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Moist and soft, dry and stiff: a review of neutron experiments on hydration-dynamics–activity relations in the purple membrane of *Halobacterium salinarum*☆

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Abstract

Twenty-five years of neutron experiments on hydration and thermal dynamics in purple membranes of *Halobacterium salinarum* are reviewed. Neutron diffraction, elastic and quasielastic scattering, allowed to map the distribution of water and lipids and to measure thermal fluctuations and correlation times in the membranes, under various conditions of temperature, hydration and lipid environment. Strong correlations were established between dynamics parameters and the activity of bacteriorhodopsin (the purple membrane protein), as a light driven proton pump supporting the hypothesis that the influence of hydration on activity is in fact due to its effects on membrane thermal dynamics. Hydrogen–deuterium labelling experiments highlighted stiffer and softer parts in the bacteriorhodopsin structure. The soft parts would allow the conformational changes involved in activity, while the stiffer ones may control a valve-like function in vectorial proton transfer.

Keywords: *Halobacterium salinarum*; Quasielastic scattering; Hydrogen–deuterium labelling; Bacteriorhodopsin

1. Introduction

A major part of the human genome is expected to represent membrane proteins. Yet their structural study remains a difficult enterprise and of the more than 12 000 protein structures now in data banks, only approximately 30 are for membrane proteins. In this context the purple membrane of *Halobacterium salinarum* is an exceptional model for study, because it is naturally organised as a highly ordered two-dimensional lattice. Because of this, it remains unique in that

☆ Dedicated to Henryk Eisenberg.

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it is the only natural biological membrane whose structure has been studied to high resolution — this structure, of course, referring to all membrane components: protein, lipid and water molecules, and not just to the protein alone.

The purple patches in the plasma membrane of the extreme halophile, *H. salinarum* (then named *H. halobium),* were discovered and first characterised by Stoeckenius and his collaborators in the 1960s (reviewed in [1]). They are made up of a 26 000 molecular weight protein (75% wt.) and various specific lipids (25%). The colour is due to a molecule of retinal covalently bound to a lysine residue in the protein. Retinal is the chromophore in rhodopsin, the light sensitive protein in animal eyes. Its discovery in the purple membranes (PM) was the first indication that retinal existed also in microbial cells and the protein was named bacteriorhodopsin (BR). A few years after the discovery of PM, it was shown that, associated with a photocycle of colour changes in the retinal, BR functions as a light-driven proton pump. For each photon absorbed by the retinal, it transfers one proton across the membrane, from the cytoplasmic side to the exterior. The photocycle and proton pump activity were studied extensively by various spectroscopic methods. The natural organisation of PM as a two-dimensional crystal, however, presented an extraordinary opportunity to solve the structure of a membrane protein in its lipid environment to high resolution (PM is ordered to better than 3 Å resolution) and to understand the molecular and atomic bases of energy transduction and proton translocation. The 7 Å resolution structure of BR, published in 1975 and showing the seven transmembrane helices, was the first of a membrane protein [2]. This was a landmark study in electron microscopy (EM). Neutron diffraction is a powerful method to study hydration, because neutrons can distinguish between hydrogen (H) and its isotope, deuterium (^{2}H or D) and at about the same time, a neutron diffraction study using H_2O–D_2O exchange was initiated with the aim to explore the hydration and proton pathway in the membrane [3]. Neutron scattering is also uniquely suited to the study of thermal fluctuations and experiments to measure PM dynamics were initiated by neutron spectroscopy almost a decade later [4]. It is interesting to note that neutron contributions to understanding PM have continued, with a number of recent papers in high impact journals [5–11]. The structural study of PM has also progressed enormously, and the three-dimensional structure of BR is now known to relatively very high resolution. To the results from electron microscopy [12,13] have now been added X-ray crystallography data from BR three-dimensional crystals grown in lipid cubic phases [14–19]. Progress in understanding BR function has been reviewed recently by different authors [20–22].

Henryk Eisenberg made major contributions to the understanding of the relationship between thermodynamics and structure and to the study of extreme halophiles. This critical discussion of the neutron experiments that established relations between hydration, thermal dynamics fluctuations and function in a specialised membrane from halobacteria is dedicated to him.

