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Biochimica et Biophysica Acta 1508 (2000) 129–145

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

Customizing model membranes and samples for NMR spectroscopic studies of complex membrane proteins[1]

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Received 10 February 2000; received in revised form 15 June 2000; accepted 3 August 2000

Abstract

Both solution and solid state nuclear magnetic resonance (NMR) techniques for structural determination are advancing rapidly such that it is possible to contemplate bringing these techniques to bear upon integral membrane proteins having multiple transmembrane segments. This review outlines existing and emerging options for model membrane media for use in such studies and surveys the special considerations which must be taken into account when preparing larger membrane proteins for NMR spectroscopic studies.

Keywords: Nuclear magnetic resonance; Membrane protein; Micelle; Vesicle; Amphipol; Bicelle

1. Introduction

As the transition is made to the post-genomic era of biological science, membrane proteins remain one of the great frontiers of structural and chemical biology, particularly when viewed from the standpoint of what is known about individual molecules. For example, there is not a single high resolution structure available for a G protein-coupled receptor as of mid-2000. Progress in the area of membrane protein structural determination has been relatively slow because of the difficulties of applying solution nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallographic methods to membrane proteins. This has provoked innovation in these areas as scientists find ways to bring these classical methods to bear upon a greater number of membrane proteins. It has also led to innovation in the form of non-classical approaches to structural determination such as solid state NMR and high resolution electron microscopy [1].

In this review, we shall not focus upon the details of NMR spectroscopy. Instead, the focus shall be upon how appropriate and sometimes innovative sample preparation methods can enhance the spectroscopic accessibility of membrane proteins. Because of the predisposition of the authors, this review has been written specifically with NMR spectroscopy in mind. However, much is applicable or extendible to other forms of spectroscopy. From a molecular taxonomic standpoint, the focus of this review is upon complex integral membrane proteins: proteins having multiple transmembrane segments, sometimes with substantial extramembrane domains. It is for proteins of this class that progress is slowest and,

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[1] This review is dedicated to the fond memory of Professor Gitte Vold of the University of California, San Diego, CA, USA.

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00308-4

from the standpoints of basic and applied biomedical science, most needed. It is noted with regret that the scope of this review does not encompass the many published NMR studies of single-span or peripheral membrane proteins (cf. [1,2]). Also not covered are isotopic labeling methods or strategies. In writing this review, the authors are attempting to address both NMR experts with little membrane protein experience and membrane biophysicists with little NMR experience. It is hoped that specialists in either area will put up with sections presenting information which is elementary to them.

It is a presupposition of this review that the 'golden age' of NMR as applied to membrane proteins will arrive in the not so distant future. Solid state NMR technology is in an extremely rapid phase of development, such that assignment of the spectra of relatively small (<100 residues) proteins now appears to be within reach for both static sample and magic-angle-spinning approaches [2–7]. Breakthroughs in solution NMR technology, including the availability and exploitation of very high magnetic fields, have led to the claim that it is now possible to tackle structural analysis of proteins and complexes even as large as 100 kDa [8]. This leads one to contemplate whether it may soon be feasible to use solution NMR to tackle the three-dimensional structures of membrane proteins such as the G protein-coupled receptors (which have seven transmembrane spans and whose molecular weights (MW) typically fall into the 35–50 kDa range [9]).

In general terms, choice of suitable sample conditions for a membrane protein represents a much more difficult problem than for a water soluble protein. Consider the case of a soluble protein which one would like to subject to solution NMR analysis. In screening for optimal sample conditions, one might test three temperatures, two salt concentrations and four values of pH. If all possible combinations are screened, a total of $3\times2\times4=24$ preliminary samples is required. Now, consider the case of a membrane protein. In addition to the variables which pertain to water soluble proteins, one might also want to test three different detergent micelle types, each at two different detergent concentrations, each in the presence and absence of added phospholipid. With these two additional variables, an exhaustive screen of conditions would require $3\times2\times4\times3\times2\times2=288$ different samples. Thus, optimizing sample conditions for membrane proteins will typically require either extraordinary fortitude (and the accompanying resources to support it), combinatorial methods [10], or well-informed guesses regarding which sample variables can be safely chosen without systematic testing. It is the goal of this review to lay out the full range of membrane-related variables and to review how rational choices can be made which are most likely to yield positive results in various spectroscopic situations.

2. Basic difference between solution NMR and solid state NMR

'Solution NMR' can be defined as the spectroscopy of molecules which tumble rapidly and isotropically on an NMR time scale. Isotropic tumbling means that the molecule tumbles in three dimensions in solution such that it has no net average orientational preference with respect to an imposed magnetic field. Rapid motion on an NMR time scale means that motions must be faster than the frequency range spanned by relevant static dipolar and chemical shift tensors. Generally, motions more rapid than very approximately 200 000 s^{-1} (Hz) will satisfy this condition. Molecules which do not satisfy the requirements of rapid isotropic motions fall into the regime of solid state NMR. Note that molecules such as bilayer lipids may execute very rapid motions which are not isotropic. Also, molecules may populate an isotropic distribution of orientations but fail to execute rapid motional averaging over all orientations (as in powders). Such molecules behave as solids from an NMR standpoint.

While a review of NMR methods is not the goal of this paper, it should be noted that both solid state and solution NMR can, in principle, be used to effect total high resolution structural analysis. However, because the technology of solid state NMR is in a relatively early state of development relative to solution NMR, the largest protein whose structure has been determined to high resolution as of mid-2000 by solid state NMR is a homodimeric polypeptide of 10 residues per subunit, gramicidin A [4]. This is in contrast to solution NMR methods which have been

used to solve hundreds of (water soluble) protein structures, some well in excess of 25 kDa. However, success to date in applying solution NMR methods to complex membrane proteins has been modest: the largest structures determined to high resolution have only two transmembrane segments and are in the 5–10 kDa range [11–13]. Moreover, solid state NMR has at least one tremendous advantage over solution NMR: methods of this class can be applied to proteins in lipid bilayers.

