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Communication

Assigning solid-state NMR spectra of aligned proteins using isotropic chemical shifts

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Abstract

A method for assigning solid-state NMR spectra of membrane proteins aligned in phospholipid bicelles that makes use of isotropic chemical shift frequencies and assignments is demonstrated. The resonance assignments are based on comparisons of ^{15}N chemical shift differences in spectra obtained from samples with their bilayer normals aligned perpendicular and parallel to the direction of the applied magnetic field.

Keywords: Bicelle; Lanthanide; Chemical shift anisotropy; Membrane protein

1. Introduction

Many resolved resonances can be observed in one- and two-dimensional solid-state NMR spectra of ^{15}N labeled membrane proteins in magnetically aligned bicelle samples [1,2]. The structures of the proteins are mapped onto the spectra by the anisotropy of the nuclear spin interactions. As a result, the frequencies associated with the resonances provide orientational constraints as input for structure calculations [3]. Assignment of the resonances to specific residues is a prerequisite for calculating the three-dimensional structures of the proteins.

The assignment of resonances in two-dimensional PISEMA spectra of membrane proteins is facilitated by analysis of the characteristic patterns of resonances observed for residues in regular secondary structures. However, comprehensive assignment methods are essential for resonances from residues in the terminal and loop regions with irregular three-dimensional structures and for dealing with the greater number of resonances observed in the spectra of larger membrane proteins [4].

A wide variety of methods have been used to assign resonances in solid-state NMR spectra of aligned samples [5]. The most direct method is through the preparation of a specifically labeled sample where only a single residue is isotopically labeled through bacterial expression [6], which is feasible when the polypeptide contains only one copy of a type of amino acid, or through chemical synthesis [7], since the observed signal can only come from the known labeled site. Selective isotopic labeling by residue type results in simplified spectra [8] that provide valuable, albeit partial, assignment information, since the only signals that are observed are from the labeled residues. Both specific and selective labeling contribute to the identification of overlapped resonances and the assignment of the resonances in spectra of uniformly labeled samples through comparisons.

In solid-state NMR spectra of aligned samples of membrane proteins, the resonances from residues in the trans-membrane helices are segregated from those in the inter-helical loop and terminal regions without regular secondary structure. In combination with specific and selective isotopic labeling, this enables the simultaneous resonance assignment and structure determination of smaller membrane proteins, and provides substantial input for making resonance assignments in larger membrane proteins. When combined with PISA wheel analysis [9,10], this

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provides sequential assignments for those residues in the regular secondary structure elements of the protein. Resonances from proximal residues, whether from the same or different types of amino acids, can be identified by incorporating intervals that allow either dilute-spin (i.e., ^{15}N or ^{13}C) or abundant-spin (i.e., ^{1}H) homonuclear spin-exchange into multi-dimensional experiments [8,11,12]. It is also possible to implement triple-resonance methods on ^{13}C and ^{15}N labeled samples. Most directly, double selective labeling can be used to identify the resonance from the selectively ^{15}N labeled amino acid Y in a unique XY pair in the sequence when the amino acid X is selectively $^{13}C'$ labeled [13]. In this situation, the ^{15}N NMR spectrum of a $^{13}C'$ X-labeled and ^{15}N Y-labeled sample has a doublet due to the $^{13}C'$-^{15}N dipolar coupling. In more sophisticated experiments, the ^{15}N bonded to a ^{13}C can be identified through selective editing or cross-polarization procedures [14].

Combinations of homonuclear and heteronuclear experiments have yielded complete resonance assignments for the solid-state NMR spectra of several aligned proteins. However, they required the use of multiple labeled samples. Additional assignment methods are needed in order to reduce the number of samples that must be prepared and to improve the efficiency of the entire process so that the increased number of resonances in larger proteins can be assigned.

In this Communication we demonstrate a general method for making resonance assignments that is applicable to samples whose direction of alignment relative to that of the applied magnetic field can be changed by 90°. This approach is particularly well suited for samples of membrane proteins in magnetically aligned bicelles where the alignment of the bilayer normal can be "flipped" by 90° by adding small amounts of lanthanide ions [15]. It is based on the measurement of ^{15}N chemical shift differences in parallel and perpendicular bicelle samples, since ^{15}N NMR spectra can be obtained from a single sample by recording the spectrum in perpendicular bicelles first and then adding the lanthanide to switch to the parallel orientation. In cases where it is undesirable to include lanthanide ions in the sample the method can, in principle, still be used by preparing samples of mechanically aligned bilayers on glass plates and varying their orientation manually [16].

