidin A synthesized with individual isotopically labelled amino acids (Fig. 2). The side chain torsion angles were determined from ^{2}H quadrupole splittings of the deuterated amino acid residues [29,30•]. The initial structure determined solely from NMR orientational restraints is sufficient to define

the molecular fold, helix sense and pitch of the channel, and the orientation of the tryptophan side chains at the bilayer surface. Refinement against all of the structural restraints, together with energy refinement, additionally yields a unique set of side-chain torsion angles.

The gramicidin channel in the lipid bilayer consists of a head-to-head dimer composed of right-handed, 6.3 residues per turn β-helices (Fig. 3). The orientation of the tryptophan residues is of particular interest [30•,31]. The indole N–H bonds are all oriented to the membrane surface and serve to anchor the channel at the polar/apolar interface, as is found also for tryptophan and tyrosine residues in certain integral membrane proteins. The orientation of the dipole moments of the indole rings is such as to stabilize cation binding near the channel entrance.

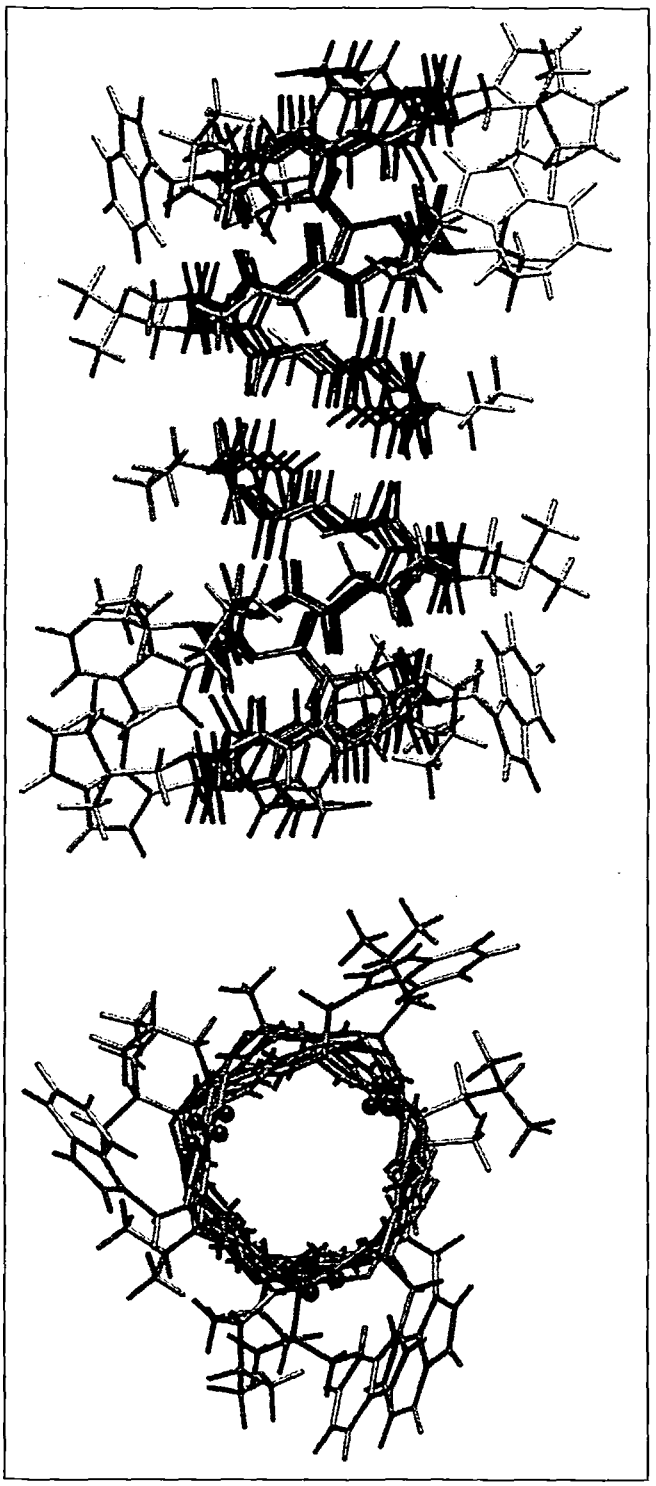
Figure 2



A dipeptide unit; the diagram indicates the linked peptide planes of the backbone. Each plane can be defined by two bond vectors, N–H and N–C_1', relative to the magnetic field direction, B_0. These orientations are obtained from the principal elements of the corresponding dipolar splittings, $\nu_{\parallel NH}$ and $\nu_{\parallel NC}$. The (ϕ,Ψ) torsion angles are then determined by the covalent geometry of the C_α carbon linking the planes. The ^{15}N chemical shift tensor (σ_{11}) and the C_α-^2H quadrupole splittings provide additional restraints. Published with permission from [28••].

Solid state NMR techniques also allow characterization of the peptide dynamics. Local motions of the tryptophan residues in gramicidin preserve their orientation relative to the membrane interface, but cause significant fluctuations in the dipolar stabilization energy that may be related to channel gating [30•]. Some of the valine side chains of gramicidin are in fixed rotomeric states, whereas others undergo three-state jumps [29]. Of most interest are the dynamics of the peptide backbone which forms the lining of the gramicidin ion channel. Field-dependent spin-lattice relaxation time measurements on the ^{15}N-labelled amides revealed that local librational motions about the C^α–C^α axes occur with correlation times in the nanosecond range [32••]. Librational motions on this relatively slow timescale suggest extensive correlations

Figure 3



Complete structure of the gramicidin A channel dimer in lipid bilayers determined solely from solid state NMR orientational constraints. Published with permission from [28••].

between peptide segments, resulting in concerted motions at a frequency that is comparable with the rates of ion translocation. Librational motions about the C^α–C^α

axes can tilt the peptide C=O groups into the channel, hence providing a solvation environment for translocating ions. Therefore a functional correlation between backbone dynamics and channel conductance is possible.

