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The present and future of solution NMR in investigating the structure and dynamics of channels and transporters

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Membrane channels, transporters and receptors constitute essential means for cells to maintain homeostasis and communicate with the surroundings. Investigation of their molecular architecture and the dynamic process of transporting substrate or transmitting signals across the membrane barrier has been one of the frontiers in biomedical research. The past decade has seen numerous successes in the use of X-ray or electron crystallography in determining atomic-resolution structures of membrane proteins, and in some cases, even snapshots of different physiological states of the same protein have been obtained. But there are also many cases in which long-standing efforts to crystallize proteins have yet to succeed. Therefore we have practical needs for developing complementary biophysical tools such as NMR spectroscopy and electron microscopy for tackling these systems. This paper provides a number of key examples where the utility of solution NMR was pivotal in providing structural and functional information on ion channels and transporters.

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Introduction

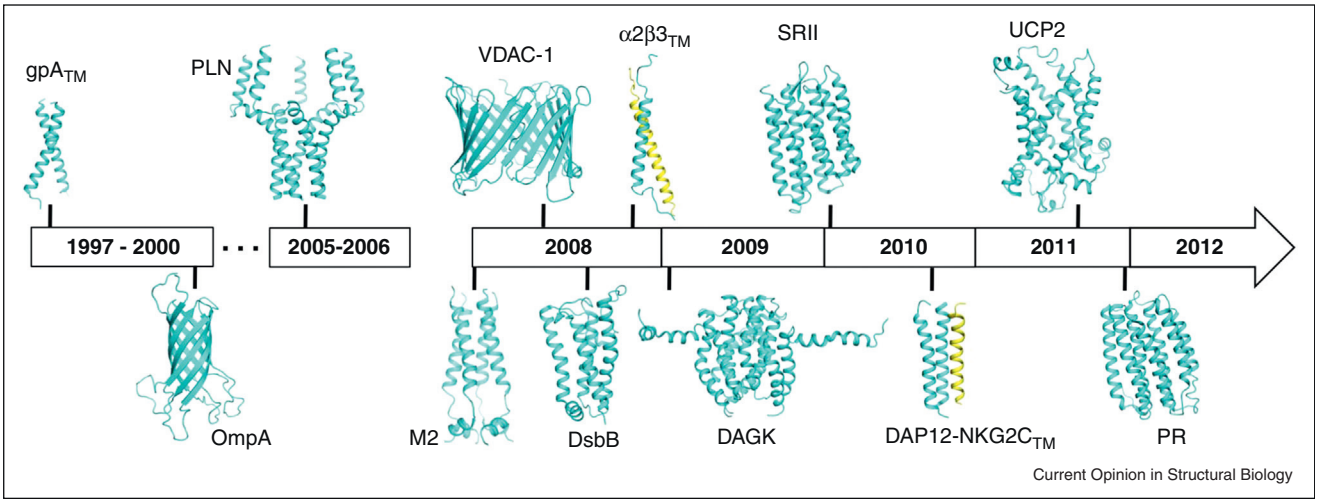
Since solution NMR was used to determine the structure of the transmembrane (TM) domain of the glycophorin A dimer in 1997 [1], there has been steady progress in its application to membrane proteins. As illustrated by the timeline of achievements in Figure 1, full-scale structural characterization by solution NMR has been carried out for a variety of membrane systems, including the membrane-associated regions of surface receptors [2,3,4••], membrane-embedded porins [5], regulatory proteins [6], ion channels [7–9], intramembrane enzymes [10,11], the seven TM helix receptors [12••], and transporters [13••]. These advances have coincided with and benefitted from the enabling methods that have gradually matured in the past decade, such as relaxation-optimized spectroscopy [14], dipolar coupling measurement [15], paramagnetic relaxation enhancement (PRE) protocols [16], as well as methods for characterizing msec–μsec time-scale protein dynamics [17]. In addition to solution NMR, solid-state NMR has also illustrated great potential, as represented by recent studies of the influenza proton channels [18] and the more complex G-protein coupled receptor [19]. Although detailed structure determination of membrane proteins NMR remains challenging, the versatility of the technology permits to generate a wide variety of data including structure, dynamics, and ligand interaction which are particularly useful when investigating dynamic systems such as ion channels and transporters.

