Solid-state NMR applied to membrane proteins

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Solid-state NMR spectroscopy applied to membrane proteins

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One major remaining problem in structural biology is to elucidate the structure and mechanism of function of membrane proteins. On the basis of preliminary information from genome projects, it is now estimated that up to 50,000 different membrane proteins may exist in the human being and that virtually every life process proceeds, sooner or later, through a membrane protein. Solid-state NMR spectroscopy in high magnetic field is rapidly developing into a widely applicable tool to describe the structure and help understand the mechanism of function of a membrane protein. Recent work in applied solid-state NMR spectroscopy crosses the boundary between the biological and the physical sciences, and aims at increasing the predictive range of this biophysical method.

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Abbreviations

CP	cross polarisation
MAS	magic angle spinning
MD	molecular dynamics
PSII	photosystem II

Introduction

The chemistry of the enzymatic function of membrane proteins allows them to perform some of the most demanding tasks in life. For instance, in photosynthesis the photosystem II (PSII) membrane protein complex is capable of tuning the electronic structure of chlorophyll and increasing its redox potential by approximately 0.5 V. As a result, the chlorophyll in PSII can use photochemistry to split water and provide our planet with an oxygen atmosphere. The rhodopsin G-protein-coupled receptor in our eye operates at room temperature as a highly efficient single photon counter and energy converter that initiates the visual signal transduction cascade.

To gain insight into these and other functional mechanisms of membrane proteins, solid-state NMR, in conjunction with isotope labelling and computational methods, is increasingly important. There have been several breakthroughs in elucidating mechanisms of function of membrane proteins and the principle focus of this short review will be on the different opinions as to how solid-state NMR can provide information about mechanisms that is otherwise inaccessible. One particular example is that it is possible to obtain insight with genuine predictive range. It transpires that scaling effects and the associated nonlinearities are very important to arrive at a proper understanding of mechanisms of function of membrane proteins. It is proposed that solid-state physics can help to identify, at various length and timescales, emerging properties that make membrane proteins work — the structure/function relation.

Describing structure using solid-state NMR

Structural biology generally considers the question of how biological constructs like proteins are built and how they function. Structural biology is, by and large, derived from the conceptual 'central dogma' of molecular biology: DNA→mRNA→protein→function. 'A' protein is here primarily described by its (dynamic) structure. This has triggered enormous effort in solving protein structures and, currently, about 10,000 protein structures have been described. However, membrane proteins form an exception here. Membrane proteins are generally considered to be difficult, are left aside by the majority of structural biologists and, by consequence, are largely unexplored. With an estimated 30% of all proteins being membrane proteins and with virtually every life process passing, sooner or later, through a membrane protein, the importance of such proteins is hard to overestimate. Driven by demand from the molecular biology field for structural information about membrane proteins, there is continuous effort among physical chemists to develop solid-state NMR techniques for determining the structure of membrane proteins and membrane-bound biological assemblies. Initially, pairs of isotopes were introduced in membrane proteins to verify existing structural models by measuring distances using solid-state NMR [1]. However, a generic concept for structure determination requires a distribution of NMR isotopes. In an intermediate approach, a high-resolution structure of the gramicidin dimer interface in the membrane was determined from distance measurements on a set of label pairs [2].

In a novel development, it has been shown that a concept for structure determination using multispin labelling and magic angle spinning (MAS) NMR exists and can be evaluated [3]. The (protein-free) structure of a biological membrane-bound light-harvesting assembly, the chlorosomal antenna of the green bacterium *Chlorobium tepidum* chlorosome, was partially resolved. Recently, a new bilayer model for the tubular antenna was developed on the basis of proton chemical shift constraints obtained using high-field 2D and 3D MAS NMR dipolar correlation spectroscopy (BJ van Rossum, DB Steensgaard, K Schaffner, AR Holzwarth, HJM de Groot, unpublished data). It has been known for quite some time that the inside of a membrane protein is often a highly ordered solid, yielding surprisingly narrow MAS NMR lines [4]. In this environment, local interactions can be very strong. An essential point then is that a concept for MAS structure determination can be based, in part, on chemical shift

constraints [5]. Recent studies exploring the Ca^{2+}-binding site in the oxygen-evolving complex of PSII and the secondary structure determination of a fibrous protein have emphasised this point [6•,7].