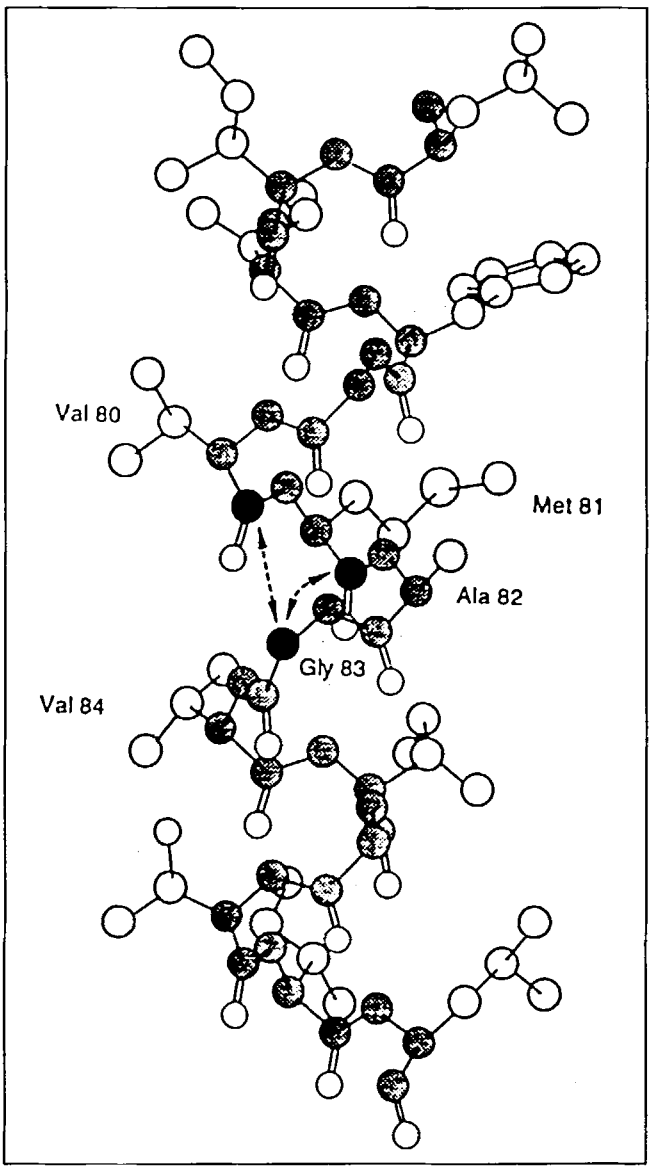
Glycophorin A and other proteins

The structure of the transmembrane segment of glycophorin A, the major glycoprotein and antigenic determinant of red blood cell membranes, has been investigated by solid state NMR methods. Rotational resonance was used to measure the rate of magnetization exchange between pairs of specific ^{13}C labels in a peptide corresponding to the transmembrane domain which was incorporated in dimyristoyl phosphatidylcholine bilayer membranes [33]. This resulted in intrapeptide internuclear distance measurements with resolutions of the order of 0.3 Å. Backbone distances between [$^{13}C^{\alpha}$]Gly83 and [^{13}C=O]Val80 or [^{13}C=O]Met81 in the middle of the transmembrane section were both ~4.5 Å. These correspond to the distances expected across one turn and across the diameter, respectively, of an α-helix (Fig. 4). Measurements of the distance between residues Ile91 and Gly94 were also consistent with an α-helical geometry, but the distance between Ile95 and Gly98 was longer, suggesting that the transmembrane helix unravels at the membrane interface.

More recently, the interpeptide magnetization exchange rates were measured between ^{13}C-methyl groups of the side chains and ^{13}C-carbonyl groups of the backbone in the hydrophobic sequence –Gly^{79}–Val^{80}–Met^{81}–Ala^{82}–Gly^{83}–Val^{84}– that is located in the middle of the transmembrane domain of glycophorin A [34•]. Transmembrane peptides with a single side-chain label were mixed with backbone-labelled peptides in order to probe distances across the dimer interface in reconstituted membranes. Significant interpeptide magnetization exchange was observed only between [$^{13}CH_3$]Val80 and [^{13}C=O]Gly79 and between [$^{13}CH_3$]Val84 and [^{13}C=O]Gly83, indicating that these residues are packed closely at the dimer interface (Fig. 5). This method represents a general way of investigating helix–helix interactions in the assembly of transmembrane protein segments.

REDOR has been used for structural investigations of the ligand-binding site of the glutamine-binding protein that is part of the glutamine transport system in *E. coli* [35]. REDOR distances of up to 6.2±0.2 Å were measured between ^{13}C-labels in the L-glutamine ligand and ^{15}N-labels on amino acid residue His156 of the binding site. This method has previously been used to measure the distance between ^{13}C and ^{15}N isotopic labels in the backbone of gramicidin A incorporated in lipid bilayers [36].

Figure 4

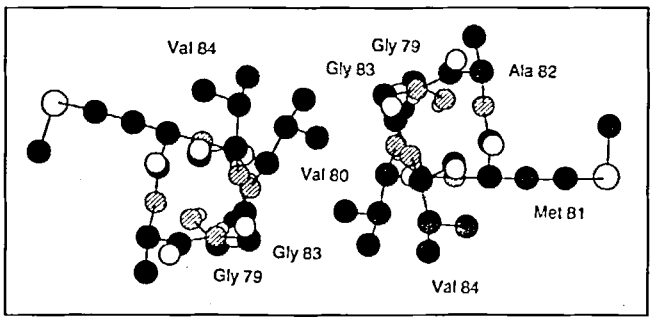


The region between Leu75 and Leu89 in the transmembrane domain of glycophorin A. The arrows connect pairs of labelled ^{13}C-atoms, across the diameter (Met81–Gly83) and across one turn (Val80–Gly83) of the α-helix for which magnetization exchange is observed by rotational resonance. Reproduced with permission from [33].

