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'*q*-Titration' of long-chain and short-chain lipids differentiates between structured and mobile residues of membrane proteins studied in bicelles by solution NMR spectroscopy

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ABSTRACT

'*q*-Titration' refers to the systematic comparison of signal intensities in solution NMR spectra of uniformly ^{15}N labeled membrane proteins solubilized in micelles and isotropic bicelles as a function of the molar ratios (q) of the long-chain lipids (typically DMPC) to short-chain lipids (typically DHPC). In general, as q increases, the protein resonances broaden and correspondingly have reduced intensities due to the overall slowing of protein reorientation. Since the protein backbone signals do not broaden uniformly, the differences in line widths (and intensities) enable the narrower (more intense) signals associated with mobile residues to be differentiated from the broader (less intense) signals associated with "structured" residues. For membrane proteins with between one and seven trans-membrane helices in isotropic bicelles, we have been able to find a value of q between 0.1 and 1.0 where only signals from mobile residues are observed in the spectra. The signals from the structured residues are broadened so much that they cannot be observed under standard solution NMR conditions. This q value corresponds to the ratio of DMPC:DHPC where the signals from the structured residues are "titrated out" of the spectrum. This q value is unique for each protein. In magnetically aligned bilayers ($q > 2.5$) no signals are observed in solution NMR spectra of membrane proteins because the polypeptides are "immobilized" by their interactions with the phospholipid bilayers on the relevant NMR timescale ($\sim 10^5$ Hz). No signals are observed from proteins in liposomes (only long-chain lipids) either. We show that it is feasible to obtain complementary solution NMR and solid-state NMR spectra of the same membrane protein, where signals from the mobile residues are present in the solution NMR spectra, and signals from the structured residues are present in the solid-state NMR spectra. With assigned backbone amide resonances, these data are sufficient to describe major features of the secondary structure and basic topology of the protein. Even in the absence of assignments, this information can be used to help establish optimal experimental conditions.

1. Introduction

Proteins exhibit a wide range of dynamics, including global reorientation, local backbone fluctuations, and side chain motions of various types. Often the motions are correlated with specific structural features of the protein, and this is certainly the case for helical membrane proteins, which typically have mobile residues near their N- and C-termini, and sometimes associated with surface loops and amphipathic helices. NMR spectroscopy is adept at describing the dynamics of proteins because both relatively slow motions (>10^5 Hz), which average static powder patterns, and fast motions (10^8–10^9 Hz), which have strong affects on relaxation rates, can be detected and characterized experimentally. A thorough description of the amplitudes, directions, and frequencies of motions requires the analysis of many experimental measurements obtained at multiple field strengths. However, our understanding of the fundamental principles of nuclear spin relaxation is well established [1], and qualitative conclusions can be derived from the simplest measurements; for example the spin–spin relaxation time, T_2, which can be determined from the resonance line width, and indirectly through its effect on the intensity of signals. A major benefit of using uniformly ^{15}N labeled protein samples is that each backbone amide nitrogen has a single covalently bonded ^{1}H, therefore the motional averaging of powder patterns and the induction of nuclear spin relaxation result from fluctuations of the same heteronuclear dipole–dipole interaction. This enables complementary results to be obtained from solution NMR of micelle/isotropic bicelle samples and from solid-state NMR of bilayer samples [2].

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The situation is further simplified by the assumption that individual signal intensities, which can be readily measured in two-dimensional spectra, are determined principally by their line widths since the area for each backbone amide signal is the same. Consequently, the combined effects of local backbone and global reorientation amplitudes and rates are the principal determinants of signal intensities in solution NMR spectra of uniformly ^{15}N labeled proteins. For soluble globular proteins, the global isotropic reorientation rate is determined primarily by the size of the protein, and is effectively measured by the backbone ^{1}H–^{15}N or ^{1}H–^{13}Cα relaxation times [3]; most backbone sites are structured and have the same rotational correlation time as the protein itself. Larger globular proteins reorient more slowly than smaller monomeric proteins at the same temperature; consequently, the signals of larger proteins have broader line widths and lower signal intensities in the spectra [1]. The situation is more complex for helical membrane proteins; their global reorientation rate is affected by not only the number of residues in the polypeptide chain, but also the overall shape of the protein, i.e. whether is it "I", "L", or "H" shaped, or compactly folded. Moreover, the global reorientation also depends on the properties of the solubilizing detergent molecules and their assemblies [4].

The '*q*-titration' experiment relies on the measurement of relative signal intensities in two-dimensional ^{1}H/^{15}N heteronuclear single quantum correlation (HSQC) spectra of uniformly ^{15}N labeled membrane proteins solubilized in isotropic bicelles over a range of *q* values, typically between 0.1 and 1.0. When the global reorientation is slow at a relatively high *q* value, the resonances from structured residues disappear into the baseline because of the very limited capability of solution NMR experiments and spectrometers to detect the rapidly decaying free induction decays associated with resonances with very broad line widths. Local dynamics also affect signal intensities, and even quite large membrane proteins typically yield spectra that have some narrow, intense signals from mobile residues near the N- or C-termini [5].