3. Available model membrane systems

3.1. Vesicles

The variables distinguishing vesicle types from each other are degree of bilayer multilayering, size, lipid composition, net charge, shape and lyotropic phase. A vast literature exists which describes the preparation and characterization of lipid vesicles (liposomes) and for reconstituting membrane proteins into them [14–25]. Because even the smallest vesicles are very large from a solution NMR point of view (MDa aggregate weights), vesicles are not used in direct solution NMR studies of integral membrane proteins [26]. However, they are broadly employed in solid state NMR. In most cases, large multilamellar vesicles are used because these are easily formed and because multilayering makes it possible to prepare samples with relatively high concentrations of lipid and membrane proteins, a fact which is critical for optimizing NMR signal intensity. It may be particularly important to choose bilayers in which the transbilayer thickness matches the span of the hydrophobic domain of the membrane protein of interest [27]. Principles for choosing among other variables for specific experimental situations are described in Section 4.

3.2. Native membranes

There are a number of published examples of NMR studies using samples involving non-purified membrane proteins in native membranes. One set of studies involves titrating membranes rich in the protein of interest with ligands specific to that protein. The NMR spectrum of the ligand is monitored. For solid state NMR, the ligand is generally labeled with ^{13}C or some other observable heteroatom [28]. Solution NMR studies of the conformation of membrane protein-associated ligand may be possible in cases where exchange between free (isotropic) ligand and receptor-bound ligand is rapid on the NMR time scale. In such cases, transferred nuclear Overhauser effect (NOE) measurements may be possible ([29], see also [30]). In this latter regard, it should be noted that the NOE effect in solids may be extensively complicated by spin diffusion: great care should be exercised in interpreting NOE-like measurements when the ligand is binding to native membranes or large vesicles which lie in the solid state regime of NMR.

A second class of studies involves detection of the membrane protein itself. This generally requires a biosynthetic isotopic labeling method. Unless great care is taken, many other proteins in the membrane will also be labeled. In some cases, this is not a serious problem because the protein of interest is in vast excess to other proteins within a given membrane preparation as a result of natural or induced overexpression [28,31–34]. In other cases, double isotopic labeling schemes and accompanying pulse technology can be employed to filter out unwanted signals and to focus upon specific protein sites of interest (for a solution NMR example of this approach, see [35]).

3.3. Bicelles

Bicelles are 'binary, bilayered mixed micelles bearing a resemblance to the classical model for bile salt–phosphatidylcholine aggregates' [36,37]. Bicelles represent an intermediate between lipid vesicles and classical mixed micelles, being composed of phospholipid bilayer discs which are edge-stabilized by an annulus of detergent. Unlike lipid vesicles, bicelles do not have inner aqueous compartments and are optically clear; unlike classical mixed micelles, they retain a bilayered domain which maintains a number of key dynamic and conformational properties of liquid crystalline phase bilayers. Bicelles can be oriented by magnetic fields such that their bilayer normals are orthogonal to the direction of the applied field (Fig. 1). It is also known that by doping bicelles with certain paramagnetic ions, aromatic molecules, and some membrane proteins, it is possible to change

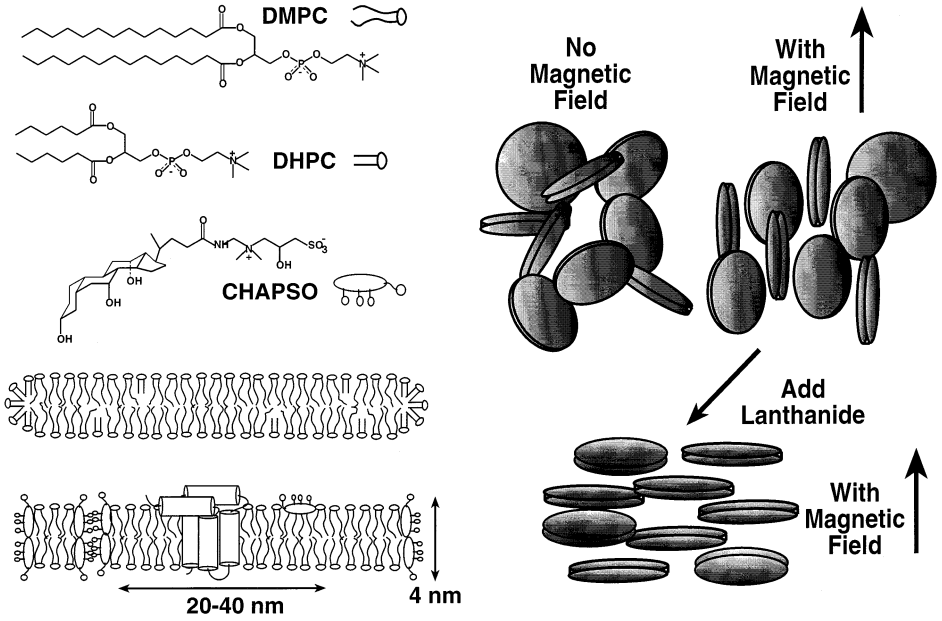

Fig. 1. Components and orientational properties of common bicelles. The 2-D cross-section slice models are drawn approximately to scale (adapted from [36]).

the sign of aggregate magnetic susceptibility such that alignment occurs with normals parallel to the direction of the applied field (see review in [36]).

The best-characterized bicellar systems are composed of mixtures of dimyristoylphosphatidylcholine (DMPC) and either dihexanoylphosphatidylcholine or CHAPSO as the detergent component. For these mixtures, bicelles form over a fairly wide detergent: lipid ratio and over a fairly wide range of temperatures, but only above the phase transition temperature of the lipid component. There is most likely a wide range of lipid/detergent type combinations which will form bicellar assemblies under appropriate conditions.

While the existence and biological relevance of bicelle-like aggregates has been appreciated for many years [38,39], only recently have bicelles been employed as a medium in which to reconstitute and characterize integral membrane proteins [40,41]. In some cases, membrane proteins can be integrated into bicelles in a manner consistent with maintenance of the bicellar morphology and with native protein structure and function. However, it is known that some membrane proteins interact with bicelles in a manner such that the aggregate morphology is grossly perturbed and also that some bicelles disrupt native folding of some membrane proteins [40,109]. It is quite possible that through continued bicelle system development, systems may be found which are compatible with proteins which presently seem to be incompatible with DMPC-based bicelles. For example, obvious areas of development include making bicelles more like native membranes by using lipids having longer acyl chains and some degree of chain unsaturation, by imposing a net negative charge on bicellar assemblies, and by including some cholesterol. Work is in progress in these areas (cf. [42,43]).

Larger bicelles are potentially useful for solid state NMR studies of membrane proteins. Smaller isotropic bicelles are still much too large to be effectively employed in solution NMR studies of complex integral membrane proteins. The use of bicelles as a medium in which to study water soluble proteins is reviewed in [36].