Receptor tyrosine kinase, rhodopsin and bacteriorhodopsin

Direct chemical shift measurements by magic-angle spinning (MAS) solid state NMR can also provide important structural and functional information on membrane proteins [27••]. This approach makes use of the sensitivity of the chemical shift, particularly the chemical shift tensor, to the local electronic environment. A particularly striking

Figure 5



The region between Gly79 and Val84 in the transmembrane domain of the glycophorin A dimer. The helices are oriented such that the side chains of Val80 and 84 are in close proximity with the backbone carbonyls of Gly79 and 83, respectively, across the dimer interface, as is indicated by magnetization exchange in rotational resonance NMR experiments. Reproduced with permission from [34•].

structure-function correlation has been obtained from determination of the pK_a and ^{13}C chemical shift anisotropy of the glutamic acid residue Glu664 that is located in the transmembrane domain of a receptor tyrosine kinase encoded by the neu/erbB-2 proto-oncogene [37••]. Replacement of the valine residue, normally at this position, by glutamic acid leads to constitutive activation of the receptor and hence to cell transformation in the absence of ligand. From MAS measurements on the 5-[^{13}C]-Glu664 transmembrane peptide reconstituted in phospholipid bilayers containing a physiological level of negatively charged lipid, it was found that the Glu664 carboxyl side chain is protonated and strongly hydrogen bonded. These solid state NMR studies provide strong evidence for the direct involvement of glutamate–glutamate hydrogen bonding in the receptor dimerization and activation.

Molecular orbital calculations have been used to interpret the ^{13}C chemical shifts obtained by solid state NMR for the retinal chromophore of the visual receptor protein rhodopsin in its native membrane environment [38,39,27••]. In this way, it was possible to position the 11-*cis* retinal relative to the charged counterion (amino acid residue Glu113) of its protonated Schiff base linkage to Lys296, and relative to the transmembrane helices of the protein. Generation of a twisted 11-*trans* retinal conformer in the binding site then leads to ^{13}C chemical shifts that are strikingly similar to those of bathorhodopsin, the first photointermediate of the visual cycle. Relaxation of the chromophore to the fully all-*trans* retinal then requires displacements of the α-helices forming the binding site.

Along similar lines, the ^{13}C chemical shifts (or rather their tensor elements) of retinal in the light-driven proton pump bacteriorhodopsin have previously been used to obtain structural information on the protein-bound chromophore in native bacterial membranes (reviewed in [27••]). More recently, orientational constraints derived from the quadrupole splittings of specifically deuterated retinals have been applied to this same problem [40,41], as has also rotational resonance of retinal containing specific ^{13}C label pairs [42].

Solution state protein NMR

The nuclear Overhauser effect (NOE)-based ^1H-NMR techniques used for determining the structures of soluble proteins may be applied to integral membrane proteins if they are solubilized in deuterated organic solvents or detergents that form small micelles [1]. This is necessary to obtain high resolution spectra and to limit spin diffusion. The success of this approach depends partly on how well the solubilizing system is able to mimic the membrane environment and also on the size of the protein.

Phage coat proteins

The major coat proteins of the filamentous bacteriophages have been studied extensively in detergent micelles [43]. These are composed of approximately 50 amino acid residues with a single hydrophobic domain and are stored as integral proteins in the membrane of the host bacterial cell prior to phage assembly. The structure of the M13 bacteriophage coat protein has been studied recently in micelles of dodecylphosphocholine [44•]. This single-chain detergent bears a zwitterionic phospholipid polar group. The structure obtained by using both homonuclear and heteronuclear multidimensional experiments was similar to that found previously in micelles of the charged detergent sodium dodecylsulphate (SDS) [45]. The structure of the zwitterionic micelles was, however, less distorted than that obtained for SDS, because of the charge interactions between the lysine side chains and the anionic detergent in the latter case. The protein consists of a hydrophobic α-helix that spans the micelle and which is separated by a hinge region around residue 21 from an N-terminal amphiphilic α-helix that lies in the micelle surface. The locations of the two helices relative to the micelle were determined by the effects of electron spin-labelled fatty acids on the NMR resonances.

The structure of the closely related fd bacteriophage coat protein in SDS micelles was previously found to be similar to that described here for the M13 coat protein [46]. In this case, the orientation of the hydrophobic and amphiphilic helices was established in aligned bilayer membranes by solid state NMR of single ^{15}N-labelled residues in the two regions.