Small to medium sized channel proteins

The influenza proton channels

The influenza M2 protein forms a pH-modulated proton channel that equilibrates pH across the viral membrane during viral entry and across the trans-Golgi membrane of infected cells during viral maturation [20]. The viral channel has been the subject of intense NMR research owing to its biomedical importance; it is a target of the adamantane-family antiviral drugs, but drug resistance has become a critical problem [21]. In 2008, back-to-back X-ray crystallographic and solution NMR studies independently determined the structures of the closed states (pH ~7.5) of the M2 channel using a peptide containing residues 24–46 and a longer construct containing residues 18–60, respectively [7,22]. The NMR structure was determined using a combination of inter-strand nuclear Overhauser enhancements (NOEs), backbone residual dipolar couplings (RDCs), and sidechain dihedral angles [7]. The structure shows a homo-tetrameric assembly in which left-handed packing of four equivalent TM helices forms the channel pore and four amphipathic helices constitute the C-terminal base of the channel (Figure 2a). This architecture provides a channel-like scaffold for positioning the critical HxxxW sequence element inside the pore where four imidazole rings of His37, which receive and transport protons, are about midway inside the pore and four indoles of Trp41 are packed within van der Waals (VDW) distances to form the channel gate. In addition to M2, the structure of a M2 functional homolog from influenza B (BM2) was also determined using solution NMR methods [8]. The BM2 channel, as revealed by the NMR structure, is very different from AM2 of infulenza A; its channel domain is an inverse coiled-coil tetramer in membrane that does not contain the amphipathic helices

Figure 1



Membrane protein structures determined by solution NMR that represented technological advances. Protein abbreviations are: gpA TM – the transmembrane domain of the glycophorin A surface protein; OmpA – bacterial outer membrane protein A; PLN – human phospholamban pentamer (unphosphorylated); M2 – the M2 proton channel of influenza A virus (closed state); VDAC-1 – human voltage-dependent anion channel 1; DsbB – bacterial intramembrane disulfide oxidoreductase; $\alpha 2\beta 3_{TM}$ – the transmembrane domain of the α2b-β3 integrin receptor; DAGK – bacterial diacylglycerol kinase; SRII – bacterial sensory rhodopsin II; DAP12-NKG2C$_{TM}$ – the transmembrane domain of immunoreceptor type-II triad assembly between the DAP12 signaling dimer and NKG2C receptor; UCP2 – mouse mitochondrial uncoupling protein 2; PR – proteorhodopsin.

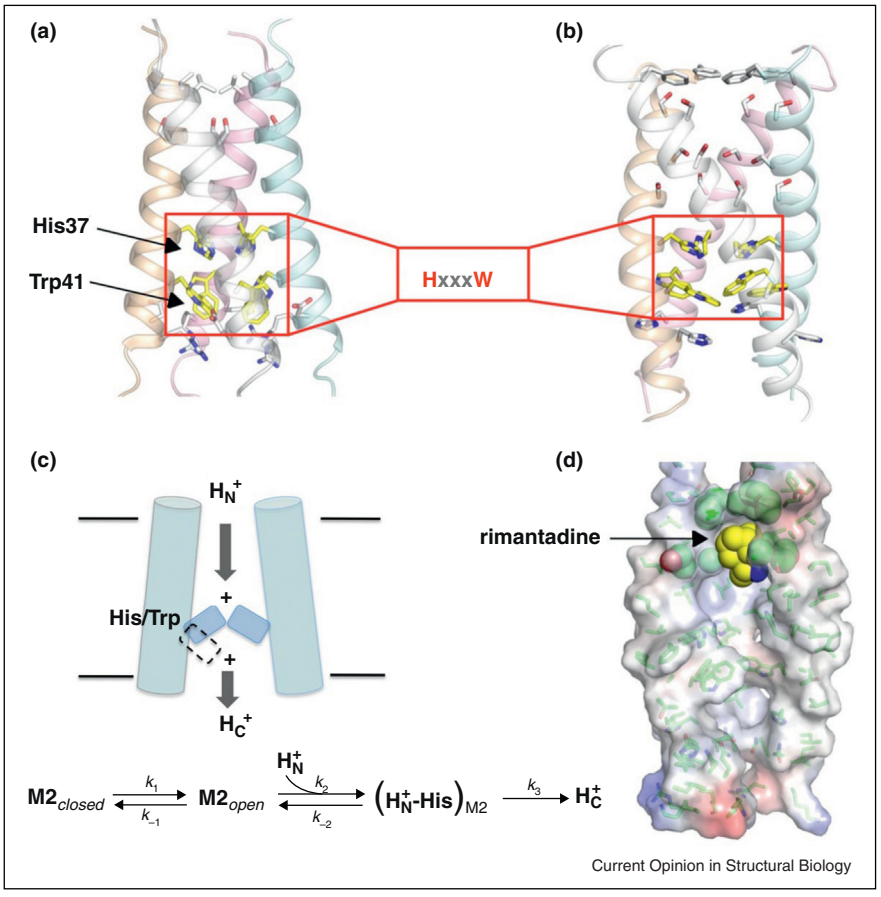
(Figure 2b). The arrangement of HxxxW inside the pore is however very similar to that of M2.