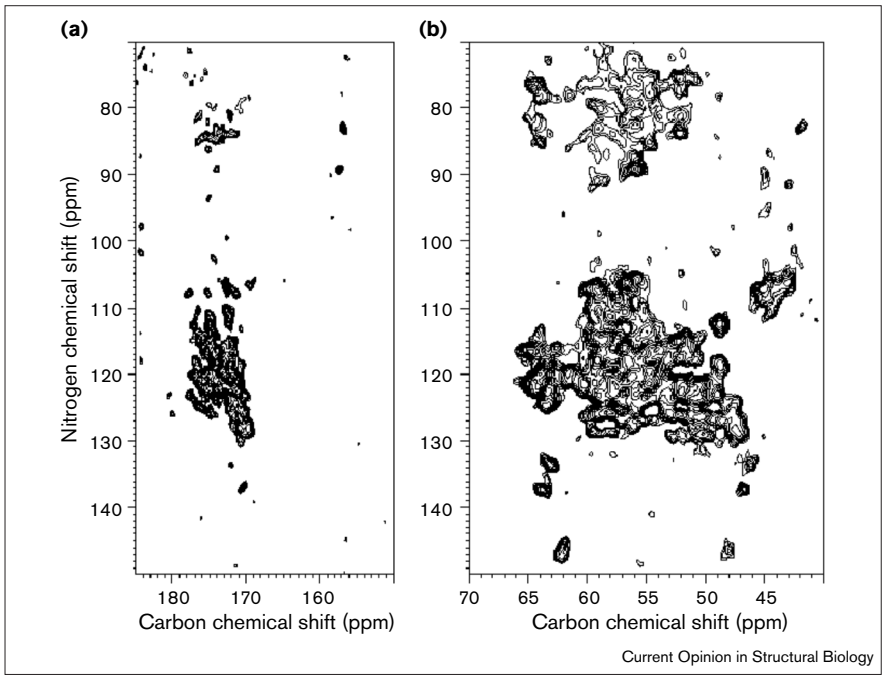
Currently, tools are being forged to establish a versatile methodology for structure determination. At a technical level, many pulse sequences for dipolar correlation spectroscopy have been developed and are now routinely applied in MAS NMR laboratories worldwide [8]. In a novel route to formulating a rigorous theoretical basis for the multispan MAS and recoupling effects, a formalised many-particle classical quantized field interpretation of MAS NMR rotor-frequency-driven dipolar recoupling has been proposed. The essential step here is the use of a 2D space-time continuum, two degrees of freedom, in a nonrelativistic quantum rotor dynamics description [9•]. In this way, it was calculated that the radio frequency dipolar recoupling is driven by the MAS rotor frequency, not by the radio frequency, as the name of the pulse sequence suggests. This illustrates the lack of knowledge regarding the fundamentals of spin diffusion processes and the need for extending the theoretical basis of the MAS NMR of multispin systems. Another important point is access to the protons. For this purpose, frequency-switched Lee–Goldburg heteronuclear dipolar correlation methods were implemented at high field and with fast MAS [10]. It has been shown that it is possible to determine heteronuclear intermolecular distance constraints in the solid state using Lee–Goldburg decoupling [11]. In the long run, this may offer significant advantages over homonuclear methods, as hydrophobic structures are frequently stabilised by nonbonding interactions between methyl groups and aromatic residues. Subsequently, it has been shown that hydrogen bonds can be characterised with excellent accuracy by probing the ^{1}H–^{13}C interactions with Lee–Goldburg methods and with chemical shift anisotropy analyses [12•,13]. It has been demonstrated that dipolar interactions in the solid are strong and that intermolecular polarisation transfer between ^{13}C labels using spin diffusion techniques can be used to characterise global distances up to approximately 100 nm [14,15,16•]. To determine the backbone conformation of peptides and proteins in the solid state, torsion angles can be measured and pattern labelling techniques are currently being investigated [17,18•]. Finally, a solid is intrinsically an object with a suprastructure. In a study aimed at determining a 3D structural model of solid self-assembled chlorophyll *a*–H_2O using multispin labelling and MAS NMR 2D dipolar correlation spectroscopy in high magnetic field, the problem of the propagation of modelling errors is effectively circumvented by exploring a homology approach involving consistency checks against complementary information obtained for related systems (BJ van Rossum, EAM Schulten, J Raap, H Oschkinat, HJM de Groot, unpublished data).