Mitochondrial proton ATPase subunit c

The structure of the proteolipid subunit *c*, which forms the proton channel of the mitochondrial F_1F_o-ATPase, has been investigated in $CHCl_3$–MeOH–H_2O by 2D spin-label difference spectroscopy [47••]. It was previously shown that, in this aqueous–organic solvent mixture, the protein folds as an anti-parallel pair of extended helices similar to the hairpin fold expected in the membrane [48,49]. The approach using spin-label difference spectroscopy is particularly useful in detecting the tertiary fold of the

protein from the paramagnetic broadening of the NMR resonances. Here, a spin-labelled maleimide was attached covalently at Cys67 in the N-terminal helix of a functional site-specific mutant protein A67C. In this way, it was possible to map out the 3D structure of a 30 Å region of the protein from the reduced (diamagnetic) minus oxidized (paramagnetic) spin-label 2D NMR difference spectra. The structure defined by residues 10–25 and 52–79 consists of two gently curving helices which cross at an angle of 30°. The Asp61 residue in the C-terminal helix that undergoes protonation-deprotonation during H^+ transport was found to be in van der Waals contact with residue Ala24 of the N-terminal helix, in agreement with mutagenesis experiments.

Ion-channel peptides

^{1}H-NOEs have been used to compare the structures in trifluoroethanol and in dodecylphosphocholine micelles of peptides representing transmembrane regions from the voltage-gated sodium channel [50•]. The 260 kDa α-subunit of the Na channel contains four homologous repeats (I–IV), each of which is thought to have six transmembrane segments (S1–S6). Simplification by synthesizing the corresponding transmembrane peptides is necessary for study by solution state NMR methods, because of the size of the intact protein. Previous studies of channel proteins by using this strategy have been reviewed recently [7]. Single-spanning segments IS2, IS4, IVS4, as well as a double-spanning segment, IS34, and a putative link region, ISlink56, were investigated. All peptides were found to be predominantly α-helical in both solvent systems, but well defined tertiary structure was not detected for the two longer peptides, IS34 and ISlink56, which were composed of two helices linked by a central hinge region (cf. ATPase subunit *c*, above). The ISlink56 peptide is important because it is thought to form the channel lining, for which a nonhelical β-structure has been proposed in the intact protein [see 7].

Solution state NMR studies are also being carried out to investigate the structure of the transmembrane segment of glycophorin A in micelles [51]. These will provide an interesting complement and comparison with those studies undertaken in bilayer membranes by solid state techniques (cf. above).

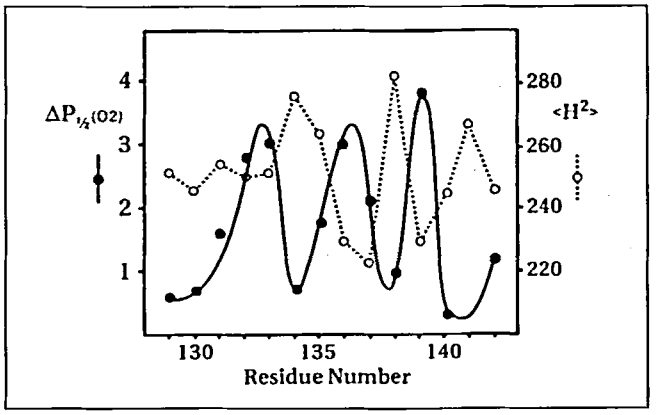
Other aspects

Interest is now moving to the dynamic information contained in the high resolution solution state 2D-NMR spectra, for instance from the heteronuclear ^{1}H–^{15}N NOEs. Determinations of high frequency fluctuations of the peptide N–H bond orientation have been used in conjunction with molecular dynamics simulations to detect mobile link regions between helical segments [52].