3.4. Micelles and mixed micelles

Detergent micelles are useful as a medium in which to solubilize membrane proteins for solution NMR work because of their relatively small size (usually 10–100 kDa) compared to any available bilayered

assemblies. There are, of course, a host of different detergent types of varying charges and sometimes distinctly varying molecular topologies [44–47]. A number of papers have compared the biochemical compatibility of various detergent types [48–52]. For NMR experiments, the primary class employed to date are those having a single polar head group and a single extended apolar tail type.

Detergent micelles are most often thought of as spherical assemblies. However, in actuality most micelles (even those formed from only a single detergent type) are probably somewhat cigar-shaped (prolate ellipsoid) or discoidal (oblate ellipsoid) [46,47,53–56]. It should also be kept in mind that when lipids or proteins are added to micelles, both the critical micelle concentration (CMC) (the concentration of detergent below which detergent is monomeric in solution and beyond which all additional added detergent forms micelles) and the aggregation number (the average number of detergent molecules per micelle) can be perturbed [47,48,50,57,58] sometimes dramatically. This is probably especially true for membrane proteins when the size of the protein approaches or exceeds the normal size of the protein-free micelle. In these cases, it is probably the properties of the protein which are the primary determinant of the final detergent protein aggregate size. For example, the 40 kDa diacylglycerol kinase (DAGK) homotrimer forms 100 kDa protein–detergent micelles with octylglucoside, even though protein-free octylglucoside micelles are only about 20 kDa [58]. Indeed, for DAGK in a variety of micelle types, it has been observed that there is little correlation between the size of detergent–DAGK micelles and the corresponding protein-free micelles [48].

The term 'mixed micelles' usually implies a lipid component. For micelle size to remain small enough for solution NMR studies, the lipid:detergent ratio must remain low. The usual reason for having lipid present in studies of membrane proteins involving detergent micelles is to enhance membrane protein stability and/or functionality. For example, DAGK is only marginally active in most detergent micelles. However, when micelles are doped with 5–20 mol% of various lipids, DAGK's activity is typically increased by about 50-fold [60,61], such that its specific activity becomes similar to that in membrane bilayers.

3.5. Amphipols

Amphipols are *amphi*pathic *pol*ymers which were recently introduced by Tribet, Audebert and Popot as a new way of solubilizing membrane proteins in aqueous solution [62–64]. While many amphipathic polymers have previously been prepared [65] and many others can be envisioned, the specific amphipols prepared by Tribet et al. are based upon partial random amidation of polyacrylic acid with isopropylamine and/or octylamine to generate random graft co-polymers having MW of 8–35 kDa (Fig. 2). Thus, polar side chains along the polymer backbone (i.e. carboxylate) are randomly dispersed with non-polar side chains (i.e. alkylamides), giving the polymer an amphipathic character. Because preparations of polyacrylic acid will contain a range of MW (distributed around a mean) and because the derivatization of carboxylic acids is partial and random, any given preparation of amphipols represents a highly heterogeneous mixture of similar but usually non-identical molecules.

In principle, a single amphipol molecule can maintain the solubility of a single integral membrane protein in aqueous solution by wrapping itself around the transmembrane domain such that apolar side chains of the polymer interact with the protein, while the polar side chains interact with water and confer solubility to the complex. Matrix porin (OmpF) from *Escherichia coli* has been shown to maintain its trimeric structure when complexed by amphipols [62]. In the case of bacteriorhodopsin, it has also been found that complexation with amphipols does not seem to perturb its native structure [62]. For example, the 14-meric form of cytochrome b_6/f solubilized by amphipol A8-35 or A8-75 molecules has been shown to retain its ability to catalyze electron transfer reactions [62,64]. The average MW of such a complex is about 300 kDa compared to 370 kDa when b_6/f is solubilized in mixed micelles.

Amphipols represent a very exciting development from an NMR point of view because they may ultimately provide a means of solubilizing membrane proteins for solution NMR which is as good as detergent micelles from the standpoint of maintaining protein fold and functionality, but which form aggregates of lower effective MW than is possible using detergent micelles. While not yet extensively tested

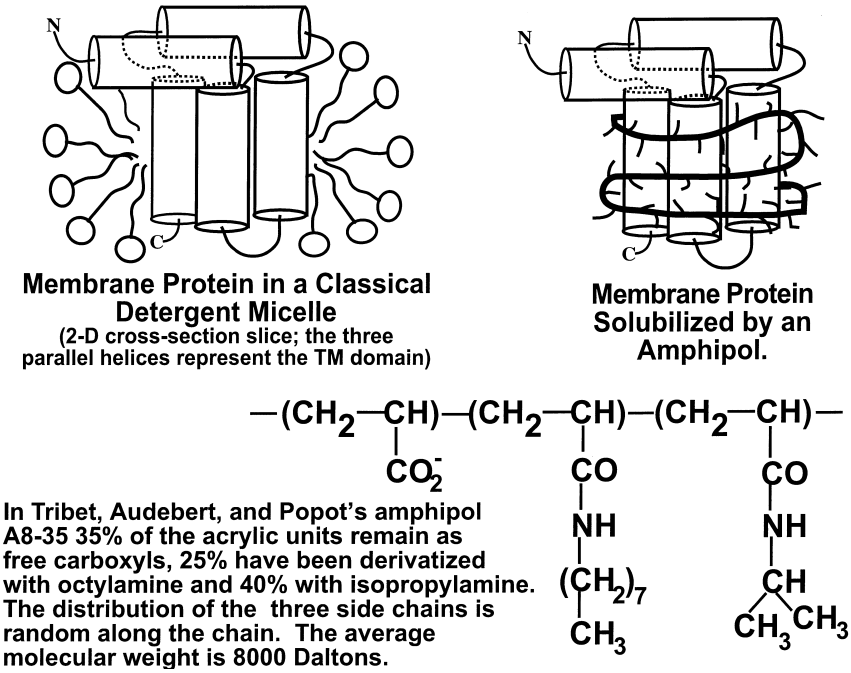

Fig. 2. Solubilization of a hypothetical membrane protein in detergent micelles or in amphipols.

for use in NMR experiments, it is possible that the present class of polyacrylate-based amphipols could prove to be sub-optimal for NMR of some proteins because of their anionic nature (which in some cases might destabilize proteins in a manner analogous to sodium dodecylsulfate (SDS)) and because of their molecular heterogeneity. However, even if these issues do prove problematic, it is highly feasible that future classes of amphipols can be rationally fashioned which may eliminate possible drawbacks.

