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Multiple Alignment of Membrane Proteins for Measuring Residual Dipolar Couplings Using Lanthanide Ions Bound to a Small Metal Chelator

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While the field of structural biology is advancing rapidly with over 40 000 structures currently in the Protein Data Bank, progress on membrane proteins, which comprise nearly 30% of the human genome, has been much slower; only about 110 of solved structures represent unique membrane proteins. NMR is an attractive alternative to X-ray crystallography for these proteins that are difficult to crystallize and has the added advantage of studying their structure and dynamics in solution.

For membrane protein NMR, the solubilizing detergent micelle or phospholipid bicelle adds significantly to the size of the system and, hence, to the rotational correlation time. Thus, the protein behaves as a much larger particle, resulting in weak or missing signals in multidimensional spectra due to rapid R_2 relaxation. Deuteration and TROSY[1] pulse sequences reduce the experimental relaxation rates, permitting resonance assignments, but fully deuterated proteins pose a problem for structure determination using traditional NOE analysis. Residual dipolar coupling orientational constraints[2] have seen extensive use in the determination and refinement of deuterated globular protein structures.[3] However, there are few reports of RDCs being used to define the structures of any membrane proteins by NMR.[4] Most alignment media—phage, phospholipid bicelles, and organic liquid crystals[5]—are incompatible with the detergents or lipids used to solubilize these proteins. Strained polyacrylamide gels are inert and provide different alignment orientations by varying the composition of copolymers[6] or the method of straining.[7] In practice, however, strained gels performed poorly with polytopic helical membrane proteins. With two- and four-helix membrane proteins, we have seen poor quality spectra in all tested gels, with weak or missing cross-peaks resulting from the lower achievable protein concentration and restricted tumbling in uncharged gels, and significant interactions between proteins and the matrix in charged gels. Furthermore, neutral acrylamide gels lose alignment over the course of a few days (Figure S1), making the measurement of RDCs by 3D NMR methods impractical. Hence an alternative alignment method was sought that ideally would not reduce the achievable protein concentration, would not significantly increase resonance line widths, and would yield samples with significant alignment and high-quality NMR spectra that were stable for multiple days.

Several reports have described the partial alignment of proteins induced by the binding of lanthanide ions.[8] Chimeric proteins have been constructed by fusing the target protein to a calcium-binding protein or peptide, such as calmodulin[9] or an EF-hand.[10] A similar approach employed small metal-chelating compounds with thiol-reactive groups, linked to a unique cysteine side chain in the protein.[11] Several compounds of varying linker lengths have been synthesized, and some are commercially available.[12] Here we show that the thiol-directed approach overcomes the shortcomings of other alignment media with polytopic helical membrane proteins, providing a robust method for multiple, stable alignments.

Alignment methods were tested using the two transmembrane helix subunit c of the *E. coli* F_1F_o ATP synthase in micelles. An A79C mutation introduced a unique cysteine at the C-terminus of the protein, which should be the most innocuous location for introducing a chemical modification. Modification was achieved by adding 30 μL of 100 mM *N*-[*S*-(2-pyridylthio)cysteaminyl]-EDTA (Toronto Research Chemicals, Inc.) to 500 μL of 600 μM subunit c in 5% LPPG (1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-RAC-(1-glycerol)], Avanti Polar Lipids, Alabaster, AL), 100 mM Tris pH 8. The reaction proceeded overnight at 42 °C. To incorporate metals, it was necessary to preload the *N*-[*S*-(2-pyridylthio)cysteaminyl]EDTA with the lanthanide ion prior to protein modification, as the free ions caused excessive detergent precipitation. The lanthanides were incorporated by adding a 3-fold molar excess of $LnCl_3$ solution to the *N*-[*S*-(2-pyridylthio)cysteaminyl]EDTA sample in the Tris buffer without detergent. After 30 min, sufficient EDTA was added to leave a slight (~5−10%) $LnCl_3$ excess, resulting in negligible precipitation of the NMR sample.

NMR spectra of high quality were obtained for the modified and metal-bound subunit c (Figures 1, 2, and S2). The spectra of the mutant and EDTA-modified mutant were largely unchanged with respect to the wild-type subunit c spectrum. Upon addition of lanthanide ions, such as $TbCl_3$, significant pseudocontact shifts were observed for residues 66−78, with smaller shifts for residues 61−65. Some perturbation of the resonances for residues 2, 6, 7, and 8, which are located on the N-terminal helix directly opposite Cys79, were also observed.

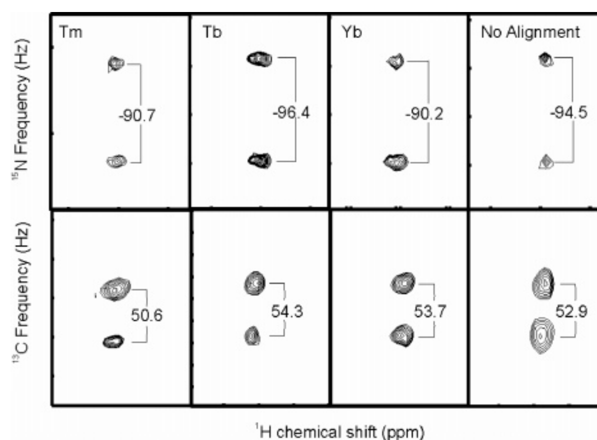

Figure 1. Selected upfield and downfield components of a 3D-IPAP HNCO experiment measuring (A) $^1H^{15}N$ splittings for Gly18 or (B) $^{13}C'^{13}C^{\alpha}$ splittings for Phe54. The top panel indicates the lanthanide ion used for alignment or no alignment. Tm^{3+} and Yb^{3+} spectra were measured at 800 MHz, and Tb was measured at 900 MHz. The observed splitting in hertz is shown.