Site-directed spin labelling

The introduction of single cysteine residues in the sequence of membrane proteins by site-directed mutagenesis has opened up the possibility of specific spin-labelling for structural studies by ESR [2,53,54]. Structural information comes primarily from the accessibility to paramagnetic agents with preferential solubilities in polar or apolar environments, determined from enhancements in the spin-lattice relaxation rate of the spin label [2,55]. The overall transmembrane fold can be mapped from the accessibility to water-soluble paramagnetic ion complexes, and the secondary structure of the assembled transmembrane segments can be determined from the characteristic periodicity of exposure to paramagnetic oxygen concentrated in the hydrophobic phase of the lipid. A 3.6-residue periodicity is particularly well illustrated by helix-E of bacteriorhodopsin [53]. Here, high accessibility to lipid-dissolved oxygen correlates with higher mobility of the corresponding spin-labelled residues exposed to lipid, whereas at other positions of labelling mobility is restricted by tertiary interactions in the protein (see Fig. 6). Spectral lineshape analysis to determine spin label mobility is therefore a valuable and developing adjunct to the methods of structure elucidation [56,54].

Figure 6



Accessibility to oxygen (solid line) and motional restriction (dotted line) of the residues 129–142 in helix-E of bacteriorhodopsin obtained by spin-label scanning. Oxygen accessibility is obtained from the microwave saturation properties ($\Delta P_{1/2}$) and motional restriction from the second moment of the spectral lineshape (<H²>) of the spin-labelled residue. Reproduced with permission from [53].

Rhodopsin

Spin-label scanning mutagenesis has been applied to 20- and 30- contiguous residue sequences that contain the C-D and E-F interhelical loops, respectively, which link transmembrane segments in rhodopsin [57••,58••]. Both these cytoplasmic loops form part of the binding site for the G-protein transducin in visual transduction by photoactivated rhodopsin. The C-D loop was found to cross the aqueous/hydrophobic boundary near Val138 and near His152, and the E-F loop in the range Val227–Lys231 and again in the range Val250–Val254. Several of the transmembrane helices extend beyond the membrane surface: the C-helix to at least residue 140, and the E and F helices by about 1.5 and 3 turns, respectively.

Photoactivation of rhodopsin results in changes of mobility at specific sites that are best interpreted as a rigid-body movement of helix-C relative to the rest of the protein, and generally in terms of helix movements that extend into the aqueous loop regions.

Lactose permease

In the sugar transport system, lactose permease of *E. coli*, site-specific labelling has been used to demonstrate that the acidic residues Asp237 and Asp240 are located in transmembrane domains, either near to the periplasmic surface or in a water-filled cleft [59]. Scanning of both oxygen accessibility and spin label mobility has been performed in the region between residues 387 and 402 [60•]. This transmembrane domain XII is found to be in an α-helical conformation and to be situated at the periphery of the permease, exposed to lipid. A different ESR approach has also been used to determine the proximity of the intramembranous segments in lactose permease by engineering in a potential interhelical paramagnetic ion binding site using His replacement mutants [61–63]. From Mn^{2+} binding assays using ESR, transmembrane helices VIII and X, helices VII and XI, and helices IX and X are found to be closely apposed in the tertiary structure of this polytopic membrane protein. In addition, a paramagnetic ion binding site inserted in one of the periplasmic loops of lactose permease has been used to determine the distance to specific spin-labelled residues in the putative transmembrane helix IV [64•,65].

Diphtheria toxin

Site-directed spin labelling has also been applied to the hydrophobic helix-9 (TH9) in the transmembrane (or translocation) domain of diphtheria toxin [66••,67]. The TH9 helix, which is buried within the hydrophobic interior of the soluble toxin, is thought to be involved in translocation from the low-pH interior of the endosome to the cytoplasm in the final stages of toxin entry into the cell. Spin-label scanning mutagenesis indicates that, at low pH, the TH9 segment is inserted in lipid bilayers with a transmembrane orientation in which its helical structure is preserved. Accessibility to NiEDDA suggests that one face of the helix is exposed to an aqueous channel.

Other aspects

The membrane insertion of the voltage-gated channel-forming peptide alamethicin has also been studied by site-directed spin labelling [68]. The resting conformation and location limits possible models for the voltage gating. Spin-labelled cysteine mutants have also been used to study the membrane binding of a 25-residue highly basic peptide from the MARCKS protein [69]. This region contains both the calmodulin-binding domain and the phosphorylation sites in this substrate of protein kinase C. In contrast to alamethicin, this peptide binds at the membrane interface in an extended conformation with the N-terminal directed out from the surface.