In order to analyse the structure of a membrane protein, the sequence-specific assignment of NMR signals is a prerequisite. It was realised at an early stage that future structural investigation of proteins using solid-state cross polarisation (CP) MAS NMR will rely on uniformly labelled, structurally intact protein samples showing spectra with excellent resolution in high magnetic field [19,20••]. This was confirmed by studies involving a precipitated model protein showing considerable disorder, which leads to lack of resolution [21–23]. In contrast, the spectrum of an optimised microcrystalline [U-^{13}C,^{15}N]-labelled sample generated by precipitation shows very narrow ^{13}C signals and resolved scalar ^{13}C–^{13}C couplings [20••]. The backbone and sidechain ^{13}C and ^{15}N resonances of the solid 62-residue SH3 peptide were assigned by 2D MAS ^{15}N–^{13}C and ^{13}C–^{13}C dipolar correlation spectroscopy at 17.6 T (J Pauli, M Baldus, BJ van Rossum, HJM de Groot, H Oschkinat, unpublished data). It was found that, for the microcrystalline biopolymer in high field, proton-driven ^{13}C–^{13}C spin diffusion (i.e. without a dipolar recoupling sequence) performs remarkably well. In addition, inter-residue correlations leading to sequence-specific assignments were obtained from 2D NCOCX experiments. The solid-state assignment is now nearly complete, although the signals of N-terminal residues 1–6 and C-terminal residue 62 outside the domain boundaries are not detected in the MAS spectra. This confirms early information that the signals from surface residues are either not cross polarised or broadened beyond detection at high resolution [4].

In addition, the first heteronuclear dipolar correlation experiments had already been obtained for a fully labelled membrane protein complex in ultrahigh magnetic field. Backbone and sidechain correlations for a uniformly ^{13}C,^{15}N-labeled version of the 150 kDa LH2 light-harvesting nonameric complex reveal a good resolution at low temperatures and with MAS (T Egorova-Zatchernyuk, J Hollander, P Gast, HJM de Groot, M Baldus, unpublished data). Tentative assignments of some of the observed correlations have been obtained and attributed to the membrane-spanning part of the protein (Figure 1). The resolution observed in these spectra strongly suggests that moderately sized, intrinsic membrane proteins and peptide ligands bound to their membrane protein receptor targets will be assigned in the near future.

In addition to the recent developments in the field of MAS NMR methodology, structure determination using orientational constraints is making continuous progress. A recent review of this topic is presented in [24]. One major issue that has been revisited in the past year is that solid-state NMR has been used to establish that the structure of gramicidin in the membrane is single stranded [25••,26]. This has attracted considerable attention, as two double-stranded structures were found using X-ray crystallography attempts from saturated solutions and were erroneously promoted as the channel structure in the membrane. Thus, an essential point is that the lipid environment is, in this case, a strong factor in establishing the functionally intact

Figure 1



Heteronuclear dipolar (N,C) correlation spectra obtained on the [U-^{13}C,^{15}N] LH2 photosynthetic light-harvesting complex, illustrating the resolution that can be obtained for the transmembrane region. In **(a)** and **(b)**, the ^{13}C carrier frequency was placed slightly outside the CO and C_α regions of the ^{13}C spectrum, respectively. For backbone resonances, correlations of the type (a) N_iCO_{i-1} and (b) $N_iC\alpha_i$ are expected. For the strongest sets of signals, additional C_δ and CO resonances between a residue and its preceding amino acid are expected, leading to sequence-specific assignments of signals from the membrane-spanning segments of the protein complex.

structure of the protein! Several groups have recently reported the determination of helix tilt angles in a membrane, leading to functional models [27•,28,29]. Orientational changes of methyl bonds associated with the mechanism of function can be characterised [30]. Finally, in a combination of MAS and orientational methods, the MAOSS approach has been proposed and has yielded the first structural results for the M13 coat protein, bacteriorhodopsin and rhodopsin [31,32••,33].