3.6. *Perfluorinated surfactants*

Largely through the efforts of Jean Riess and his co-workers, a host of alkyl chain perfluorinated analogues of common lipids and detergents have been synthesized and characterized [66]. Perfluorinated alkanes are even less soluble in water than the corresponding hydrocarbons [67]. As a result, bilayers formed by chain-perfluorinated lipids are generally much more stable than the corresponding hydrocarbon-based lipids [66,68]. Chain-perfluorinated detergents assemble into micelles, but usually have CMCs which are about one order of magnitude lower than hydrocarbon-based detergents of similar chain lengths [66]. Perfluorocarbons are not very soluble in hydrocarbons [67,69]. Perfluorocarbon chain alkanes have a distinct conformational preference for stiff twisted helical conformations [67] such that the interiors of bilayers and micelles dominated by these chains can be expected to have different properties than corresponding hydrocarbon assemblies.

It is known that some membrane proteins can be solubilized using perfluorinated detergents, and that in some cases native structure may be maintained [70–73]. It is not generally known whether membrane proteins are more or less soluble in perfluorinated phases than in hydrocarbon phases or whether the native structure can generally be expected to be maintained. From an NMR standpoint, perfluorinated phases have yet to be exploited as a medium in which to solubilize membrane proteins, but this possibility is worth pursuing. In this regard, it should be noted that the cesium salt of perfluorooctanoic acid (CPO) is known to form bicelle-like assemblies in which both the bilayer and the micelle-like edge components of the assemblies are composed exclusively of CPO [74]. Because perfluorocarbons tend to align in a magnetic field with their long axes parallel to the field, CPO bicelles orient with their bilayer normals parallel to the field [74]. CPO bicelles

have been used as a medium for some studies of membrane-associating polypeptides and lipid molecules [75,76]. It is interesting to speculate that it may be possible to use perfluorinated phospholipids which can form bicelles suitable for use with complex membrane proteins and which may align with bilayer normals parallel to the field direction without the need for additives such as lanthanides.

3.7. Organic solvent mixtures

Complex membrane proteins can often be solubilized in organic solvent mixtures. The concept of 'naked' membrane proteins in such mixtures is appealing for NMR because the effective MW of the protein is not increased by the association of detergents or other amphiphiles. Girvin and co-workers have elegantly shown that subunit c of the F1-F0 ATPase (two transmembrane helices with a short loop connector) adopts a stable, native-like fold in at least one organic solvent system and have determined its structure at high resolution [12,13]. However, one can argue that most complex membrane proteins cannot be expected to behave as well as subunit c. While secondary structure may often be retained, it seems probable that significant perturbations of protein tertiary structure will usually accompany solubilization by this route. A thorough case study has been carried out [59] for DAGK (trimer of 13 kDa subunits, each with three transmembrane helices). While the authors do not wish to discourage investigators from trying organic solvent mixtures, great caution is encouraged.

3.8. A call for creativity

There remain entire classes of detergents which have been subjected to very little testing for use with membrane proteins. These include detergents having non-straight chain saturated hydrocarbon tails (cf. [77]) and bipolar detergents with two polar head groups separated by a long connecting apolar chain. Some of these detergents may have lyotropic properties which are unique and which may be spectroscopically exploitable. They deserve to be tested.

There is also room and impetus for the development of novel classes of model membrane media. Two concepts for how membrane proteins might be solubilized in low aggregate MW forms are extensions of established strategies. First, there may be advantages to working with cyclized amphipol-like molecules. Formally, cyclodextrins fall into this class (cyclized molecules with a hydrophobic cavity and a polar surface). However, existing cyclodextrins [78,79] have cavity diameters which are too small for more than one transmembrane helix (at the most). The potential advantages of such cyclized molecules rather than open chain amphipols include enhancement of stability for the solubilized protein and for the protein–polymer complex. A disadvantage might be the need to match the polymer cavity size to that of the protein of interest (if the polymer is rigid).

A second potential method for solubilizing membrane proteins would be the use of reversed micelles in very low viscosity organic solvents. Joshua Wand and co-workers recently demonstrated [80,81] that it is possible to solubilize water soluble proteins in the interior of reversed micelles and that when these are then bathed in a very low viscosity organic solvent that the tumbling rate of the entire reversed micelle/protein complex is more rapid than for the protein alone in water. This is an extremely exciting development in solution NMR, promising to help extend the upper MW limit to total structural analysis by NMR methods. As of the writing of this review, Wand and co-workers are working to see if it is possible to utilize reversed micelles to solubilize membrane proteins for the same purpose. It has previously been proposed that membrane proteins can be solubilized such that the two water-exposed domains are encapsulated in separate reversed micellar units with the intervening transmembrane domain being bathed by the apolar solvent, with the whole complex having a dumbbell shape [82].

Finally, for no model membrane medium (including vesicles) is it presently possible under NMR conditions to impose a stable and constant transmembrane voltage, as is present across the membranes of many living cells. Many membrane proteins are regulated by variations in transmembrane voltage. The structural consequences of applied transmembrane voltage and its variations may be profound [83]; it is possible that some membrane proteins require the presence of a transmembrane electrical potential to adopt their physiologically relevant conformation.

This represents an area which is almost completely untouched in the realm of high resolution structural determination because of the technical difficulties in establishing and maintaining such potentials in model membrane systems within samples appropriate for NMR or crystallographic experiments. Some preliminary work in this area has been reported [84,85], but obstacles remain.

4. Considerations for sample preparation

4.1. Desired orientational state

One family of solid state NMR methods relies upon the use of samples in which molecules are uniformly aligned, usually parallel to the direction of the magnetic field. The net degree of molecular alignment of lipid vesicles with normals parallel to the field can be quite high if vesicles can be forced to adopt a pancake shape. There are two methods for accomplishing this. First, multilamellar vesicles can be sandwiched between glass plates which are often then stacked [86–88]. The vesicles are forced to 'pancake' between the plates by shear/mechanical forces such that the vast majority of bilayer normals are aligned perpendicular to the plates. A second method relies upon the use of an ultracentrifuge to prepare essentially flat and uniformly aligned multilamellae through controlled centrifugal forces [89].