Establishing a relevant NMR measurement protocol of ion channels allows for investigating the dynamic aspects of these proteins. The M2 protein conducts protons in a pH-dependent manner [23]. Its slow rate of conduction (~15 H^+/s) [24] is consistent with its function as a transporter that binds protons from on low pH side and turn them over to the high pH side of the channel (Figure 2c) [24]. In this mechanism, the rate-limiting step (k_3) must involve a conformational change, coupling histidine protonation to opening of the tryptophan gate. Indeed, the relaxation-compensated Carr-Purcell-Meiboom-Gill (CPMG) experiment [17] showed that as the pH was lowered from 7.5 to 6.0, the conformational exchange rate of Trp41 indole increased by more than four-fold, suggesting that the gate is being 'unlocked' and becomes more dynamic upon channel activation [7]. The structure of the open state of M2 is not yet determined due to severe resonance broadening at low pH. Recently, NMR measurements of an AM2-BM2 chimera showed extremely high quality NMR spectra at both pH 7.5 and 6.0 [25•]. This hybrid construct, which was inspired by earlier functional studies [26], contains residues of the N-terminal half of the AM2 channel, followed by residues of the C-terminal half of the BM2 channel; it has been shown to recapitulate the channel properties of the native AM2 channel [26]. We remain hopeful that this new system will eventually provide detailed structural and dynamic information of the open on conducting state of the M2 channel.

Another utility of NMR is in investigating ligand interaction. Solution NMR study of the wild type M2 identified a rimantadine binding site external to the channel near the tryptophan gate [7]. This site was, however, subsequently proven not the primary site of amantadine inhibition observed *in vivo* [26]. The functional site is inside the channel as suggested by the low resolution density in the crystal structure [22] and solid-state NMR measurements [18]. Recently, the precise structure of the rimantadine binding site inside the channel has been determined by solution NMR using the AM2–BM2 chimera construct mentioned above [25•]. The structure shows that the adamantyl cage of the drug fits snuggly into a hydrophobic pocket inside the pore while the drug amino group is in polar contact with protein backbone oxygen. Furthermore, NMR studies showed that drug binding significantly altered the channel structure, suggesting that mutations could confer drug resistance by altering conformational equilibrium between the apo and the drug-bound states.

The technological know-how's established through the application of NMR to influenza M2 channels have set a bigger stage for future investigations of the more complex viroporins such as the hexameric p7 channel of the Hepatitis C virus [27] and the 3a channel of the SARS corona virus [28].

Figure 2



Structural and functional characterization of influenza proton channels by solution NMR. Detailed NMR structures of the transmembrane domains of the M2 protein of influenza A virus **(a)** and the BM2 protein of influenza B virus **(b)**, both determined in DHPC micelles and at pH 7.5. The amino acid sidechains are displayed for functionally important residues, in particular, the pore-lining histidine and tryptophan of the HxxxW sequence motif. **(c)** A model for proton transport, illustrated both schematically and by an enzymatic reaction equation, in which protons are accepted by the histidines from the low-pH side and histidine protonation induces changes in both conformation and dynamics of the tryptophan gate that allow protons to be turned over to the high pH side. **(d)** The precise NMR structure of rimantadine bound in the internal pocket of the AM2–BM2 chimeric channel determined in DHPC micelles and at pH 7.5. One of the four subunits is omitted for drug visibility.

NMR of larger channels

When it comes to solution NMR of polytopic membrane proteins, β-barrels are usually more amenable to spectroscopic analysis than α-helical proteins. This is due to a generally higher structural stability and better spectral dispersion of β-barrels in detergent micelles. Bacterial outer membrane proteins OmpA, OmpX, enzyme PagP and porin OmpG are examples of structures determined by solution NMR [29–32].

