Visiting order on membrane proteins by using nanotechnology

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During the past decade, there has been spectacular progress in membrane protein structural biology, with the number of determined structures increasing by an order of magnitude to more than 100 (1). Most structures of multispan integral membrane proteins (IMPs) have been determined by using x-ray crystallography. However, solution NMR methods have also provided a modest number of structures for both α -helical and β -barrel IMPs, ranging up to 30 kDa in protein molecular mass (2, 3). Moreover, momentum in both solution and solid-state NMR (4, 5) of IMPs is growing, thanks in part to iterative innovation between NMR pulse technology and sample preparation/manipulation. In the latter category is an important new method to achieve marginal alignment of membrane proteins, as introduced in this issue of PNAS by Douglas *et al.* (6). Marginal alignment is a prerequisite for experimentally accessing a rich source of NMR structural restraints: residual dipolar couplings (RDC). This novel method can be predicted to significantly extend the number of membrane proteins for which RDC can be accessed as well as to enhance the ease and accuracy by which RDC are measured.

Pairs of NMR-active nuclei in a magnetic field undergo through-space magnetic dipole–dipole interaction (“coupling”) that is experimentally manifested in the form of NMR peak splitting. The measured coupling constant is directly related to the time-averaged orientation of the internuclear vector with respect to the static magnetic field and is proportional to the inverse cube of the distance separating the coupled nuclei (Fig. 1). Because the internuclear distance is typically known in advance, it is the orientational dependence of dipolar couplings that makes these couplings a potent source of structural information. Indeed, there is growing consensus that high-quality 3D structures of biomacromolecules can be determined from dipolar coupling data alone (7, 8). However, until approximately 10 years ago, there were formidable barriers to widespread acquisition of dipolar coupling data.

In solids and semisolids such as membranes, dipolar coupling constants can

Fig. 1. At the top is an alignment of a micellar membrane protein within an aqueous matrix of magnetically aligned DNA nanotubes. The nanotubes are actually more flexible than illustrated here and are also not necessarily 100% aligned: they most likely undergo orientational excursions around their preferred parallel orientation with respect to the magnetic field and are also much longer (800 nm) relative to the micellar IMP complex (≈ 5 nm) than is implied by this cartoon. At the lower left is an example of an NMR-active pair of nuclei (^1H and ^{15}N) that will give rise to dipolar coupling when there is net molecular alignment that favors a particular internuclear orientation (θ) with respect to the magnetic field. The brackets in the applicable equation indicate that averaging of the orientation-dependent term occurs, provided that orientational changes are rapid relative to the breadth of the relevant static dipolar tensor (typically on the order of 10^3 Hz). At the lower right, a dipolar coupling is experimentally manifested as anti-phase (as shown) or in-phase splitting of the NMR peak from either nucleus, with the frequency separation of the peaks being equal either to the observed dipolar coupling constant (D) or, when the two nuclei are linked through 1–3 bonds, $J + D$, where J is the orientation-independent scalar coupling constant.

be in the range of thousands of hertz, and a nucleus may be strongly coupled to dozens of other proximal nuclei, making the measurement and assignment of individual dipolar couplings very difficult. In isotropic solutions, where molecules randomly sample all possible

orientations with respect to the external magnetic field, all dipolar couplings are averaged to zero. A landmark development in the mid-1990s was the demonstration that if conditions can be construed where biomolecular tumbling rates are effectively the same as in isotropic solutions, but in which the molecule of interest is forced to adopt a marginal net degree of alignment with respect to the magnetic field ($\approx 0.1\%$ net preference for a certain orientation), then dipolar couplings are uniformly scaled down by orientational averaging to the point where couplings between directly bonded pairs of nuclei can be conveniently measured by using standard solution NMR methods. At the same time, couplings between more distal spin pairs are effectively zero, thereby avoiding resonance line-broadening (9, 10). Coupling constants measured under these conditions are referred to as RDC. The ability to conveniently measure dozens or even hundreds of RDC for well behaved biomolecules has significantly advanced NMR as a tool for studying biomolecular structure, interactions, and dynamics, providing restraints that supplement more traditional NMR data, often leading to significantly enhanced precision and quality for NMR-determined structures (7, 8). This is especially important for studies in which access to classical NMR restraints may be limited by obstacles such as slow molecular tumbling rates. Micellar membrane proteins often fall into this category, and methods that facilitate acquisition of RDC for IMPs are expected to significantly advance membrane protein structural biology.

Membrane proteins in bicelles or bilayers have long been aligned for solid-state NMR with magnetic or mechanical forces (5). However, the degree of imposed orientational order is too high to permit RDC to be measured by using solution NMR methods. Solution NMR studies of multispan membrane proteins are, like x-ray crys-

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tallographic studies, almost always conducted by using detergent micelles as the model membranes. Although the detergent component of IMP-micelle complexes increases the effective molecular weight of the included membrane protein, access to very high magnetic fields and the use of TROSY-based pulse sequence technology (11) often allows high-quality solution NMR spectra to be acquired even for >20-kDa IMPs (2, 3). It is therefore IMP/detergent micellar complexes that must be marginally aligned if RDC are to be measured for membrane proteins.