Bicelles represent an alternate method of achieving model membrane alignment. In order to achieve the parallel alignment of the bicelle normals with respect to the field that is required for studies of complex integral membrane proteins (for reasons described in [36]), it is necessary to change the sign of aggregate magnetic susceptibility for the bilayered discs. This can be accomplished by doping the bicelles with certain lanthanide ions, with lanthanide ion–lipid chelate complexes, or certain aromatic molecules [36]. Of these mechanisms, the use of the lanthanide/lipid chelates may be the most biochemically compatible [90,91]. In some cases, the presence of the membrane protein alone may be adequate to effect the change in sign of susceptibility [92,93]. One potential advantage of bicelles is that they can easily be employed in ligand titration experiments, unlike the case of mechanically aligned bilayers.

4.2. Motion and solid state NMR

Solid state NMR studies of membrane proteins will normally be carried out in bilayers (native membranes, vesicles or bicelles). A range of different (non-isotropic) aggregate and molecular motions may be present in some samples, with each motion having its own rate and amplitude. Some of these motions can result in serious problems for solid state NMR. When motional frequencies are similar to the frequencies spanned by anisotropic spin tensors or pulse radiofrequency fields, resonance line broadening and/or reduced signal to noise may result from interference or intermediate exchange affects [94–100]. Accordingly, motion should be regarded as an important experimental variable which will often need to be managed in order to acquire quality solid state NMR data. Fortunately, strategies exist for experimentally modulating motions in membrane systems.

Liquid crystalline phase bilayers (at temperatures above T_m) are highly dynamic environments and generally permit both whole-protein axial rotation about the bilayer normal and, most likely, whole-protein wobbling and local conformational dynamics [14,101–103]. Bilayer domain undulations may also be present in some cases [15]. Below T_m, bilayers adopt the highly ordered semi-crystalline gel phase where both lipid and protein dynamics are dramatically dampened. When the temperature is lowered still further, sample freezing can be induced and motions can be dampened further. Alternately, samples may be freeze-dried. It is possible that when working with freeze-dried samples or low temperature hydrated samples that it may sometimes prove advantageous to employ additives (such as cryoprotectants) to reduce potentially disruptive phenomena resulting from bilayer dehydration or ice crystal formation [104,105]. The conformational states of bilayer lipid and constituent membrane proteins can be a function of the freezing method employed. In principle, by very rapid freezing of liquid crystalline phase samples, it may be possible to preserve in 'snap-shot' form the heterogeneous conformational states of the liquid crystalline phase at low temperatures [106,107]. This is in contrast to samples prepared by slower freezing, where proteins and lipids will (ideally) anneal into their thermodynamically pre-

ferred conformational states determined by the final temperature.

The presence of lipids having distinct topologies and dynamics such as cholesterol [108], glycolipids [97,98] or cardiolipin may also be used to provide some degree of modulation of protein motions in bilayers.

At the present state of technology, bicelles have a disadvantage with respect to vesicular systems in that they form an uncharacterized isotropic phase below the T_m of the lipid component. Preliminary experiments to freeze samples so that bicelle morphology and orientation is maintained at low temperatures have been described [109], but work in this area remains in an early stage of development. It should also be noted that whole-bicelle wobbling motions likely occur, but that the typical amplitudes and rates are not known. Such motion is yet another potential source of spectroscopic line broadening.

4.3. Motion and solution NMR

Overall protein–amphiphile aggregate tumbling is rapid and isotropic within micelles, detergent-rich mixed micelles and amphipols. However, protein conformational motions may vary considerably from system to system. It is quite likely that when proteins are removed from the quasi-two-dimensional (2-D) environment of a lipid bilayer and solubilized in a more disordered environment such as that of a detergent micelle, that internal protein conformational motions may be dramatically enhanced (Fig. 3). Crudely, one might think of this phenomenon in terms of the protein acquiring some degree of 'molten globular' [110,111] character. This may be a disadvantage, particularly if the amplitudes of such motions are large and/or if the rates for such motions are on an intermediate NMR time scale. This can lead to serious spectroscopic problems including loss of spectral dispersion, dramatic line broadening and loss of observable NOE transfers between normally proximal spin pairs. The presence of such motions may be minimized in some cases by employing detergents known to be mild to proteins, by using lipid-containing mixed micelles in which protein–lipid interactions may enhance maintenance of native-like structure, and by adding ligands (such as substrates or inhibitors if the protein is an enzyme) which may bind and thereby stabilize the protein.

There are examples of complex membrane proteins for which high quality NMR spectra have been acquired in micelles or organic solvents, but for which structural determination has yet to be achieved [112–114]. In some of these cases, it is likely that the proteins under examination adopt their correct secondary structure, but do not have stable tertiary structures. Thus, while high quality spectra may be acquired and possibly even assigned, long range NOEs may not be observed, making structural determination very difficult. This suggests that when seeking to acquire NMR data demonstrating feasibility of structural determination for membrane proteins, observation of long range NOEs should be regarded as a critical test.

4.4. Consistency with protein fold, function and stability

As human observers, it seems very obvious that some model membrane systems better mimic the structural, dynamic and morphological properties of native bilayers than others. In general, the resemblance to native membranes decreases in the order: vesicles > bicelles > mixed micelles > micelles > amphipols. However, from the standpoint of a membrane protein under spectroscopic conditions, what matters most is the degree to which a given system mimics native membranes in terms of maintaining a protein's native conformational, dynamic and functional state (Fig. 3). From this point of view, even micelles may in some cases represent perfectly respectable model membrane systems. Accordingly, when possible, assessment of specific conditions should be made from the specific protein's point of view. This can be accomplished fairly easily for proteins having a function which can be specifically assayed under spectroscopic sample conditions. For example, enzyme activity can typically be measured regardless of the model membrane medium used. This has been a powerful tool in the case of DAGK [48,59]. However, even in such favorable cases results are sometimes ambiguous. If an enzyme's activity in a particular medium is 50% of the known activity in native membranes, does one interpret this observation as reflecting a 'significant