Understanding structure using biomolecular spectroscopy

Although most biologists will probably argue that the central dogma is based on the most comprehensive principle of nature, it has the drawback that there is little or no predictive range regarding, in particular, mechanisms of function. Every protein device is essentially a separate issue, following from its own corresponding piece of DNA. In particular, it is a challenge to understand the mechanism of function, not in terms of the central dogma, but by crossing the boundary between biology and the physical sciences, and by working out descriptions in terms of the universal laws of nature and the theories based on these laws. This is actually a matter of strongly different current opinion: Socrates versus Darwin. Among solid-state NMR spectroscopists working on membrane proteins, there has always been a strong focus on universal principles and inductive reasoning based on the laws and theories of the physical sciences, while aiming at cross-fertilisation with the modern molecular biology approaches and the dogma. The goal, in this case, is to improve the predictive range of current understanding. There is a strong feeling in the community that an increasingly bright future exists for solid-state NMR techniques through exploring their spectroscopic capabilities, complementary to describing structures.

Membrane proteins are highly sophisticated solid-state electronic and electromechanical devices that are biologically ordered, that is, participating in hierarchical order and without translational symmetry. Many concepts from hard and soft condensed matter physics and chemistry exist that can, in principle, contribute to understanding mechanisms of membrane protein function. For instance, have you ever asked the question: How does photosynthesis manage to provide the earth with an oxygen atmosphere? A central problem here is to increase the oxidative power of a chlorophyll, while maintaining its green colour by increasing its redox potential, to allow the extraction of electrons from water molecules. In over-simplified terms, chlorophyll in the PSII photosynthetic membrane protein complex does not rust, but can generate oxygen instead! Recently, the first ^{13}C MAS NMR observation of photochemically induced dynamic nuclear spin polarisation of the PSII complex has been reported [34•]. The light-enhanced NMR signals of the natural abundance ^{13}C provide information on the electronic structure of the primary electron donor, chlorophyll P_{680}, and on the p_z spin density pattern in its oxidised form, $P_{680}^{\bullet+}$. The data reveal significant asymmetry of the electronic spin density distribution within $P_{680}^{\bullet+}$. The spin density appears to be shifted in a different way than in monomeric chlorophyll *a* radical cations in solution. In this way, evidence is obtained for the presence of a local electrostatic field over the chlorophyll *a* macroaromatic cycle that raises the electronic chemical potential