Recent advancements in NMR spectroscopy, such as four-dimensional NOESY in combination with non-uniform sampling [33] and methyl labeling schemes [34], allowed structure determination of a 30 kDa voltage-dependent anion channel from mitochondria, VDAC-1 [5]. It is the first structure of a eukaryotic β-barrel membrane protein. The study found that unlike bacterial porins, this human protein consists of an odd number of β-strands. The structure contains a hydrophilic pore capable of conducting small electrolytes. The N-terminal tail was found to be located inside the pore, consistent with its presumed role in voltage gating [35].

It is known that cholesterol is important for proper functioning of VDAC [36]. Using chemical shift mapping, the authors of Ref. [5] showed that cholesterol binds to two distinct sites on the outside of the barrel. Same approach allowed them to identify sites of interaction with ligands, such as NADH, and a pro-apoptotic binding partner Bcl-x_L. Understanding these molecular interactions will be crucial for unraveling the mechanism of VDAC involvement in mitochondrial homeostasis.

As mentioned above, structure determination of polytopic helical channels by NMR remains a serious challenge. When crystal structure is available, however, NMR can be extremely valuable for elucidation of conformational rearrangements associated with channel function, as exemplified in the studies of a pH-dependent potassium channel KcsA. The crystal structure of KcsA provided the first atomic resolution view of ion conduction and selectivity [37], yet molecular mechanism of pH-gating of this channel is still a matter of intense research [38,39].

Solution NMR has recently been used to elucidate K^+ and pH-dependent changes in the conformation of KcsA. Backbone assignment of the 68 kDa tetrameric KcsA was achieved by assigning separately solvent-exposed and solvent-inaccessible regions of the protein in two different samples [9]. pH-induced chemical shift perturbations showed that the largest effect (residues Phe114, Val115) is in the intracellular gate region which is presumed to open upon activation. Moreover, the measurement of residue-specific NMR relaxation rates of KcsA showed that the C-terminal helices that form the intracellular gate are indeed more dynamic than other regions of the protein [40].

Another NMR investigation of KcsA analyzed effects of pH-induced inactivation on the extracellular gate [41]. By comparing methyl TROSY spectra of wild type, inactivated and non-inactivating mutants, the authors were able to make conformation specific assignment of a number of Ile, Leu and Val methyls in key regions of the structure. They demonstrated that protonation of His25 on the intracellular side of the channel caused a conformational change in the extracellular gate on the opposite side of the membrane (Val76). In addition, chemical shift perturbation and methyl-water NOE measurements suggest that pH-dependent inactivation of the channel results from a change in the conformation of the selectivity filter [38] coupled with the replacement of potassium ions by water molecules. These studies demonstrate that NMR can be used successfully to investigate conformational rearrangements that play a key role in the functioning of relatively large and complex membrane channels.

Application to membrane transporters

Compared with ion channels, transporters generally have more distinct conformational states that are specific to substrate binding and release in each cycle of substrate transport. In the case of a small multidrug resistant transporter EmrE, the two states were found to be related by a pseudo-twofold symmetry and NMR chemical exchange analysis helped confirm the antiparallel dimer model [42••]. In a much more frequent situation of nonequivalent states, however, in addition to obtaining the structure of the transporter, it would be very important to characterize conformational differences between those states for understanding the transport mechanism. A highly tempting possibility is to use NMR to generate structural and dynamics information on membrane transporters under various conditions of substrate or inhibitor binding that would complement the atomic resolution crystal structures. It should be emphasized here that the synergy of the two approaches can greatly enhance our understanding of conformational plasticity exhibited by many other membrane proteins, including the all-important G-protein coupled receptors [43••].