Further developments in membrane applications of site-directed spin labelling are likely to come from the link between structure and dynamics, as has already been demonstrated with T4 lysozyme [56], and also from direct distance measurements by using paramagnetic dipolar interactions [54].

Membrane protein assembly and translocation: ESR

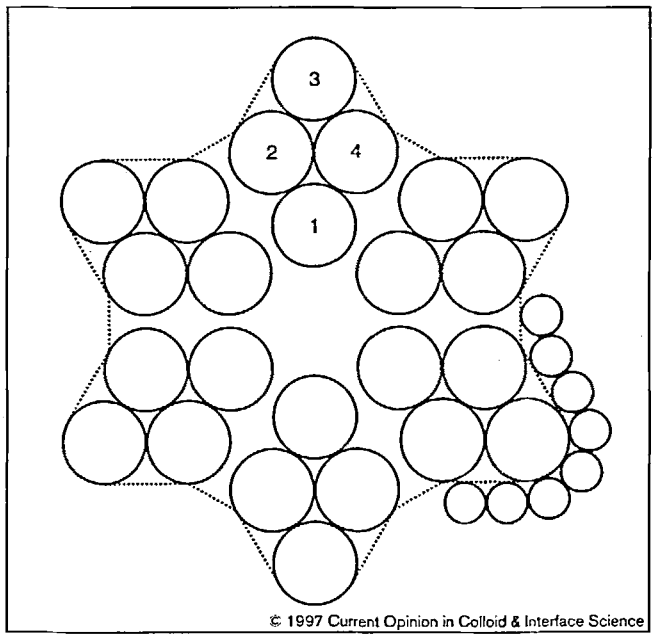
Membrane properties such as lipid–protein interactions and protein rotational diffusion, that are readily investigated by spin label ESR spectroscopy, are directly related to the state of membrane protein association [70,7]. Such studies give valuable information about the more global aspects of membrane structure. Important are the oligomeric assembly of integral proteins, the insertion and translocation of presequences and precursor proteins, and the lipid interactions with surfactant proteins.

Integral proteins

The number of lipids at the intramembranous perimeter of integral membrane protein assemblies and their specificity of interaction with the protein side chains can be determined from the ESR spectra of spin-labelled lipids [70]. This method has been used to study the assembly of a 16 kDa proteolipid that belongs to a class of membrane channels that includes those of the vacuolar ATPases [71•]. The perimeter of the protein determined in this way is consistent with a hexameric assembly of transmembrane 4-helix bundles that was proposed on the basis of molecular modelling and electron microscopy (see Fig. 7). The selectivity of interaction with anionic lipids is also consistent with the placement of the basic amino acid side chains in the model. The assembly in lipid bilayers of a 26-residue peptide corresponding to the single putative transmembrane sequence of the IsK voltage-gated potassium channel has been studied by similar methods [72•]. The low stoichiometry of the lipid interaction (ca. 2.5 phospholipids per peptide monomer) is consistent with an extended β-sheet structure, in agreement with infrared spectroscopy. The selectivity of interaction with negatively charged lipids locates the basic residues at the N- and C-terminals of the peptide close to the lipid headgroups, indicating that the β-strands are strongly tilted relative to the membrane normal. Recently, spin label studies of the lipid interactions with phospholamban have been used to study the role of phosphorylation in its intramembranous association and dissociation relevant to regulation of the cardiac Ca-ATPase [73]. Lipid–protein interactions and protein rotational diffusion investigated by spin label ESR have been used to determine conditions under which removing the extramembranous domains of the Na,K-ATPase by extensive proteolysis preserves the structural integrity of the transmembrane domains [74]. Such characterization is necessary to validate the use of proteolytically simplified systems for structural investigations on complex integral proteins. For the intact Na,K-ATPase, variation of the

aqueous viscosity has been used to determine the size of the extramembranous domains by rotation diffusion measurements using saturation-transfer ESR spectroscopy, which normally yields information only on the size of the intramembranous section [75]. The effect of the unique proline-rich C-terminal extension of squid rhodopsin on the assembly of cephalopod photoreceptor arrays has also been studied by rotational diffusion measurements using saturation-transfer ESR [76].