2. Results

The resonance frequencies observed in one-dimensional solid-state NMR spectra of aligned samples of membrane proteins are determined by the orientationally dependent chemical shift interaction at each isotopically labeled site [17]. As a result, when the direction of alignment of the bilayer normal is changed by 90°, the observed chemical shift frequency for each resonance crosses over its isotropic chemical shift frequency, δ_{iso}. The relationship between the resonance frequency observed in perpendicular bicelles ($\delta_\perp$) and that in parallel bicelles ($\delta_\parallel$) is given in Eq. (1).

$$\delta^i_\parallel - \delta^i_{iso} = -2\left(\delta^i_\perp - \delta^i_{iso}\right)\left(\frac{S_\parallel}{S_\perp}\right) \tag{1}$$

S denotes the bicelle order parameter [18], and the $S_\parallel/S_\perp$ ratio compensates for any (minor) variations between perpendicular and parallel bicelles. The frequency difference between resonances from two different ^{15}N labeled sites in parallel bicelles can be predicted from the difference observed in the spectrum obtained from perpendicular bicelles by Eq. (2).

$$2\left(\delta^1_\perp - \delta^2_\perp\right)\left(\frac{S_\parallel}{S_\perp}\right) + 2\left(\delta^2_{iso} - \delta^1_{iso}\right)\left(\frac{S_\parallel}{S_\perp}\right) + \left(\delta^2_{iso} - \delta^1_{iso}\right) = \delta^2_\parallel - \delta^1_\parallel \tag{2}$$

In spectra obtained from parallel bicelles, the frequency difference between two resonances is exactly twice that in perpendicular bicelles only if the two sites have the identical isotropic chemical shift. Since most sites have unique isotropic chemical shifts, this accounts for the different spectral patterns observed in perpendicular and parallel bicelles; they are not simply reflections of each other [20]. Indeed, resonances that overlap in spectra obtained from perpendicular bicelles can be resolved in spectra of parallel bicelles as long as their isotropic chemical shift values are different. If the sign of the term $(\delta^2_{iso} - \delta^1_{iso})$ is the opposite of that for $(\delta^1_\perp - \delta^2_\perp)$, then the right hand side of Eq. (2) may be zero, and changing the direction of alignment by 90° may result in two signals going from well-resolved to overlapping. Because the sign of the isotropic term $(\delta^2_{iso} - \delta^1_{iso})$ depends on the resonance assignments, it is possible to resolve the ambiguity in the assignment of δ^1 and δ^2 in the solid-state NMR spectrum simply by measuring the ^{15}N chemical shift differences between two signals, provided that the isotropic ^{15}N chemical shifts for these residues are known. Thus, this provides a mechanism for assigning the aligned sample solid-state NMR spectrum on the basis of known assignments of an isotropic chemical shift spectrum, which can be obtained either by magic angle sample spinning solid-state NMR on bilayer samples or by solution NMR on micelle or small "isotropic" bicelle ($q < 1$) samples. Relatively small differences in isotropic chemical shifts resulting from variations in the lipid environments are taken into account by including generous error estimates in the various frequency measurements. The value of ±1 ppm used here is substantially larger than the actual uncertainties in the experimental measurements. The method is only applicable to proteins whose structures do not change in going between bilayer and micelle environments, if solution NMR spectra are used to obtain the isotropic chemical shift frequencies and assignments.

