# Lanthanide Ion Binding to Adventitious Sites Aligns Membrane Proteins in Micelles for Solution NMR Spectroscopy

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Lanthanide ions can be used to weakly align membrane proteins in lipid micelles, enabling the measurement of residual dipolar couplings, RDCs, for use as orientational constraints in structure determination.[1,2] This is an important application of weak macromolecular alignment because the difficulty in observing "long-range" NOEs in membrane proteins has hindered the determination of their three-dimensional structures by solution NMR spectroscopy. Although solid-state NMR of membrane proteins in oriented lipid bilayers is an effective alternative approach,[3] there are a number of reasons for determining the structures of membrane proteins in lipid micelles. In this Communication, we demonstrate the selective binding of lanthanide ions and resulting weak magnetic alignment of three small membrane proteins. The coat proteins from the filamentous bacteriophages Pf1 (46 residues) and fd (50 residues), which reside in the membrane prior to viral assembly, and MerF (80 residues), a membrane transport protein from the bacterial mercury detoxification system, are typical membrane proteins with hydrophobic trans-membrane and amphipathic in-plane α-helices as their principal structural elements.

Membrane proteins require lipids for structural and functional integrity. Lipids that assemble into small micelles, such as dihexanoyl phosphatidylcholine (DHPC), dodecyl phosphatidylcholine (DPC), and dodecyl sulfate (SDS), solubilize membrane proteins, allowing them to undergo isotropic reorientation in solution. However, even with careful optimization of sample conditions, the combination of broad resonance line widths, due to the relatively slow reorientation of the protein containing micelles, and limited chemical shift dispersion, due to the predominantly helical secondary structure, results in extensive overlap in crucial regions of NOESY spectra. This is especially true for the many similar hydrophobic side chain resonances, making the determination of the global folds of these proteins problematic.

RDCs provide direct long-range angular constraints with respect to a molecule-fixed reference frame.[1,4] They overcome limitations resulting from having few "long-range" NOEs available as distance restraints. The parametrization of RDCs in NMR structure refinement protocols[5] has proven to be highly effective in improving resolution,[6] determining the relative orientations of structural elements,[7] as well as global folds.[8]

Aqueous solutions containing bicelles,[2] "purple membrane" fragments,[9] rod-shaped viruses,[10,11] and recently cellulose crystallites[12] have all been successfully employed to obtain RDCs in soluble proteins and other macromolecules. However, none of these media has been demonstrated to work with membrane proteins in micelles, since the hydrophobic proteins and lipids interact with and disrupt bicelles, membrane fragments, and virus particles. Moreover, membrane proteins associated with even very small bicelles, which would directly cause weak alignment, reorient too slowly to give adequately resolved spectra. However, the binding of lanthanide ions ($Ln^{3+}$) has been shown to result in the weak alignment of a calcium-binding protein,[13] and in a related study, we have shown that lanthanides bound to an "EF-hand" added to the N-terminus result in weak alignment of the protein.[14]

The membrane-bound form of Pf1 coat protein has been characterized by NMR spectroscopy;[15] the relatively short amphipathic helix lies in the plane of the bilayer and is connected to the hydrophobic trans-membrane helix by a flexible loop. An adventitious lanthanide-binding site is present, because the protein contains a 12-residue sequence similar to that found in the loop portion of an "EF-hand" calcium-binding site.[16] The data in Figure 1C show that the addition of $Yb^{3+}$ ions results in resonance shifts for only those residues involved in metal binding. Figure 1B presents the values of the RDCs, which are calculated as the difference between $J_{HN}$ and $J_{HN} + D_{HN}$ and reflect the induced orientation of the protein. At low [$Ln^{3+}$]:[protein] ratios, the RDCs do not have a simple relationship with lanthanide concentration, possibly due to competition between the EF-hand sequence and the C-terminus, although the measured values of the RDCs are of similar magnitude (−3 to +4 Hz) to those observed with paramagnetic metals tightly bound to single sites in calcium-binding proteins[13] and other metalloproteins.[17,18] At [$Ln^{3+}$]:[protein] ratios near 10:1, the RDCs have values between −15 and +20 Hz, comparable to those measured for diamagnetic proteins in the presence of bicelles or phage particles.[2,6] At higher [$Ln^{3+}$]:[protein] ratios, the amide resonances broaden and undergo substantial pseudocontact shifts. The RDCs and the chemical shifts induced by the lanthanide ions are fully reversible; addition of the chelating agent EDTA reestablishes the metal-free values of both $J_{HN}$ and chemical shifts in the spectra.

The three-dimensional structure of the membrane-bound form of fd coat protein has been determined in micelles by NMR spectroscopy.[19] Even though it has little sequence similarity to Pf1 coat protein, its overall structural properties are the same. It also has an adventitious lanthanide-binding site, consisting of residues 2 through 9 (EGDDPAKA) (Figure 1F), which has 38% identity with the binding site in Pf1 coat protein (DGNGDMKA).