2. Theoretical and experimental background

2.1. Neutron diffraction, molecular structure, deuteration, hydration

Diffraction from oriented PM samples appears as a powder pattern from the two-dimensional lattice because the membranes pack in a stack with rotational disorder. The pattern is for a P3 hexagonal lattice of spacing close to 65 Å, with peaks extending to approximately 7 Å resolution (the 7, 1 reflection). The D16 diffractometer at the Institut Laue Langevin (ILL) was designed, using a cold neutron beam (wavelength 4–5 Å) and good angular resolution, to measure neutron diffraction from this type of sample [23]. A similar design was later adopted for the V1 diffractometer at the Hahn Meitner Institute, Berlin Neutron Scattering Centre (BENSC), with improvements such as a focusing monochromator and a larger detector to compensate for the lower neutron flux compared to ILL beams [24]. In the past year, a focusing monochromator was installed on D16 and a larger detector is currently under study.

The basis for the analysis of powder diffraction from PM was essentially established by Zaccai and Gilmore [3]. Two-dimensional Fourier projection map information is obtained by using electron microscopy data to split the peaks in the one-dimensional intensity pattern into two-dimensional structure factor moduli, $[F(h,k)]$, as well as to assign them the appropriate phases. In contrast to X-rays, for which atomic scattering lengths are proportional to the number of electrons, the coherent scattering length (b_{coh}) of an atom for neutron diffraction is a property of its nucleus. C, O, N, P, S atoms all have similar b_{coh} values, in the range of 5–10 Fermi units. Natural abundance H has a negative b_{coh} value (-3.74 Fermi units); the isotope ^{2}H (deuterium or D), however, has a b_{coh} value of 6.7 Fermi units, making neutron diffraction, by using H–D labelling a powerful method to study hydration and structure. Neutron diffraction experiments on PM were therefore based mainly on H–D labelling, either of the water and exchangeable atoms (by H_2O–D_2O exchange) or of non-exchangeable membrane components by specific deuteration. H_2O–D_2O exchange for different relative humidity values was effected by vapour diffusion in sealed temperature-controlled cans over appropriate saturated salt solutions. *H. salinarum* lends itself well to culture in fully deuterated or specific deuterated media, and non-exchangeable H–D labelling was performed both by reconstitution of membrane samples using deuterated and natural abundance components, and by biosynthesis to incorporate labels in specific groups. Popot et al. [25] is a good example of the combination of the two approaches to label BR, while Weik et al. [9] labelled the glycolipid headgroups, simply by feeding the organisms deuterated glucose.

In a typical experiment, data would be collected from the labelled and unlabelled PM and the standard crystallographic difference Fourier method or model fit approaches would be used to locate the labels in the two-dimensional projection structure. Both approaches were used, for example, to locate deuterated retinal [26]. It was also usually possible to calibrate the maps so as to measure the extent of H–D exchange represented by the peaks in the difference Fouriers [27]. This allowed to quantify membrane hydration in H_2O–D_2O exchange samples [3,28] and provided a good check of deuterium incorporation in the case of specific labelling [9].

2.2. Neutron spectroscopy, molecular forces

The forces that maintain functional biological structure at the molecular level are known. They include H-bonding, electrostatic interactions, van der Waals interactions and pseudo-forces arising from the hydrophobic effect. Biological matter is 'soft' because they are 'weak' forces. Their associated energies are similar to thermal energy at usual temperatures (≈ 1 kcal/mol) with atomic fluctuations in the 0.1-nm range. Quite literally, (even though etymologically wrong) motions in this energy range represent protein *thermodynamics,* since dynamics, from the Greek $\Delta\upsilon\nu\alpha\mu\iota s$ (strength) concerns forces.

Debye–Waller factors in X-ray crystallography provide information on atomic displacements from average positions but no information on frequencies; IR, NMR and fluorescence spectroscopies provide frequencies or relaxation times but no amplitude information. A neutron beam, however, presents a particularly well-adapted dispersive relation for the study of thermal motions. Neutrons of energy ≈ 1 kcal/mol have wavelengths of ≈ 0.1 nm, which makes them uniquely suitable for the simultaneous characterisation of the frequency and amplitude of motions — they can tell us how far an atom goes in how long. Furthermore, neutron experiments rely on incoherent scattering and can be performed on any sample (that need not be crystalline or even monodisperse). The coherent and incoherent scattering amplitudes of nuclei can be quite different. The incoherent cross-section of ^{1}H is much higher than that of other nuclei in usual biological samples and than that of deuterium. So that, in incoherent scattering experiments, specific deuterium labelling allows to mask certain parts of a complex structure. The incoherent neutron scattering experiments provide information on protein dynamics because, in the energy range examined, H atoms reflect the motions of the side

chains and backbone atoms to which they are bound. The mass of D is twice that of H; does deuteration not affect molecular dynamics? In an important control experiment, the scattering of a fully deuterated purple membrane sample was measured to show that the mean atomic fluctuations are essentially identical to those in the natural abundance sample [6]. This observation confirmed that it is the dynamics of larger chemical groups (for which the relative change in mass due to deuteration is small) that is probed and not of the H atoms with respect to these groups.