Figure 1 shows selected upfield and downfield $^1H^{15}N$ and $^{13}C'^{13}C^{\alpha}$ components from a 3D in-phase anti-phase HNCO

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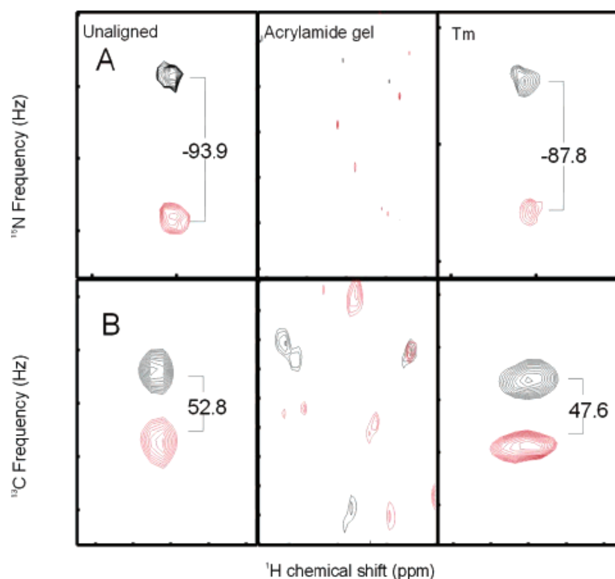

Figure 2. (A) $^1H^{15}N$ and (B) $^{13}C'^{13}C^{\alpha}$ splittings for Val15 in an unaligned sample, polyacrylamide gel, or Tm^{3+} alignment. All spectra were measured at 800 MHz. The observed splittings in hertz are indicated.

experiment, with changes in the observed splittings demonstrating lanthanide-induced alignment of the protein in the magnetic field. The alignment tensor depends on the metal used.[13] Here, for example, the Gly18 $^1H^{15}N$ RDC was +3.8 Hz with Tm^{3+} and −1.9 Hz with Tb^{3+} (Figure 1A), with the opposite sign clearly demonstrating the different alignments with the different metals. Similarly the $^{13}C'^{13}C^{\alpha}$ RDCs for Phe54 were −2.3 Hz with Tm^{3+} and 1.4 Hz with Tb^{3+} (Figure 1B). The degree of lanthanide-induced alignment increases with increasing magnetic field strength;[13] the observed range of $^1H^{15}N$ RDCs for Yb^{3+} was −6.6 to 5.3 at 800 MHz and −8.1 to 5.9 at 900 MHz.

Multiple sets of orientational constraints from RDCs will likely be essential for high-resolution NMR structures of polytopic helical membrane proteins. Unlike β-barrels, long-range backbone NOEs are vanishingly scarce, and long-range side chain NOEs are difficult to identify unambiguously for these proteins due to extensive chemical shift degeneracy in the side chains. Alignment using strained polyacrylamide gels has yielded useful RDCs for several membrane proteins,[4] but for helical proteins, only measurements for $^1H^{15}N$ vectors using 2D experiments have been reported. The 3D experiments typically required to fully resolve the $^1H^{15}N$, $^{13}C^{15}N$, and $^{13}C'^{13}C^{\alpha}$ cross-peaks appear to be impractical in polyacrylamide gels, due to the reduced sample concentrations and restricted rotation in the gels, as well as the lower sensitivity of the 3D experiments themselves. Additionally, relaxation of uncharged gels reduces the usefulness of any measurement that cannot be completed within 1 or 2 days. As shown in the middle panels of Figure 2A and B, many of the $^1H^{15}N$ and $^{13}C'^{13}C^{\alpha}$ are simply missing from the 3D data sets. Using lanthanide ions to induce alignment, however, we were able to use 3D experiments to readily measure nearly all of the $^1H^{15}N$ and $^{13}C'^{13}C^{\alpha}$ RDCs in subunit c (Figure 2A and B, right panels, and Table S1) with multiple orientations induced by varying the identity of the metal.

Protein alignment via lanthanide metals may be crucial for the application of RDCs to solving the NMR structures of polytopic helical membrane proteins such as subunit c. For the partial alignment of such membrane proteins, lanthanide ions proved to be far superior to acrylamide gels. For proteins that naturally contain a cysteine residue, this method is quite simple and easily yields RDCs for full sets of the $^1H^{15}N$, $^{13}C^{15}N$, and $^{13}C'^{13}C^{\alpha}$ vectors, with partial alignment that does not decay over time. Multiple alignments are easily obtained by using different metals or different mutations.

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Supporting Information Available: $^1H^{15}N$ spectra of modified and unmodified subunit c in detergent micelles, and tables of RDCs from alignment with Tm^{3+}, Tb^{3+}, and Yb^{3+}. This material is available free of charge via the Internet at http://pubs.acs.org.

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