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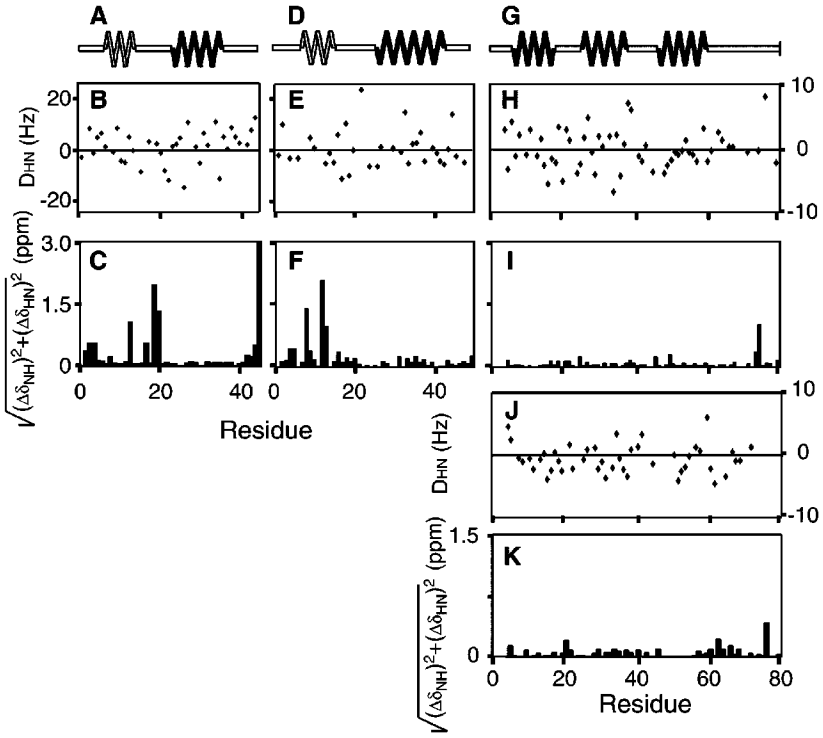


**Figure 1.** Panels A, D, and G are schematic representations of the (gray for amphipathic and black for hydrophobic) helical secondary structure of Pf1 coat protein, fd coat protein, and MerF, respectively. Panels B, E, and H show the values of the RDCs (in Hz) of the backbone amide N−H sites of the proteins in lipid micelles. Panels C, F, and I are plots of the chemical shift changes (in ppm) resulting from addition of $Yb^{3+}$ ions to solutions of the same three proteins. Panels J and K are the plots of the RDCs and chemical shift changes measured for MerF with $Dy^{3+}$ ions added. Pf1 coat protein was in DPC micelles, fd coat protein in SDS micelles, and MerF in SDS micelles. The NMR data were obtained on a Bruker DMX 750 spectrometer using the IPAP approach.[25]

The RDCs values measured for fd coat protein in micelles at a $[Ln^{3+}]$:[protein] ratio of 10:1 are shown in Figure 1E.

MerF is an inner membrane protein associated with transport of Hg(II) into the cell.[20] The chemical shift data in Figure 1I demonstrate that $Ln^{3+}$ ions bind at or near one of the mercury-binding sites in the protein. The RDCs measured for MerF are somewhat smaller than those for Pf1 and fd coat proteins, possibly because of the higher sample temperature and lower $[Ln^{3+}]$:[protein] ratio of 5:1. The comparison of data in Figures 1H and 1I to those in Figures 1J and 1K demonstrates that the binding of two different lanthanide ions to the same sites yields different orientations of the protein in solution.

These results demonstrate that the addition of lanthanide ions to samples of membrane proteins in lipid micelles provides a mechanism for measuring RDCs. The lanthanide ions bind to specific sites, and it is possible to find experimental conditions where substantial RDCs can be observed without perturbing the structure of the protein, as monitored by chemical shifts, or degrading the quality of the spectra. It is likely that many globular proteins also have adventitious lanthanide binding sites that can serve this same purpose. As is the case for other approaches to weak orientation of proteins, the extent of the alignment can be varied by changing the experimental conditions. Since the magnetic susceptibility tensors of $Dy^{3+}$ and $Yb^{3+}$ are not collinear and their axial and rhombic component are rather different,[24] the binding of different lanthanides results in different net orientations of the protein. This enables data from two or more orientations of the protein to be used in structure determination, which is useful in reducing ambiguities.[21,22]

Not all membrane proteins have adventitious lanthanide binding sites. For example, the 80-residue membrane protein Vpu from HIV-1, which has one hydrophobic trans-membrane helix and a cytoplasmic domain with two amphipathic in-plane helices,[23] could not be oriented by the simple expedient of adding lanthanide ions to the samples of protein containing micelles, as demonstrated here for three other small membrane proteins. However, it was possible to orient Vpu in micelles by adding a specific lanthanide-binding site in the form of 12-residues corresponding to an "EF-hand" to its N-terminus.[14]

The ability to weakly orient membrane proteins in micelles through the binding of lanthanide ions makes it possible to measure RDCs. These data are essential for overcoming the limitations resulting from having few "long-range" NOEs and it is now feasible to determine the three-dimensional structures of membrane proteins in lipid micelles by solution NMR spectroscopy. It may be possible to utilize many other spectral perturbations from the lanthanide ions in structure determination as well.[24] The fundamental similarities between structure determinations based on RDCs measured from weakly aligned samples using solution NMR experiments and those based on heteronuclear dipolar couplings measured from immobile and fully aligned samples with solid-state NMR experiments may presage a merging of these two complementary approaches to protein structure determination.

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