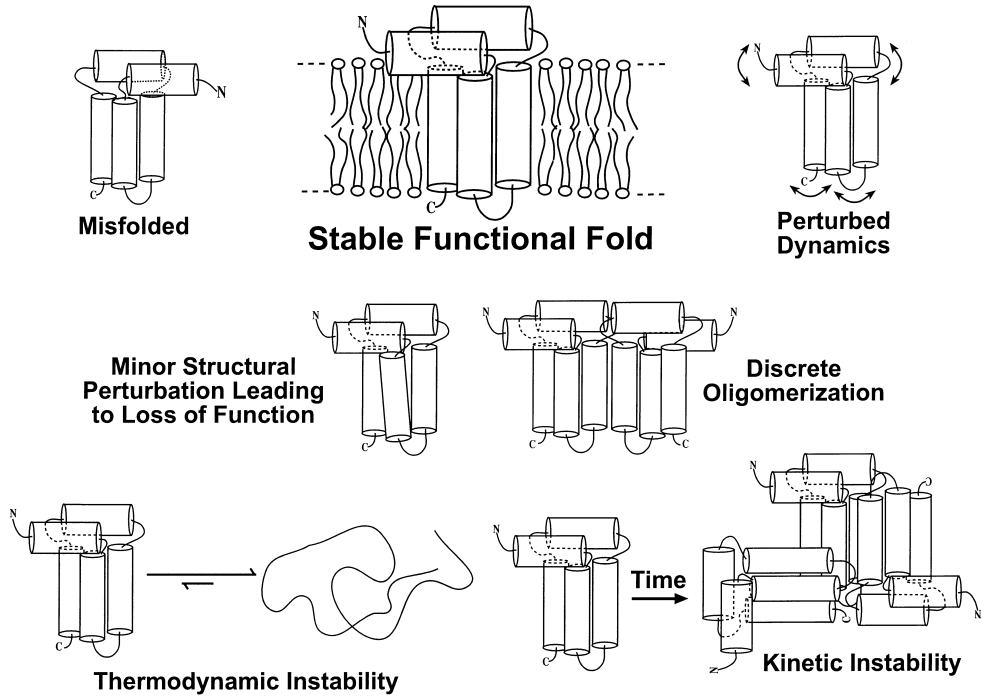

Fig. 3. Undesired fates for a hypothetical membrane protein in model membrane media (membrane-mimetic phases not shown).

perturbation' of native structure or as reflecting 'only minor perturbation'? In this case, additional characterization beyond activity measurement is required to establish the validity of structural studies.

For some classes of proteins in some media, functional assay is not possible. For example, channel activity cannot be assessed in micelles. In such cases, evidence for native structure must be acquired through indirect methods such as measurement of ligand binding capacity (for example, a channel may have a site for an allosteric effector) or testing for native oligomeric state. Evidence for or against maintenance of native state may also be acquired in the form of structural 'finger-prints' using methods such as chemical modification rate measurements, chemical cross-linking, amide H–D exchange measurements, or spectroscopic methods such as near-UV circular dichroism or tryptophan-based fluorescence. In these cases, the goal is to compare structurally sensitive data acquired for the protein under conditions in which it is known to be correctly folded (but which may be unsuitable for NMR) to spectra acquired under the NMR-relevant conditions. In this regard, it should be pointed out that methods which yield the degree of protein secondary structure (far-UV CD and FT-IR) must be used with particular care, since unfolded or misfolded membrane proteins often retain the same secondary structure composition as in the native fold [59,115–117]. The possibility that the protein may adopt a 'molten globule'-like structural state which reflects the functional fold, but in which there are considerable conformational dynamics not present in the true native structural state of the protein, must also be taken into account (Fig. 3).

Thermodynamic protein stability (Fig. 3) is primarily of relevance to the question of whether a protein can be considered to be properly folded under spectroscopic conditions or whether the unfolded population in equilibrium exchange with the folded population is high enough that NMR data analysis is complicated (see end of Section 4.2). Assessment of the thermodynamic stability of complex membrane proteins is not trivial. Bowie and co-workers have shown that SDS can be used as a denaturant in titrations of at least one micellar membrane protein, leading to denaturation curves akin to those typically obtained for water soluble proteins using urea or

guanidine hydrochloride [116]. Differential scanning calorimetry has also been applied to a number of membrane proteins [115].

Kinetic protein instability typically leads to the formation of irreversibly aggregated, un- or misfolded protein with time (Fig. 3). This is a particularly worrisome phenomenon in cases where very long NMR experiments are required, sometimes at elevated temperatures. Such instability is often detectable in the form of a visible aggregate, in the form of loss of NMR signal intensity with time, or in the appearance of unexpected peaks with time. In general, conditions which promote a protein's thermodynamic stability will promote kinetic stability, but this is not always true.

From a practical sample preparative standpoint, finding conditions in which protein stability is enhanced and maintained over long periods of time involves optimizing the usual parameters of pH, ionic strength and temperature. Choice of specific model membrane media (for example detergent type) is also critical, as is the amphiphile:protein ratio. Some detergents are general protein denaturants and should usually be avoided, including SDS and *N*-laurylsarcosine [48]. The presence of lipid in mixed micelles can often enhance stability (e.g. [60]). As noted earlier, ligands specific for the protein of interest may be employed to enhance stability.

Bowie and co-workers have recently shown that it is possible to use mutagenesis methods to dramatically enhance both the thermodynamics and kinetic stability of DAGK in detergent micelles [118,119]. If this approach can be generally and simply applied to most membrane proteins, this represents an exciting development in the practical structural biology of membrane proteins.

4.5. Avoidance and correction of protein misfolding

Distinct from the stability issues described above is the potential that preparations of membrane proteins may be plagued by a population of protein which is stably folded, but which has adopted a fold which is not the native (usually thermodynamically preferred) conformational state of the protein (Fig. 3). Such misfolding may occur at the point of protein expression and folding in vivo or could occur at some point during protein purification and/or reconstitution. This phenomenon appears to be related to a number of diseases [120–125]. The presence of this problem for a given protein may not be easy to detect. In the case of DAGK, misfolding appears to be a serious problem and can occur both during or prior to purification and during the process of reconstitution [126]. For DAGK, symptoms include irreproducible catalytic specific activities (U/mg) from batch to batch of purified protein, irreversible activity loss following reconstitutions into vesicles by some methods, and observation of aberrant oligomeric states for the micelle- and vesicle-solubilized protein.

Avoiding misfolding altogether may be difficult if this occurs for the protein of interest at the stage of biosynthesis. Misfolding which occurs during protein purification is likely to be highly protein-specific; however, key variables are likely to include choices of methods for lysing and extracting cell membranes, and choices of buffers and detergents used during purification steps.