Technical challenges

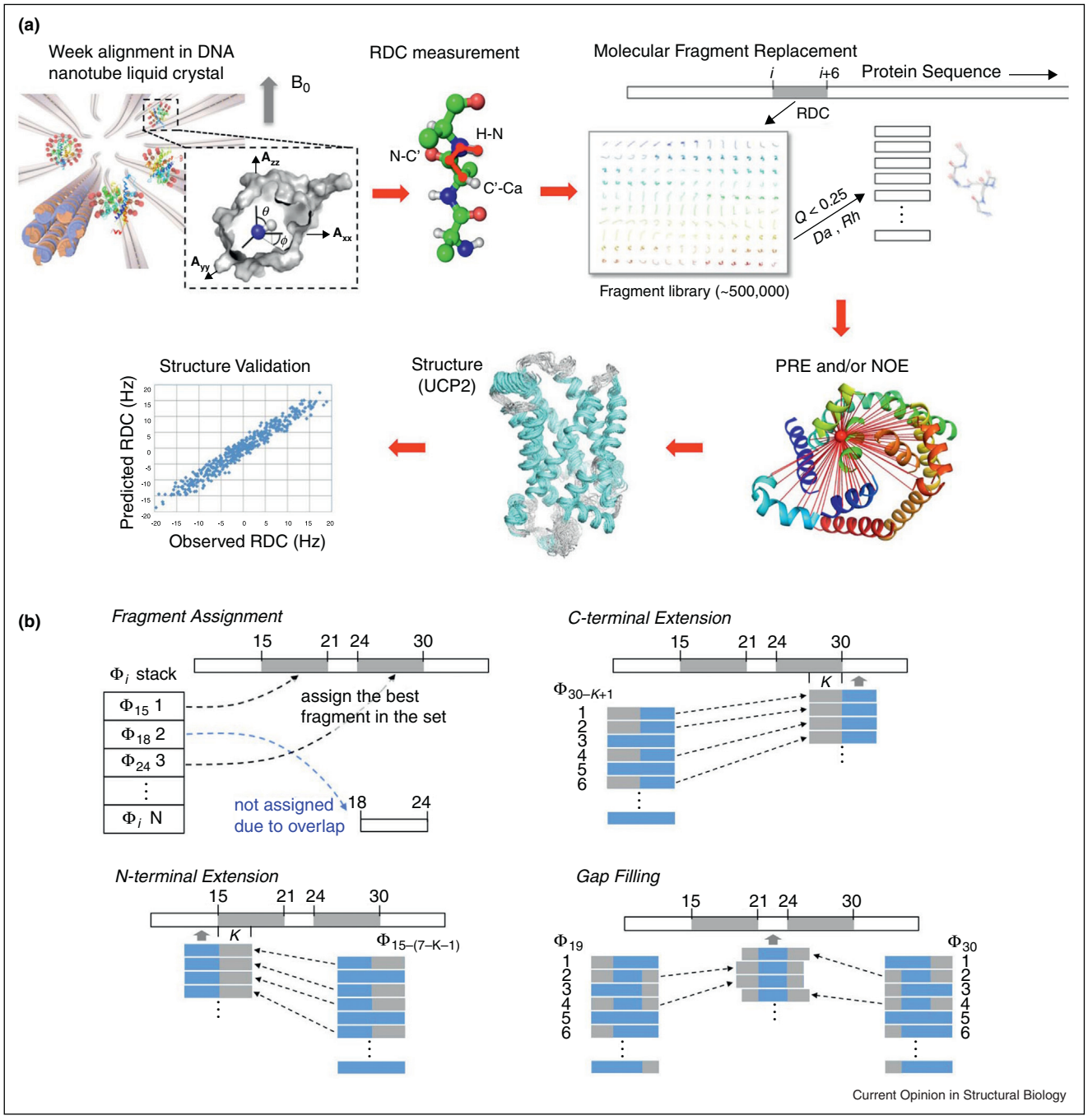
NMR studies of viroporins described above benefitted greatly from high order of symmetry in which channels can assemble from short polypeptides. But most transporters are helical proteins with much longer polypeptide chains (>300 amino acids). The long-range NOEs for helical proteins involve mostly methyl groups, and this type of NOEs are difficult to measure due primarily to two reasons. One is the poor chemical shift dispersion of the methyl resonances that precludes comprehensive assignment of methyl-methyl NOEs. The other is the higher dynamics of the methyl groups (e.g. unlocked side chain χ_1 or χ_2 rotamers) that significantly reduces the efficiency of NOE buildup.

Structural characterization of the mitochondrial uncoupling protein by NMR

Mitochondrial uncoupling proteins (UCPs) belong to a large family of *mitochondrial carriers* (~300 amino acids), which are known as solute exchangers that transport negatively charged metabolites and ions across the mitochondrial inner membrane. UCPs have adopted the capacity to transport protons in a mechanism that requires fatty acids, making them rather special members of the carrier protein family. UCP2 reconstituted in DPC/cardiolipin mixed micelles generated good NMR spectra, but spectral crowding and dynamics made conventional NOE-based methods ineffective.