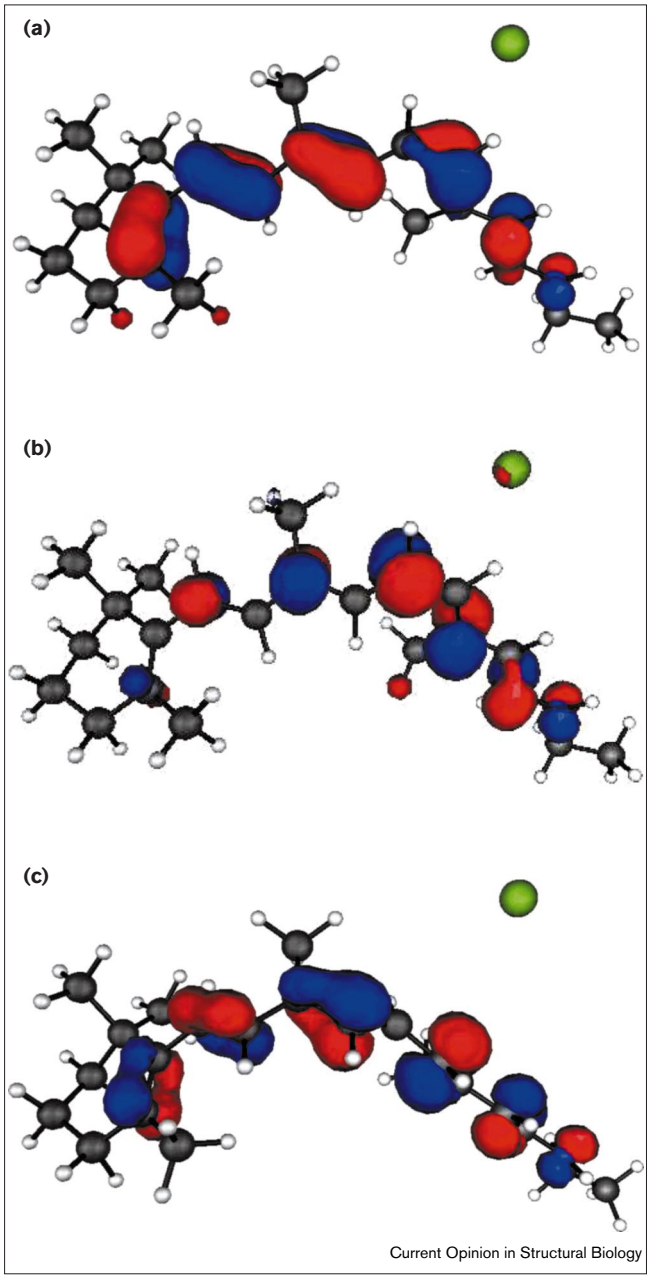


Figure 2

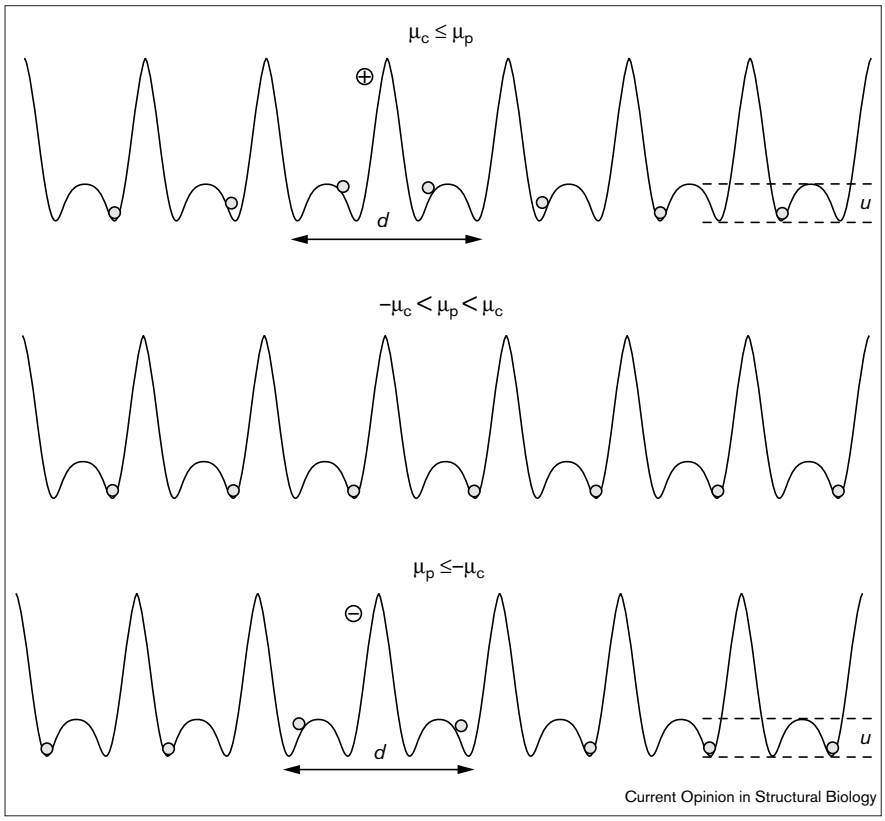
Predictive range of solid-state NMR. A classically coherent mechanism for activation of the rhodopsin G-protein-coupled receptor target by its retinylidene chromophore. The spatial and electronic structure of a minimal model for the ligand, the inverse agonist form, bound to the receptor target in the membrane was determined using solid-state NMR **(a)**. Density functional theory calculations reveal the transfer of electronic charge over the ligand upon photoexcitation **(b)**. *Ab initio* MD starting from the MAS NMR structure provided evidence for a novel classically coherent mechanism of photochemical energy conversion. The MD leads to a prediction of the spatial structure of the bathorhodopsin species, which is a highly distorted spring-loaded ligand **(c)**. The 'balloons' in (a) and (c) show the highest occupied molecular orbital, whereas in (b), the lowest unoccupied orbital is shown. Red and blue correspond to opposite signs of the electronic wave functions.

sufficiently to split water and to generate the oxidative power to provide the planet with an oxygen atmosphere.

Gramicidin A is a nice example of how structural information can be translated into knowledge about mechanisms of function in a merger of the physical sciences and the dogma. A model was put forward in which the dipoles of tryptophan indole sidechains have a direct impact on ion conductance in the gramicidin channel. It suggests that, as a rule, the existence of electrostatic interactions between the ion and the channel is essential to overcoming the high energy barrier caused by the low dielectric constant of the bilayer. This leads to detailed predictions about the physical chemistry of channel function. The quest for independent experimental verification of these predictions is currently in progress in a comprehensive structure/function study. The first step has been completed and involved direct measurement of the orientation of fluorinated indoles in gramicidin A analogues using solid-state NMR techniques [35•]. This is necessary as chemical modification of the indole can induce structural changes. Both the orientations and the positions with respect to the channel axis were determined using ^{2}H solid-state NMR of uniformly aligned lipid bilayer preparations, whereas chemical modification of the indoles by fluorination was used to manipulate both the orientations and the magnitudes of the dipoles.