Figure 7



Schematic diagram of the helix packing in the hexameric membrane assembly of the 16 kDa proteolipid protein from *Nephrops*. The transmembrane helices are represented by cylinders of 10 Å diameter, where each 4-helix bundle represents a protein monomer. The hydrophobic surface is covered by eight lipid chains of 4.8 Å diameter per protein monomer, in accordance with spin-labelled lipid ESR measurements. See reference [71•].

Protein insertion

The characteristic features of lipid interactions with integral proteins that are revealed by spin label ESR spectroscopy may be used to investigate the insertion of soluble proteins into membranes. Such studies with diphtheria toxin indicate that the low-pH triggered insertion takes place in two stages [77•]. At pH 6.2 the stoichiometry of lipid–protein interaction is consistent with insertion of only the translocation domain, whereas at pH 5.0 it increases to a level expected for insertion of the whole toxin. The nature of the membrane insertion of α-lactalbumin in its molten globule form at low pH has also been studied from the characteristic perturbations in the mobility of spin-labelled lipids [78]. Both of these studies are of direct relevance to the insertion and translocation of a protein in the molten globule state across lipid membranes. By contrast, the lung surfactant proteins SP-B and SP-C do not give rise to the perturbations in mobility of spin-labelled lipids that are typical of integral membrane proteins when these hydrophobic proteins are fully associated with lipid bilayers [79]. This qualitative difference in the lipid–protein interaction can be attributed to the function of these proteins in lowering the interfacial tension of lung surfactant.

Pre-/leader sequences and precursor proteins

Protein precursors that must be translocated across intracellular or plasma membranes are frequently synthesized with an N-terminal extension (named a presequence, leader sequence or signal sequence) that is cleaved off after translocation. The insertion in lipid membranes of these leader peptides or of the whole precursor may be studied with lipid spin labels, as above, or with the paramagnetic relaxation agents used in site-directed spin labelling.

The nucleotide-dependent membrane insertion of SecA, which is part of the protein translocation machinery in *E. coli*, has been studied with lipid spin labels [80•]. The mode of insertion of the presequence of mitochondrial cytochrome *c* oxidase subunit IV has been compared in different negatively charged lipid membranes [81]. Both spin-labelled lipids and the accessibility to paramagnetic agents of a spin label attached to the single cysteine residue in the presequence were used in this case. Lipid–protein interactions and membrane insertion of the entire precursor of the *E. coli* outer membrane protein PhoE have similarly been investigated [82]. Paramagnetic relaxation enhancements were determined for spin labels introduced by site-directed mutagenesis in either the signal sequence or the mature parts of the PhoE precursor protein.

A somewhat different approach has been used with apocytochrome *c*, the haemless precursor of cytochrome *c*, that is imported into mitochondria without a presequence. The membrane penetration was detected by spin–spin interactions between lipids spin-labelled at different positions and a spin label bound to the precursor protein at the site destined for haem attachment [83]. The membrane location of the precursor obtained from the double spin-labelling experiments was consistent with that deduced also with paramagnetic ions and oxygen as relaxants [84]. In principle, the spin–spin interactions also give dynamic information on the lipid–protein interaction, in a manner somewhat analogous to the determination of lipid exchange rates at the intramembranous surface of the integral myelin proteolipid protein [85].

Conclusion

The topics reviewed illustrate the breadth of different magnetic resonance spectroscopic methods available for study of membrane structure and dynamics. Applications range from the functional to the purely structural, with dynamics constituting an important link between the two. Future developments in the determination of three-di-

mensional structures of membrane proteins are certain. The potential to exploit relaxation measurements for investigating structure-function relationships will surely also develop further. Both forms of magnetic resonance, NMR and ESR, are likely to contribute equally and in complementary ways to these future advances.

Note added in proof

The author wishes to draw the reader's attention to some recent references that were published after this review had been written. They are numbered [86•] and [87•] in the reference list.

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