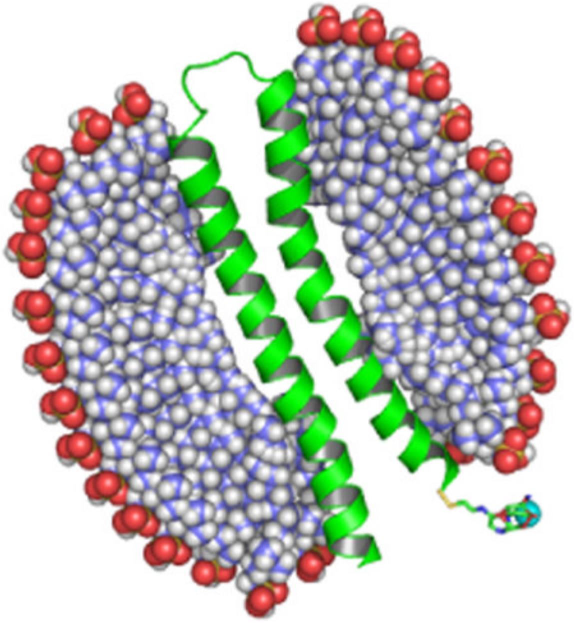
Published in final edited form as:
*J Am Chem Soc*. 2007 February 21; 129(7): 1846–1847. doi:10.1021/ja067089e.

# Multiple alignment of membrane proteins for measuring residual dipolar couplings using lanthanide ions bound to a small metal chelator

**Douglas E. Kamen**, **Sean M. Cahill**, and **Mark E. Girvin**
*Biochemistry Department, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx NY, 10461, girvin@aecom.yu.edu*

## Abstract



NMR structure determination of helical integral membrane proteins is one of the most challenging undertakings in modern structural biology. The solubilizing detergent micelle or phospholipid bicelle adds significantly to the overall size of the system, often requiring perdeuteration to obtain useable spectra. However, perdeuteration prevents structure determination using traditional NOE analysis. Residual dipolar coupling constraints are an attractive complement to NOE distance constraints, but alignment methods are limited to strained polyacrylamide gels due to the incompatibility of the solubilizing detergent or lipid with alignment media such as bicelles or phage particles. We demonstrate the use of lanthanide ions bound to the protein through a small thiol-linked metal chelator as a robust method for partial alignment of membrane proteins. This method provides multiple alignment orientations depending on the ion bound, and permits RDC measurement of multiple bond vectors. We demonstrate that using this method a large number of RDC's can be measured using 3-dimensional NMR methods where alignment using strained polyacrylamide gels results in fewer peaks due to drastic line-broadening.

---

Correspondence to: Mark E. Girvin.

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 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 RDC's 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 RDC's 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 linewidths, 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 sidechain 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 three-fold molar excess of $LnCl_3$ solution to the N-[S-(2-Pyridylthio)cysteaminyl]EDTA sample in the Tris buffer without detergent. After 30 minutes, 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, & 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 shows selected upfield and downfield $^1H^{15}N$ and $^{13}C'^{13}C^{\alpha}$ components from a 3D in-phase anti-phase HNCO 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 +3.7 Hz with $Tm^{3+}$ and –2.0 Hz with $Tb^{3+}$ (Figure 1A), with the opposite sign clearly demonstrating the different alignments with the different metals. Similarly the $^{13}C'$-$^{13}C^{\alpha}$ RDC's for Phe54 were –2.2 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 $^1H^{15}N$ RDC's 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 RDC's will likely be essential for high resolution NMR structures of polytopic helical membrane proteins. Unlike β-barrels, long-range backbone NOE's are vanishingly scarce, and long-range side chain NOE's 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 RDC's 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 one or two days. As shown in the middle panels of Figure 2A and 2B, 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 $^{13}C'^{13}C^{\alpha}$ RDC's 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 RDC's 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 RDC's 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This work was supported by NIH grants GM075026 (MG) and GM66354 (NYSBC).