Another example is rhodopsin, the G-protein-coupled visual membrane photoreceptor present in the eyes of vertebrates, including our own eye. For effective rational drug design, detailed knowledge of a drug or hormone bound to its receptor target is essential. Using rhodopsin as a paradigm for the receptor class, we have implemented a functional assay to atomic resolution that will be generally applicable in studies of ligand–protein interactions. A ligand, enriched with a stable NMR isotope, is bound to the receptor target and a 'MAS NMR snapshot' is taken of the ligand in its binding site. Starting from a low-resolution structure determined using electron diffraction and a variety of solid-state NMR data, the structure of the light-sensitive part of the retinylidene ligand and changes to the structure after photoexcitation were probed with high-resolution 1D rotational resonance and MAS double quantum techniques for a set of labelled ligands [36••,37,38••,39]. Using NMR spectroscopy, we can also probe the electron charge density that is used 'to see' [40]. This information, about the spatial and electronic structure of the ligand, was translated into a high-resolution minimal model using Car–Parrinello *ab initio* methods [41]. The electronic charge is associated with a structural deformation of the polyene backbone, caused by the predominant electron–phonon interactions in these conjugated systems. The coupling of electronic charge to an extended structural deformation is an emerging property called a positively charged polaron (sometimes known as a soliton) in molecular electronics and it can only exist and be stable at a length scale of approximately 1 nm.

Figure 3



A schematic illustration of emerging physical properties associated with a complex counterion. A complex counterion in a solid-state environment can be thought of as a chained device that operates on a length scale larger than that of an individual bond or a single amino acid. The figure sketches the background potential (solid line) of protons (grey circles) in a simplified case, an idealised 1D chain of hydrogen-bonded atoms. The potential energy maxima correspond to the positions of the heavy atoms, whereas the minima in between atoms depict the two possibilities for minimal potential energy in a symmetric hydrogen bond. The generation of positive (top) and negative (bottom) charges, or ionic hydrogen bonding defects can be governed by the local proton chemical potential (μ_p). Defects are thermodynamically stable in the ground state if $\mu_c \leq \mu_p$ or $\mu_p \leq -\mu_c$. According to general principles from solid-state physics, the charges have classically coherent behaviour at short timescales. A characteristic diameter (d ~0.4 nm) is estimated and, in the form depicted in the figure, the defects can move relatively freely around, without a large energy barrier. They can settle in regions of lowest energy and can move or be annihilated and recreated at another location if the chemical potential balance varies, for example, due to conformational changes.

In Figure 2, the molecule displayed in the top panel is the inverse agonist form of the ligand, which blocks the rhodopsin receptor. This vitamin A derivative is positively charged at its right end by protonation. The electronic charge in the highest occupied molecular orbital (HOMO) is indicated by the red and blue 'balloons', and is concentrated at the left side. The positive polaron is clearly visible at the right side of the molecule and is stable in the ground state. The middle panel shows what happens if light comes into our eye. The charge distribution is instantaneously inverted and the electron is transferred to the right-hand side of the molecule. Subsequently, it has been proposed that biology manages to circumvent the laws of quantum mechanics by 'dressing' the charge with a small distortion of the molecular framework of the vitamin, much in the way of a soliton and in line with first principles known from solid-state physics [42]. The biologically relevant rationale behind this hypothesis is that dressing increases the effective mass of the electron by two orders of magnitude. Because of its large mass, the electronic charge behaves in the molecular dynamics (MD) simulation as a classically coherent quasi-particle on the timescale of the photochemistry, which is less than 200 fs. The dressed charge can move from the ring to the Schiff base end of the chromophore, attracted by the negative counterion charge in the protein, simulated by a Cl^- ion (green ball). In the process, quantum mechanics takes over again and the molecule undergoes an ultrafast chemical reaction, known as isomerisation. It changes to the agonist (hormone) form, capable of activating the receptor. The structure of the bathorhodopsin agonist was predicted using *ab initio* Car–Parrinello MD, starting from the solid-state NMR distance and shift constraints on the ligand [36••,41]. In this way, it was established that the active form is a tiny molecular spring, which is loaded by the light energy. The positive charge is concentrated again on the right side of the vitamin (bottom panel) [43]. This entire scheme and the minimal model used for the Car–Parrinello MD simulation were revalidated recently, as it was shown that the predicted NMR/modelling structure can explain the correct circular dichroism spectrum of the primary photoproduct [44]. In the process of these calculations, the sign of the chirality of the chromophore was also revised. This presents a beautiful example of how nonlinear phenomena and scaling principles from solid-state physics fuse with modern structural biology. However, the most intriguing point is probably, in this case, the inference that the transformation of a quantum particle into a classical particle by increasing its effective mass provides a means of transporting confined energy to a region where a chemical reaction, involving quantum mechanics, can subsequently take place. This makes me wonder whether we have here a paradigm view on what may become a general principle of importance for active systems that are supplied with energy, that is, living matter.