Here we demonstrate that the measurement of signal intensities in solution NMR spectra of membrane proteins that are solubilized in DHPC micelles and DMPC:DHPC isotropic bicelles with *q* values <1.0 serves as an effective tool for the qualitative characterization of the structure, dynamics, and topology of helical membrane proteins in micelle and isotropic bicelle environments [6]. An important advantage of the '*q*-titration' experiment is that it can be applied at a very early stage of an investigation, yielding insights into a protein's properties long before its three-dimensional structure can be determined. With partial resonance assignments, the major transitions between structured and mobile regions can be identified, and as additional assignments are obtained, the structural resolution improves to that of single residues. This is particularly valuable for helical membrane proteins, which tend to have well defined transitions between secondary structure elements of helices, loops, and terminal segments. Even without assignments, the '*q*-titration' experiment provides insights into the behavior of the membrane protein in a wide range of lipid preparations, which is an extremely helpful initial step towards the optimization of sample conditions for Solution NMR, Oriented Sample (OS) solid-state NMR, and Magic Angle Spinning (MAS) solid-state NMR studies. This assumes that there are strong structural similarities between a membrane protein in micelle and isotropic bicelle environments and in bilayers.

Membrane proteins can be solubilized in micelles [7,8], isotropic bicelles [9], and nanodiscs [10–12] for solution NMR studies. However, careful optimization of the sample conditions is essential in order to obtain well resolved two-dimensional ^{1}H/^{15}N HSQC solution NMR spectra that include signals from all of the backbone amide sites [13,14]. Most successful applications of solution NMR have been performed on samples of relatively small membrane proteins where the global reorientation rate of the solubilized polypeptide is relatively rapid [15]. In general, larger membrane proteins give weaker, less well resolved spectra, regardless of the solubilization media; except for some narrow, intense signals a from mobile residues in linker regions or near the N- and C-termini [16]. There are a few exceptional cases, in particular the sensory rhodopsins [17,18], where relatively large helical membrane proteins do yield well-resolved solution NMR spectra.

In studies of membrane proteins from bacteria, viruses, and humans with between one and seven trans-membrane helices, we have found that they generally behave in a consistent and predictable manner with the full set of solubilization detergents/lipids (micelles, isotropic bicelles, and nanodiscs) used to prepare samples for solution NMR experiments. Notably, we have not studied proteins from archae, such as the sensory rhodopsins, which apparently have unique physical properties. The line widths of backbone amide resonances increase and the peak heights decrease in rough proportion to both the size of the polypeptide and the *q* of the isotropic bicelles, the molar ratio of the 'long' chain lipid (typically DMPC) to the 'short' chain lipid (typically DHPC or a detergent such as CHAPSO or Triton X-100). Smaller *q* values (0–0.5) are associated with faster global reorientation, and larger *q* values (0.5–1.0), are associated with slower global motion. We find that helical membrane proteins embedded in nanodiscs give spectra with line widths and intensities roughly equivalent to those obtained in isotropic bicelles with a *q* value of 0.5. As a result, a few spectra in samples with a range of *q* values enables a prediction of the feasibility of solution NMR structural studies of individual membrane proteins in micelle, isotropic bicelle, and nanodisc environments.

To illustrate the '*q*-titration' experiment in the simplest terms, simulated 'spectra' representative of those observed from a "typical" protein with two trans-membrane helices (Fig. 1A) are shown in Fig. 1B for *q* values between 0 and 3.2. Each 'dot' represents a single ^{1}H/^{15}N correlation signal from a backbone amide site of the protein. These dots are drawn with a range of sizes in order to explain the experiment. They are not calculated by a theoretically based algorithm. The relative intensities of the observed signals vary within each spectrum. Notably, even in the spectrum from the protein in micelles ($q = 0$), which would be expected to undergo the fastest and most isotropic global reorientation, some signals are more intense than others. This is shown clearly in Fig. 1C where relative signal intensities are plotted as a function of residue number. In this example, signals from residues near the N- and C-termini are more intense than those in the middle of the protein. The data in Fig. 1 were arbitrarily chosen to demonstrate the potential of the approach to differentiate regional dynamics within the protein by comparison of spectra using samples with *q* values of 0, 0.5, and 1.0. Notably, even for a small membrane proteins, many signals are broadened or missing in isotropic bicelles of $q = 0.5$ where there is evidence of bilayers surrounding the proteins [19], and this is reflected in the corresponding intensity versus residue number plot in Fig. 1C. The spectrum of the protein in $q = 1.0$ isotropic bicelles contains only a subset of the total number of resonances observed in the $q = 0$ micelles; the signals from the structured interior residues are 'titrated out', because they are too broad to be detected. In contrast the residues with local backbone mobility have resonances that are narrow and intense enough to be easily observed. All of the visible resonances in the Fig. 1B $q = 1.0$ spectrum are associated with residues near the N- and C-termini. When the *q* value is greater than about 2.5, a magnetically alignable bilayer phase is formed. In this case, the lipids in the bilayers immobilize the protein on the NMR timescales [20], and no amide backbone signals can be observed in solution NMR spectra of these samples (Fig. 1B).

As *q* is increased from 0 to 1.0 in an experimental titration, the intensities of the signals from structured residues, such as those in