Data measured in a diffraction experiment as a function of a reciprocal space scattering vector Q Å^{-1} (for elastic scattering, $|Q| = Q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle and λ the incident wavelength) provide information on real space fluctuations of length d Å, where $d \sim 2\pi/Q$. Similarly, data measured in a spectroscopy experiment for an energy transfer ΔE (which can be expressed in terms of a frequency in s^{-1}) provide information on a time course t (s), where $t \sim 1/\Delta E$. In incoherent neutron scattering, there is no interference between waves scattered by different nuclei and it is the sum of scattering from individual atoms that is observed. The neutron incoherent dynamical structure factor is written $S_{inc}(Q, \omega)$, where ω is an angular frequency and $\Delta E = h\omega/2\pi$ (h is Planck's constant). It represents the self-correlation function of atoms as they move about. Note that $S_{inc}(Q, \omega)$ is an intensity, in contrast to the structure factor in X-ray crystallography that represents an amplitude. Whereas the structure factor in crystallography is a spatial Fourier transform, the dynamical structure factor contains a double Fourier transformation over space and time [29]. The $S_{inc}(Q, \omega)$ value is the resultant intensity, summed over all atoms in the sample, of the wave scattered by the atom at position $R(0)$ at time 0, interfering with the wave scattered by the *same* atom at $R(t)$ at time t. The Q and ω resolution windows can be considered separately. If the instrumental resolution function of the diffractometer has a width corresponding to ΔQ, then it will not be possible to resolve distances smaller than $2\pi/\Delta Q$. If the data extends to Q_{max}, distances smaller than $2\pi/Q_{max}$ will appear 'blurred' out. These are familiar concepts in X-ray crystallography, which can be extended to the energy-time couple. An instrumental resolution of $\Delta\omega$ (ω_{min}) will not allow to resolve motions occurring in times longer than $t_{max} = 2\pi/\omega_{min}$; atoms moving slowly will appear to be immobile. If the data extends to an energy transfer of ω_{max}, motions occurring in times shorter than $2\pi/\omega_{max}$ will appear as a blur.

2.2.1. The elastic limit

In the elastic limit $\omega = 0$ and for a single atom $S_{inc}(Q, 0)$ provides information, in theory, on how far the atom has moved away from its initial position in an infinitely long time. Because of instrumental energy resolution, however, the measured value is $S_{inc}(Q, 0 \pm \Delta\omega)$ (where $\Delta\omega$ corresponds to the energy resolution of the spectrometer) and the experimental information concerns a maximum length of time, $t_{max} = 2\pi/\Delta\omega$). The magnitude, d, of the fluctuation that can be measured depends on the Q range, with d close to $2\pi/Q$. When motion is restricted to a volume of space, the single atom appears to sweep out a particle in time $t_{max} = 2\pi/\Delta\omega$). $S_{inc}(Q, 0 \pm \Delta\omega)$ in this case, can be developed in a similar way to the scattered intensity $I(Q)$ in small angle scattering and a radius of gyration (R_g) calculated for the 'particle' from the Guinier approximation [30]:

$$S_{inc}(Q, 0 \pm \Delta\omega) = \text{constant} \cdot \exp\left(-\langle x^2 \rangle Q^2\right) \quad (1)$$

where R_g^2, the radius of gyration squared of the pseudo-particle, is equal to $3\langle x^2 \rangle$; this is also the value corresponding to the mean square atomic displacement in the Debye–Waller factor of crystallography. This Gaussian approximation is valid for values of $R_g Q$ close to 1. If the atoms fall into populations with significantly different amplitudes they can be separated out by experiments in different Q ranges; this is in fact the case for the atomic fluctuations in purple membranes [5,30].

Back-scattering neutron spectrometers have an energy resolution of 1–10 μeV (corresponding to maximum times in the order of nanoseconds) [29]. In an elastic temperature scan, mean square fluctuations are calculated from the slopes of ln

$S_{inc}(Q, 0 \pm \Delta\omega)$ vs. Q^2 plots (Eq. (1)) and plotted as a function of absolute temperature, T. The low temperature part of such scans always shows a harmonic region where $\langle x^2 \rangle$ is proportional to T. Then, at approximately 200 K there is a deviation from this behaviour, with fluctuations rising strongly with T. This is called a dynamical transition. It was first revealed by neutron scattering in myoglobin [31], but has since been seen in many proteins, including BR [4]. According to the conformational substrate model of Frauenfelder and collaborators [32], it represents the transition from the protein being trapped in one of the substrate potential well at low temperature to being able to sample many wells at higher temperature. From harmonic, at low temperature, atomic thermal motions become diffusive.