Finally, in another functionally important example, it was deduced from ^{15}N MAS NMR chemical shift data on the Schiff base linkage that the charged chromophore in rhodopsin is stabilised by a complex counterion, in analogy to bacteriorhodopsin [45••]. The complex counterion is a charge storage device with some interesting physical aspects [46,47]. The complex counterion is based on emerging properties in a solid environment that are schematically depicted in Figure 3. Some of its basic physics can be understood in a simple way by adapting an established argument used for domain walls in a magnet. A hydrogen-bonded network, for instance, the 1D chain in Figure 3, can be subject to variations in the chemical potential of protons (μ_p). If the μ_p exceeds the critical value μ_c, the creation energy, a positive or negative charge, corresponding to an ionic defect, can appear in the ground state. In a simplified classical scheme, this can be viewed as the result of two competing interactions. On the one hand, the charge will have a tendency to spread over a distance d. The spreading can be associated with improper charge balance (j) between adjacent atoms, contributing a term of the order j/d. On the other hand, spreading the defect yields an energy cost of approximately $u \times d$, due to the energy barriers (u) between the minima in each hydrogen bond. To first order, $\mu_c \sim j/d + u \times d$. Stability requires that μ_c is at a minimum, with $\partial\mu_c/\partial d = 0$. This leads to $d \sim \sqrt{j/u}$, with $\mu_c \sim 2\sqrt{j \times u}$. First, the defect is expected to be relatively stable in terms of its size and energy, as the square root function lessens variations in u or j. Second, it provides a wave propagation mechanism to translocate charge without the need to overcome a series of energy barriers, similar to its electronic counterpart in Figure 2.

A first characterisation of the spatial, electronic and protonic structure of the complex counterion active site and the variations associated with the proton pumping mechanism has just been performed for the bacteriorhodopsin transmembrane proton pump [48••,49•]. Evidence has been obtained for arginine activity in concerted operation with Schiff base deprotonation, which is not readily explained from solution properties of amino acids, in particular the pK_a. It may require invoking free energy density functionals that take into account the solid-state characteristics emerging at a length scale exceeding that of individual chemical bonds or amino acids, similar to the idealised concept presented in Figure 3.

These four examples underline the importance of finding new avenues in structural biology, beyond determining just the structures, into the mechanisms of function of membrane proteins and more predictive range.

Conclusions and outlook

There is now ample evidence that solid-state NMR is conclusive in verifying structure predictions and in the partial refinement of low-resolution structures to high resolution, well beyond the capabilities of X-ray crystallography. It is anticipated that a combination of experimental techniques, in conjunction with bioinformatics, will provide a comprehensive route to structure refinement of small membrane proteins the membrane, in their natural environment.

Soon, it will be practically impossible to consider every individual protein as a separate biological entity. Bioinformatics, proteomics and functional genomics will establish periodic systems of proteins, in a similar way that the periodic system of the elements was set up about two centuries ago. Exploration of homologies in the mechanisms of function of proteins will lead to rules, laws and, ultimately, theories of function based on these laws that generate more predictive range. Thus, there is an urgent need for the continuous development of solid-state NMR techniques, both for structure determination and for the spectroscopic characterisation of the chemistry and physics of membrane protein functional mechanisms.

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- •• of outstanding interest

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