Methods have been forwarded for correcting membrane protein misfolding [126–129]. The first involves the use of protein denaturants (i.e. urea) in the presence of detergent to unfold misfolded protein bound to an affinity column, followed by the removal of that detergent and refolding into a native-like conformational state [127]. A second method which has been found to be very effective in the case of DAGK is known as 'reconstitutive refolding' [126]. This method requires that the membrane protein is purified into dodecylphosphocholine micelles. At this stage, misfolding is still present. However, when the lipid POPC is mixed with the DAGK-DPC micelles followed by dialytic removal of DPC to form DAGK/POPC vesicles, refolding of misfolded DAGK occurs. The proper fold persists even if the vesicles are redissolved into detergent micelles. This process requires the specific DPC/POPC combination; other lipid/detergent mixtures tested did not work. That many DAGK mutants could be refolded by this method was evident through observation of a dramatic increases in catalytic activities (measured under identical assay conditions before and after refolding) and through correction of previously aberrant oligomeric states following the refolding procedure [126].

4.6. Attainable protein concentration and sample heterogeneity

NMR is a notoriously insensitive technique such that it is generally necessary to conduct experiments with 'apparent' protein concentrations of at least 1 mM. We refer to 'apparent' concentration because for a membrane protein in model membranes, the critical concentration of the protein from thermodynamic and solubility standpoints will often be the concentration of the protein within the membrane-mimetic phase. This can be described in units of volume fraction (volume of membrane protein/total volume protein and model membrane) or mol fraction (mol of membrane protein/total mol of membrane protein+amphiphile). It can easily be shown that the probability of collision of two 10 kDa protein molecules in solution at 1 mM concentration is much lower than the probability for collision of two 10 kDa membrane proteins at a concentration of 1 mM in micelles or bilayers where the detergent or lipid concentration is 100 mM. Thus, at NMR-accessible apparent concentrations of membrane proteins, the probability may be high that in-membrane solubility limits will be exceeded or that aberrant oligomerization could take place (Fig. 3). There are several possible ways of keeping these problems at bay. One is to work at the highest possible lipid:protein or detergent:protein ratios (often referred to as 'surface dilution'). Another is to optimize the composition of the membrane-mimetic phase or of the aqueous phase in a fashion designed to suppress non-specific protein–protein interactions. Finally, there may be protein modifications which can be made to suppress non-specific aggregation or oligomerization (e.g. attachment of polyethylene glycol).

In addition to possible sample heterogeneity resulting from the undesired presence of protein aggregate or oligomers at high concentrations, there is another serious problem in the case of detergent micelles. Situations may exist where some micelles may have only one protein molecular per micelle while others may have more than one. Not only will the proteins in the 'multiple protein micelles' have a high propensity for aggregation, but they may yield a much broader set of signals than the protein from 'single protein micelles' or may even yield a completely different set of signals (distinct chemical shifts). This problem has been nicely identified and addressed by Opella and co-workers [130]. The solution they proposed is to work under conditions where the micelle concentration is much higher than the apparent protein concentration. The micelle concentration can be estimated as (total detergent concentration−CMC)/aggregation number. By way of example, if a detergent forms micelles with an aggregation number of 100, one should work at a much higher concentration than (100 mM+CMC) if the desired apparent protein concentration is 1 mM. There may be some proteins for which there is not an advantage to working at very high detergent concentrations (for reasons unknown). For example, we have found that the NMR spectral quality of DAGK is not detectably enhanced at high micelle concentrations (unpublished).

In the cases of bicelles and lipid bilayers used in solid state NMR, the number of protein molecules per membrane-mimetic unit will often be of less concern because the dimensions of the individual model membrane units are much larger than individual protein molecules. A more serious problem in frozen or dehydrated samples may be the conformational microheterogeneity of the protein of interest. Each conformation can yield its own distinct spectrum. When many conformations are present, individual components will usually not be resolved, but peaks may be very broad due to the overlap of spectra from the individual conformers. For this reason, it may sometimes be preferable to work with protein microcrystals (if available) or protein which has been carefully precipitated with agents such as ammonium sulfate or polyethylene glycol. While these methods are presently being explored for solid forms of water soluble proteins in several labs (e.g. [131], and unpublished work from several labs), this represents an unexplored area for membrane proteins.

4.7. Getting the protein from native membranes into a desired sample state and concentrating protein–detergent mixtures

Extracting a membrane protein from native bilayers, purifying it and reconstituting it into a model membrane medium suitable for NMR experiments generally involves a number of transfers from one medium to another. While the possible methods for

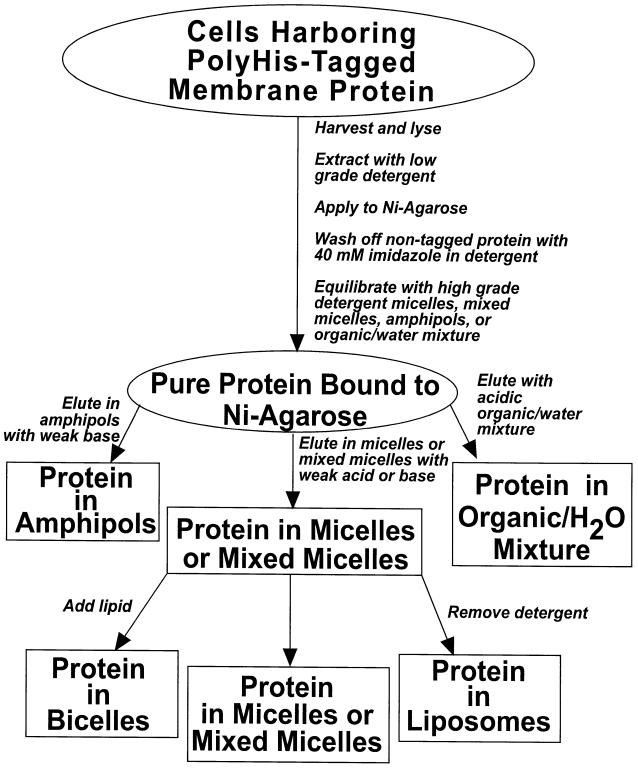

Fig. 4. Use of metal ion chelate affinity chromatography as a flexible route to membrane protein samples for NMR. The protein illustrated is hypothetical, but all of the options illustrated in this figure have been applied in the Sanders lab to DAGK (homotrimer of 13 kDa subunits, each with three transmembrane segments).

accomplishing this are legion, it should be pointed out that the use of chromatographic resins to which purified protein can be reversibly bound is ideally suited for NMR sample preparation. In our lab, we have observed that when polyhistidine-tagged membrane DAGK is bound to a Ni(II)-agarose metal ion chelate resin, it is possible to then reequilibrate the protein on the column with a host of different solvent conditions including: micelles (various detergent types), amphipols, detergent–lipid mixed micelles, denaturants (e.g. 6 M urea) and even organic solvent mixtures (Fig. 4). The protein can then be eluted in any of these solutions using 0.5% formic acid, 0.3 M imidazole or 0.5 M ammonium hydroxide ([48,59,132], Sanders, unpublished). Such methods are ideal when using perdeuterated detergents because reequilibration and elution can be accomplished with a minimal volume of (expensive) solution being required. In principle, all membrane proteins should be amenable to sample preparation using an analogous approach employing ion exchange chromatography, even without an engineered purification tag such as polyhistidine.