MerFt is a 60-residue truncated construct of the 81-residue mercury transport membrane protein MerF; it has two trans-membrane α-helices and we have determined its three-dimensional structure in both micelles [19] and magnetically aligned bicelles [20]. The protein is well aligned in both perpendicular and parallel bicelles, with the same

order parameter $S \sim 0.8$. The amino acid sequence of MerFt is shown at the top of Fig. 1. The protein has three tyrosine residues, one of which, Y60, is in a membrane spanning α-helix, while the other two, Y42 and Y45, are in the inter-helical loop. The ^{15}N NMR spectrum of selectively ^{15}N Tyr labeled MerFt in perpendicular bicelles (Fig. 1A) has three distinct resonances, and the one at 84 ppm can be assigned by inspection to Y60 based on its frequency in the spectral region associated with residues in tilted trans-membrane helices. Therefore, the signals at 124 ppm and 132 ppm are associated with Y42 and Y45, which is consistent with their location in the irregularly structured inter-helical loop. When the bicelle alignment is changed by 90° by the addition of $YbCl_3$ to the sample, the spectrum in Fig. 1B results. In this parallel bicelle spectrum, the resonances from Y42 and Y45 occur near 105 ppm, and they partially overlap because they separated by only 6 ppm. The isotropic chemical shift frequencies measured from an assigned HSQC solution NMR spectrum of MerFt in micelles are: $\sigma_{iso}^{Y42} = 119.7$ ppm; $\sigma_{iso}^{Y45} = 116.9$ ppm [19]. Since $S_{\|}/S_{\perp} = 1$, the chemical shift difference between two peaks in parallel bicelles reduces to the sum of the two terms given in Eq. (3).

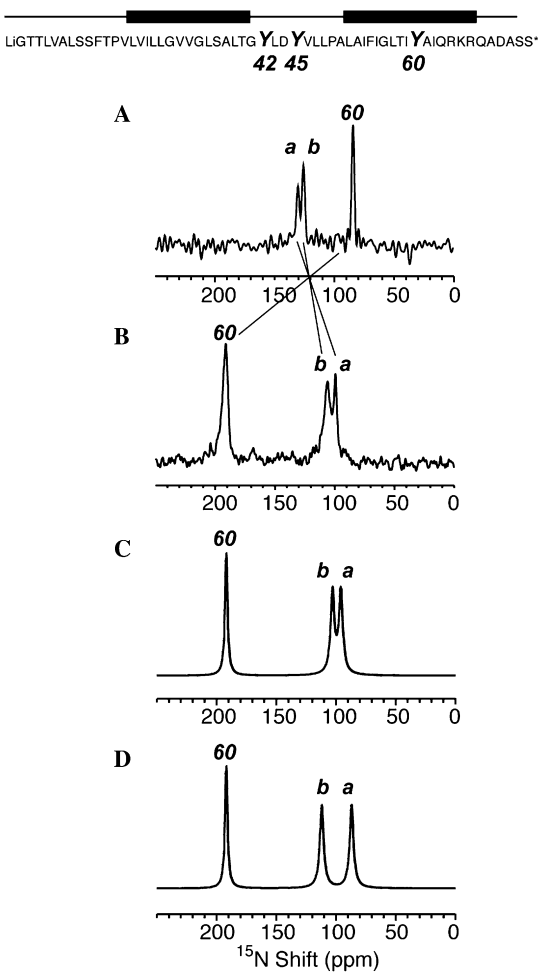

Fig. 1. (Top) The secondary structure and the amino acid sequence of the protein MerFt [19,20]. The helical segments are denoted by black bars aligned with the amino acid sequence. The three tyrosine residues are marked. Residue 60 is in the second trans-membrane helix and residues 42 and 45 are in the inter-helical loop connecting the two trans-membrane helices. (Bottom) ^{15}N NMR spectra of selectively ^{15}N-Tyr labeled MerFt in magnetically aligned phospholipid bicelles. (A) Experimental spectrum in perpendicular bicelles. (B) Experimental spectrum in parallel bicelles. (C and D) Predicted ^{15}N chemical shifts spectra in parallel bicelles obtained by applying Eq. (1) to the assigned isotropic chemical shifts in micelles and to the measured chemical shifts in perpendicular bicelles. (C) For the case where Y42 = 132 ppm and Y45 = 124 ppm. (D) For the case where Y45 = 132 ppm; Y42 = 124 ppm. The bicelle samples were prepared with ether-linked lipids with C_{14} and C_6 acyl chain lengths as described previously [1], with the long chain-to-short chain molar ratio $q = 3.2$ and lipid concentration $c_L = 28$ % (w/v). The perpendicular bicelles were "flipped" to the parallel orientation by adding 3 mM $YbCl_3{\cdot}6H_2O$. The spectra were obtained at 313 K using a spectrometer with a Bruker Avance console, a 16.4 T Magnex magnet with an ^{1}H resonance frequency of 700 MHz, and a home-built probe with a 5 mm ID double-tuned solenoid coil. Acquisition parameters were: 8 K scans, 1 ms contact time for CP-MOIST [23]; 54 kHz B_1 field; 5.12 ms acquisition time using SPINAL-16 [24] heteronuclear decoupling; 7 s recycle delay. ^{15}N chemical shift frequencies were referenced to liquid ammonia by assigning the observed external ^{15}N resonance of solid ammonium sulfate powder to 26.8 ppm.