Some of the most commonly used methods for aligning water-soluble proteins cannot be used to access RDC for IMPs. Virtually all liquid-crystal-based methods are ineffective because membrane proteins bind too tightly to the alignment matrix (magnetically aligned bicelles, for example) and acquire too high a degree of orientational order. Two other methods have proven more successful for application to membrane proteins but have drawbacks. First, paramagnetic ions have been attached to proteins through engineered metal ion binding sites or via a chelating agent that has been covalently attached to a cysteine site in the protein (12, 13). This method has the disadvantage that the protein of interest must be modified through mutagenesis and/or chemical modification and is therefore subject to the usual concerns about potential perturbations of native protein properties. A second method has been to entrap micellar membrane proteins within the aqueous pores of polyacrylamide gels, which are then strained (stretched or compressed) within the NMR tube so that the aqueous cavities in the polymer matrix become nonspherical in symmetry (14, 15). As a result, the included micellar membrane protein adopts a small net degree of alignment when the asymmetric protein/detergent complex clacks around in the now elliptical cavities. However, there are drawbacks. Sample preparation generally requires

considerable trial and error and can be difficult to reproduce. Some proteins bind polyacrylamide, leading to extensive NMR resonance broadening. Other proteins cannot easily be soaked into gels, such that final sample concentration is inadequate.

The new method of Douglas *et al.* (6) is inspired by the established phage-based alignment method (16, 17) but uses nanotechnology to work around the NMR-problematic avid affinity between phage and membrane proteins. Magnetically alignable rod-shaped bacteriophage are replaced with DNA nanotubes. The design of the nanotubes is quite sophisti-

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cated, being built around a linear series of hexameric bundles of DNA double helices that are knit together, end-to-end, with Holliday junction crossovers. The 0.8- μ m nanotubes are not absolutely rigid extended rods but have some limited worm-like flexibility. The presence of a strong magnetic field causes the nanotubes to align with their long axes, on the average, in the direction of the field. Douglas *et al.* (6) demonstrated that two different micellar membrane proteins can be marginally aligned in this system, enabling measurement of high-quality NMR spectra. An appealing feature of this method is the ideal structural homogeneity of the nanotubes, which should lead to a very uniform degree of alignment for cosolubilized IMPs. Moreover, the fact that

hydrophobic molecules and amphipathic interfaces should generally have low affinity for direct interaction with the polyanionic surface presented by the DNA-based nanotubes bodes well for widespread applicability to a range of membrane proteins (provided they do not have an intrinsic affinity for DNA or for polyanions). It should be added that the nanotube-based alignment method should also be applicable to membrane proteins that are solubilized in amphipols or in small (normally isotropic) bicelles, emerging alternatives to classical micelles (18, 19).

Nanotube-based alignment of membrane proteins represents a fine example of the productive interface between nanotechnology and structural biology, an interface that can be expected to yield more fruit in the future. Experience and further characterization will, of course, be required to fully explore the utility of the novel nanotube-based alignment approach described by Douglas *et al.* (6). For example, it will be interesting to see whether adjunct methods can be developed that will allow variation of the alignment tensor experienced by a given membrane protein, as has been shown to be a useful tool in structural analysis with RDC (7, 8, 15, 20). Like most promising new methods, nanotube-based alignment cannot be expected to completely obviate existing methods for all membrane proteins. However, it is quite possible that nanotube-based alignment will provide significantly better results relative to previously available methods for a significant fraction of membrane proteins. Given that solution NMR is now beginning to be applied with success to membrane proteins that are tantalizingly close in size and complexity to a number of high-value targets (2, 3, 21, 22), including a majority of human G protein-coupled receptors, technical advances such as nanotube-based alignment have the potential of leading directly to breakthroughs in structural biology.

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- White SH (2007) *Membrane Proteins of Known 3D Structure*, <http://blanco.biomol.uci.edu/Membrane-Proteins.xtal.html>.
- Sanders CR, Sonnichsen F (2006) *Magn Reson Chem* 44:S24–S40.
- Tamm LK, Liang BY (2006) *Prog Nucl Magn Reson Spectrosc* 48:201–210.
- Baldus M (2006) *Curr Opin Struct Biol* 16:618–623.
- Opella SJ, Marassi FM (2004) *Chem Rev* 104:3587–3606.
- Douglas SM, Chou JJ, Shih WM (2007) *Proc Natl Acad Sci USA* 104:6644–6648.
- Bax A (2003) *Protein Sci* 12:1–16.
- Prestegard JH, Al Hashimi HM, Tolman JR (2000) *Q Rev Biophys* 33:371–424.
- Tjandra N, Bax A (1997) *Science* 278:1111–1114.
- Tolman JR, Flanagan JM, Kennedy MA, Prestegard JH (1995) *Proc Natl Acad Sci USA* 92:9279–9283.
- Pervushin K, Riek R, Wider G, Wuthrich K (1997) *Proc Natl Acad Sci USA* 94:12366–12371.
- Ikegami T, Verdier L, Sakhaei P, Grimme S, Pescatore B, Saxena K, Fiebig KM, Griesinger C (2004) *J Biomol NMR* 29:339–349.
- Ma C, Opella SJ (2000) *J Magn Reson* 146:381–384.
- Chou JJ, Kaufman JD, Stahl SJ, Wingfield PT, Bax A (2002) *J Am Chem Soc* 124:2450–2451.
- Cierpicki T, Bushweller JH (2004) *J Am Chem Soc* 126:16259–16266.
- Hansen MR, Mueller L, Pardi A (1998) *Nat Struct Biol* 5:1065–1074.
- Cross TA, Tsang P, Opella SJ (1983) *Biochemistry* 22:721–726.
- Poget SF, Cahill SM, Girvin ME (2007) *J Am Chem Soc* 129:2432–2433.
- Zoonens M, Catoire LJ, Giusti F, Popot JL (2005) *Proc Natl Acad Sci USA* 102:8893–8898.
- Meiler J, Peti W, Griesinger C (2003) *J Am Chem Soc* 125:8072–8073.
- Malia TJ, Wagner G (2006) *Biochemistry* 46:514–525.
- Chill JH, Louis JM, Miller C, Bax A (2006) *Protein Sci* 15:684–698.