Once a protein is in a suitable model membrane system at a suitable protein:amphiphile ratio, it is often desirable to uniformly concentrate both the protein and the model membranes. The 'usual' methods applied to water soluble proteins can often be applied to membrane proteins. These include freeze-drying/rehydration, centrifugal or pressure-based ultrafiltration, and buffer removal through dialysis tubing in contact with impermeant hygroscopic polymers. Large protein/lipid vesicles can generally be concentrated by pelletting using an ultracentrifuge. It should be kept in mind for ultrafiltration techniques that it is usually only the buffer which is removed. While monomeric detergent can pass through ultrafiltration membranes, unless the CMC is high relative to the total detergent concentration, the amount which is removed during concentration procedures will typically be negligible unless repeated concentration/redilution (with detergent-free buffer) cycles are executed.

Selective removal of detergent from protein–lipid micellar mixtures to concentrate the protein in terms of the protein:detergent ratio can, of course, be accomplished by dialysis of the protein–detergent solution versus a detergent-free solution. Detergent removal occurs through escape of the constantly replenished monomeric detergent population in equilibrium with the impermeant detergent–protein micelles. Of course, dialysis should be halted prior to complete detergent removal or membrane protein aggregation and precipitation will occur. Because of osmotic swelling inside the dialysis compartment, the bulk protein concentration is usually lowered somewhat following partial detergent removal by this method.

Protein vesicle formation can be accomplished by a host of reconstitution methods, most of which involve the mixing of micellar protein with lipid, followed by detergent removal to induce vesicle formation. Depending on the exact method and conditions of detergent removal, different classes of vesicles (large vs. small, unilamellar vs. multilamellar) will form [14,16–18,22,23]. It should be pointed out that structural integrity of some membrane proteins can

be disrupted by some procedures. Sometimes this is not surprising. For example, when active DAGK in multilamellar vesicles is extruded repeatedly through filters to generate unilamellar vesicles, it is denatured, presumably by the shear forces (Sanders, unpublished). DAGK is also denatured when large vesicles are subjected to high power sonication. Of course, membrane proteins are notoriously finicky and in many cases much trial and error may be required to generate a correctly folded protein sample in vesicles having desired characteristics for any given experiment.

4.8. *Suitability of lipid vesicles for ligand titration experiments*

The case of lipid vesicles represents a special challenge to NMR experiments in which measurements are made as the membrane protein is titrated with membrane-impermeant ligands. In such cases, the ligand cannot freely access protein sites located on the interior of unilamellar or multilamellar vesicles. However, it has been demonstrated that if vesicles are subjected to multiple rapid freeze/thaw procedures, complete equilibration of impermeant ligands occurs [133,134]. Apparently, freeze/thawing transiently fractures or lyses membranes permitting transient permeability. Of course, one must always be concerned about whether the membrane protein of interest can functionally/structurally tolerate such repetitive freeze/thaw procedures.

4.9. *Is perdeuteration of the membrane-mimetic phase necessary in solution NMR experiments?*

Because solution NMR studies of complex membrane proteins will typically be carried out with ^{15}N- and/or ^{13}C-labeled protein, the now routine use of ^{13}C- or ^{15}N-based isotopic filtering NMR pulse methods makes it possible to avoid the use of expensive perdeuterated detergents in order to eliminate what would otherwise be an intense set of signals from the protons on the detergent. Nevertheless, there remain reasons why it may sometimes be desirable to employ perdeuterated detergents. First, for large detergent–protein complexes, the use of perdeuterated detergent will eliminate any possible spin diffusion pathways between protein and detergent peaks which could lead to spurious NOE-like cross-peaks between non-proximal spin pairs [135]. Secondly, use of perdeuterated detergents will eliminate possible line broadening of protein resonances due to relaxation through the ^{1}H–^{1}H dipolar relaxation mechanism [135,136]. Third, in cases where very high detergent concentrations are used, use of perdeuterated detergent will eliminate possible ^{1}H signals from the detergent under conditions of ^{13}C isotopic filtering because the natural abundance ^{13}C concentration is significant for the concentrated detergent component. Fourth, there are some pulse sequences in which spectral filtering to remove the detergent peaks occurs not during individual scans, but rather through resonance cancellations for multiple scans differing in associated phase cycling. In such cases, the receiver gain cannot be set very high because of the size of the detergent signal present in individual scans. This leads to sub-optimal sensitivity. Finally, any time there are huge unwanted signals which must be eliminated by a pulse sequence, there is the probability that the filtering method will be imperfect, and that artifacts will show up in the final multidimensional spectrum, sometimes in very compromising positions. The actual degree to which the above concerns translate into real problems has not been well-examined, but such concerns should be borne in mind until there is consensus based on broad experience.

Only a limited number of detergents are commercially available in perdeuterated form. Of those available in mid-2000, one can make the case that dodecylphosphocholine is the most suitable for most applications based on its structural similarity to the lipid phosphatidylcholine, its biochemical compatibility, the high degree to which its micelles have previously been characterized, the fact that it is known to actually promote refolding when used to reconstitute at least one membrane protein, and its extensive prior utilization in NMR studies of transmembrane and surface-associated polypeptides [48,126,137–140].

Acknowledgements

We thank Drs. S. Opella (and his lab), J. Prestegard, S. Prosser, L. Thompson, M. Keyes, D. Gray,

D. Cafiso, S. Smith, M. Cocco, J.-L. Popot, J. Wand, J. Bowie, M. Hong and F. Soennichsen for helpful discussion related to various topics covered in this review. This work was supported by the US National Institutes of Health (Grants R21 GM59071, R43 DK49911 and RO1 GM47485).

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