$$2\left(\delta_{\perp}^{1} - \delta_{\perp}^{2}\right) + 3\left(\delta_{iso}^{2} - \delta_{iso}^{1}\right) = \delta_{\|}^{2} - \delta_{\|}^{1} \quad (3)$$

The first term is the chemical shift difference observed in the spectrum obtained on a perpendicular bicelle sample; the second term corresponds to the difference in "isotropic" chemical shift frequencies, and its sign depends on whether Y42 = 132 ppm and Y45 = 124 ppm, or Y45 = 132 ppm and Y42 = 124 ppm. As summarized in Table 1, the absolute value of the calculated chemical shift difference in parallel bicelles is $|\delta_{\|}^{2} - \delta_{\|}^{1}| = (7 \pm 5)$ ppm, or $|\delta_{\|}^{2} - \delta_{\|}^{1}| = (25 \pm 5)$ ppm, depending on whether the peak at 132 ppm in perpendicular bicelles is assigned to Y42 or to Y45. Since the measured magnitude of the difference is 6 ppm, this unambiguously assigns the resonance at 132 ppm to Y42. This assignment was verified independently by selective ^{13}C′ and ^{15}N double labeling; Tyr 42 was assigned to the peak at 132 ppm by comparing the spectra of (^{13}C′ Gly, ^{15}N Tyr)-labeled MerFt acquired without and with ^{13}C decoupling during data acquisition [20].

3. Conclusions

In the Communication we demonstrate that the frequencies and assignments of isotropic resonances can contribute

Table 1
All frequencies are in ppm

Assignment	Experimental		Calculated
	$2(\delta_{\perp}^{1} - \delta_{\perp}^{2})$	$3(\delta_{iso}^{2} - \delta_{iso}^{1})$	$\lvert\delta_{\|}^{2} - \delta_{\|}^{1}\rvert$
Y42 = 132 ppm Y45 = 124 ppm	16 ± 3	−9 ± 4	7 ± 5
Y42 = 124 ppm Y45 = 132 ppm	16 ± 3	9 ± 4	25 ± 5

An uncertainty of ±1 ppm is assumed on all measurements. The predicted frequency in parallel bicelles is calculated as: $2(\delta_{\perp}^{1} - \delta_{\perp}^{2}) + 3(\delta_{iso}^{2} - \delta_{iso}^{1}) = \delta_{\|}^{2} - \delta_{\|}^{1}$.

to the assignment of resonances in aligned samples, as long as it is possible to change the direction of alignment by 90°.

Differences in chemical shifts observed in solid-state NMR spectra of membrane proteins in perpendicular and parallel bicelles are compared with the values predicted from the assigned isotropic chemical shifts. This method is limited to cases where the ^{15}N isotropic chemical shift differences between two peaks are sufficiently large compared to the differences measured in aligned bicelles, with respect to the experimental uncertainty. On the other hand, referencing differences between solid-state and solution NMR spectra due to susceptibility effects [21,22] are not important since only differences between chemical shifts are relevant. It is equally applicable to resonances from residues in irregularly structured loops and regular secondary structure elements, and does not require the preparation of additional labeled or modified protein samples.

Although demonstrated using one-dimensional spectra of a selectively labeled sample, this approach is also applicable to multi-dimensional spectra of uniformly labeled samples; it can be used to assign a few resonances that remain unassigned after more conventional methods have been employed and to select among ambiguously assigned resonances. More sophisticated applications to spectra with two or three chemical shift frequency dimensions can be envisaged. Appropriately applied, this method has the potential to contribute to the assignment of resonances in solid-state NMR spectra of aligned samples of membrane proteins.

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