## References

1. Pervushin K, Riek R, Wider G, Wuthrich K. Proc Natl Acad Sci U S A 1997;94:12366–12371. [PubMed: 9356455]

2. Tjandra N, Bax A. Science 1997;278:1111–1114. [PubMed: 9353189]
3. (a) Lipsitz R, Tjandra N. Annu Rev Biophys Biomol Struct 2004;33:413. (b) Prestegard J, Bougault C, Kishore A. Chem Rev 2004;104:3519–3540. [PubMed: 15303825]
4. (a) Howell SC, Mesleh MF, Opella SJ. Biochemistry 2005;44:5196–5206. [PubMed: 15794657] (b) Oxenoid K, Chou JJ. Proc Natl Acad Sci U S A 2005;102:10870–10875. [PubMed: 16043693] (c) Cierpicki T, Liang B, Tamm LK, Bushweller JH. J Am Chem Soc 2006;128:6947–6951. [PubMed: 16719475]
5. Prestegard J, Bougault C, Kishore A. Chem Rev 2004;104:3519–3540. [PubMed: 15303825]
6. Cierpicki T, Bushweller JH. J Am Chem Soc 2004;126:16259–16266. [PubMed: 15584763]
7. (a) Tycko R, Blanco FJ, Ishii Y. J Am Chem Soc 2000;122:9340–9341. (b) Chou J, Gaemers S, Howder B, Louis J, Bax A. Journal of Biomolecular NMR 2001;21:377–382. [PubMed: 11824758]
8. (a) Contreras MA, Ubach J, Millet O, Rizo J, Pons M. J Am Chem Soc 1999;121:8947–8948. (b) Ma C, Opella SJ. J Magn Reson 2000;146:381–384. [PubMed: 11001856] (c) Feeny J, Birdsall B, Bradbury AF, Biekofsky RR, Bayley PM. J Biomol NMR 2001;21:41–48. [PubMed: 11693567] (d) Dvoretsky A, Gaponenko V, Rosevear PR. FEBS Lett 2002;528:189–192. [PubMed: 12297302]
9. Feeny J, Birdsall B, Bradbury AF, Biekofsky RR, Bayley PM. J Biomol NMR 2001;21:41–48. [PubMed: 11693567]
10. Ma C, Opella SJ. J Magn Reson 2000;146:381–384. [PubMed: 11001856]
11. (a) Dvoretsky A, Gaponenko V, Rosevear PR. FEBS Lett 2002;528:189–192. [PubMed: 12297302] (b) Pintacuda G, Moshref A, Leonchiks A, Sharipo A, Otting G. J Biomol NMR 2004;29:351–361. [PubMed: 15213433] (c) Ikegami T, Verdier L, Sakhaii P, Grimme S, Pescatore B, Saxena K, Fiebig KM, Griesinger C. J Biomol NMR 2004;29:339–349. [PubMed: 15213432]
12. Ikegami T, Verdier L, Sakhaii P, Grimme S, Pescatore B, Saxena K, Fiebig KM, Griesinger C. J Biomol NMR 2004;29:339–349. [PubMed: 15213432]
13. Barbieri R, Bertini I, Cavallaro G, Lee YM, Luchinat C, Rosato A. J Am Chem Soc 2002;124:5581–558. [PubMed: 11996601]

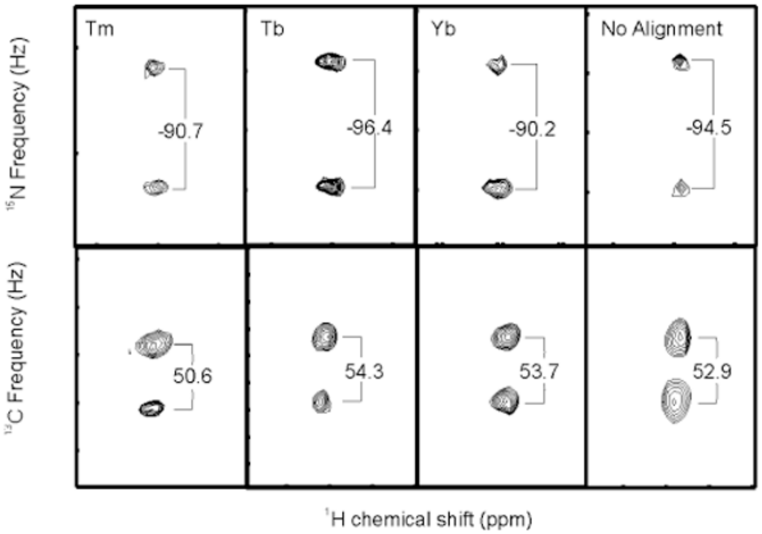


**Figure 1.**

Selected upfield and downfield components of a 3D-IPAP HNCO experiment measuring (A) $^{1}H^{15}N$ 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 Hz is shown.

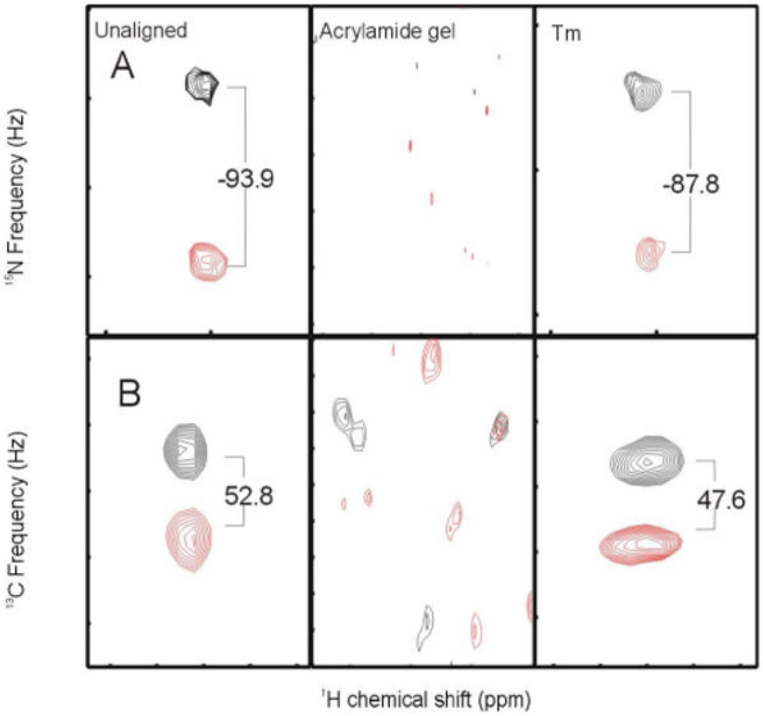


**Figure 2.**

(A) $^{1}H^{15}N$ 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 Hz are indicated.