To overcome the hurdle, an alternative scheme was developed that uses bond vector orientation restraints from RDCs and spatial restraints from PREs. In this scheme (Figure 3a), protein backbone RDCs of the NH, CαC′, and NC′ bonds were measured for UCP2-micelle complexes that are weakly oriented in liquid crystals formed by fabricated DNA nanotubes [44]. An RDC-based molecular fragment replacement (MFR) method then determined local structured segments. Having the local structures defined and their global orientation constrained by RDCs, the global fold was determined using PRE-derived distance restraints. This approach enabled determination of the backbone structure of UCP2.

Figure 3



An RDC-based molecular fragment searching protocol for determining the backbone structures of helical membrane proteins for which NOE analysis is the limiting factor. **(a)** Graphical illustration of the experimental scheme including, from the left to right, (1) weak alignment of protein–surfactant complex using the detergent-resistant liquid crystal formed with fabricated DNA nanotubes, (2) RDC measurements, (3) RDC-based MFR algorithm for determining local structured segments, (4) measurement of PRE and/or NOE for providing distance information, and (5) structure calculation and refinement. **(b)** Conceptual illustration of the operations involved in RDC-based molecular fragment searching and structural segment building, including *Fragment Assignment*, *N-terminal* and *C-terminal End Extension*, and *Gap filling*. The symbol Φ_i represents a set of fragments that agree well with the RDC data for a 7-residue sequence commencing at residue *i*. The gray regions of the fragments represent regions with matching backbone dihedrals with the structured segments of the protein that have already been determined. The blue regions represent the dihedrals to be assigned.

Determination of structured segments by RDC-based MFR method

The premise of the MFR approach is that the PDB is so vast today that its content gives an excellent coverage of local structural features of naturally occurring proteins. This approach was first applied in crystallography for building molecular fragments into crystallographically determined electron density [45]. A later NMR study demonstrated that with about four RDCs per residue, it is possible to determine the backbone structure of ubiquitin using molecular fragments that fit to RDCs [46]. Moreover, fragment searches that combine chemical shift and RDC have been implemented in the CS-Rosetta structure modeling software and demonstrated to be effective in solving water-soluble protein structures [47].

In case of the MFR study of UCP2, a large *fragment library* containing 7-residue fragments from ~1500 protein structures was constructed, and from the library, a set of *candidate fragments* with good RDC fitting (Q_{free}) and the correct alignment tensor values was found for every possible 7-residue sequence of UCP2 that has sufficiently dense RDC data. The candidate fragments were assigned to the protein sequence and this step is referred to as *fragment assignment* (Figure 3b). The next procedure was building longer structured segments based on the assigned 7-residue stretches using two operations: *end extension* and *gap filling*. *End extension* is extending the N-terminal or C-terminal ends of a structurally assigned segment along the protein sequence by searching for unused candidate fragments that overlap with the segment in backbone dihedral angles, and that provide the best Q_{free} for the final extended segment. Similarly, *Gap filling* is to fill gap regions (<4 residues) between two structurally assigned segments by searching for fragments that overlap in backbone dihedral angles with the two flanking segment and that provide good Q_{free} for the merged segment.

Sources of long-range restraints

RDCs are well suited for probing conformational rearrangements of helical membrane proteins [48]. RDC-derived structural constraints also dramatically reduce the amount of distance restraints needed for determining the tertiary fold. In the case of UCP2, 440 PRE restraints from just four spin-labeled samples were used [13••].

A major drawback of PRE is the large uncertainty (±~5 Å) associated with the restraints [16]. Therefore, effective protocols for measuring long-range methyl-methyl NOEs are desperately needed for improving the accuracy and precision of NMR structures. One potentially effective strategy is to introduce specific methyl labels in highly deuterated environment to allow for very long NOE mixing times, for example, by using recent protocols developed for labeling methyl groups of alanines [49] and valine and leucine methyl groups in a stereospecific manner [50•].

Conclusions

Recent technological advances have enabled solution NMR to tackle membrane channels and transporters of increasing complexity, and we believe it is timely to exploit this biophysical tool to gain deeper understanding of these membrane proteins from a more dynamic perspective. NMR deals with proteins that constantly tumble and 'breath' in solution due to thermal energy, and thus determining atomic-resolution structures of membrane proteins is still a substantial challenge. In a synergistic approach, a growing number of crystal structures become available for NMR as a starting point to delineate conformational switches and to investigate dynamics.

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- •• of outstanding interest

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This paper shows a method for isotopic labeling of methyl groups in a stereospecific manner. This labeling scheme enables the use of very long NOE buildup time, which can potentially solve the NOE problem in NMR structure determination of the more dynamic helical membrane proteins.