2.2.2. Quasi-elastic scattering

Mean square fluctuations measured by elastic scans on back-scattering spectrometers represent an integral over motions occurring over a time scale of approximately 1–100 ps (for < 10 μeV resolution). On time of flight neutron spectrometers, a broader energy range is examined (with more moderate resolution, however, ≥ 0.01 meV).

Diffusive motions give rise to quasi-elastic scattering that appears as a sum of Lorentzians broadening of the elastic peak in this energy range. (Vibrational motions will appear as peaks at higher energies according to their frequencies). In the elastic and quasielastic region the scattered intensity is given by [33,48]:

$$S_{inc}(Q,\omega) = \exp\left(-\langle x^2_{vib}\rangle Q^2\right)\{\mathrm{EISF}(Q)\cdot\delta(\omega) + \Sigma_n \mathrm{QISF}_n(Q)\cdot L_n(H_n,\omega)\} \quad (2)$$

where $\langle x^2_{vib} \rangle$ is the mean square fluctuation associated with vibrational motions, EISF and QISF are elastic and quasielastic structure factors, respectively, and $L_n(H_n,\omega)$ are Lorentzian functions, with H_n representing the reciprocal of a correlation time. The vibrational mean square displacements are called Debye–Waller factors in neutron scattering papers, causing some confusion with other definitions where they include all contributions to deviations from atomic mean positions. In X-ray crystallography, for example, the Debye–Waller factor also includes static disorder. Eq. (2) allows a model independent separation of the scattered intensity into vibrational elastic and quasielastic contributions. Very good data are required, however, the quasi-elastic intensity being about an order of magnitude weaker than the elastic component, limiting the number of Lorentzians that can be obtained from the fits. The interpretation of the EISF and QISF functions obtained is model dependent. For example, the QISF is often described in terms of a flat background in Q from immobile atoms whose motions are not resolved by the spectrometer and the scattering curve of a sphere. But analysis of small angle scattering curves shows that the same data might be fitted equally well by a scattering curve representing motions in an ellipsoid with a lower value for the flat background in Q.

3. Results and discussion

3.1. Structure

Neutron diffraction studies of PM were based essentially on deuterium labelling. A number of structural features in BR were precisely defined, after the publication of the 7 Å resolution structure in 1975 [2], and they contributed to the progress towards the first high resolution structure from EM published in 1990 [34]. These include the retinal [26,35] and the position and orientation of transmembrane helices, when the BR sequence became available [25,36]. In the 15 years 1975–1990, the neutron results were also extremely useful for the BR community by providing structural information for structure–function considerations. They were all fully confirmed by the high resolution EM structure, proving the reliability of the neutron diffraction/deuterium labelling approach [34]. Because of the ease with which it is purified and can be manipulated, BR became a paradigm to develop and test membrane protein structural methods and a set of small angle neutron scattering experiments were

designed to explore structural features of its incorporation into vesicles [37]. There is now a growing interest in glycolipid–protein interactions, which are postulated to be implicated in the intracellular transport and targetting of membrane proteins via the formation of patches or 'rafts'. PM provided a unique opportunity to study glycolipid–protein interactions in a natural membrane. It was shown from neutron diffraction that they occupy well-defined crystallographic positions that imply specific interactions with BR that may drive its organisation in PM [9]. Is PM an Archaeal 'raft'?

3.2. Hydration-dynamics-function

The first neutron diffraction experiments on PM showed that, at high relative humidity, hydration in the plane of the membrane is predominantly around the lipid headgroups [3]. In a dry environment, PM structure is maintained but the water is removed from around the lipids [38]. As the membranes are dried progressively, the water distribution within BR becomes apparent in the difference Fourier maps and it was possible to count the molecules in the proton channel [39]. The importance of water molecules for proton translocation was clearly established by the high resolution structures. And when the role and implication of hydration in PM function was confirmed by various studies, neutron experiments provided what remains unique structural information on the extent and location of this hydration under different conditions; this is because H_2O:D_2O exchange neutron diffraction allows to locate even disordered water molecules at relatively low resolution. No water molecules have been located in the EM studies, and only a few, well-ordered, waters in the proton channel were seen in the microcrystal studies.

A hypothesis relating hydration, dynamics and function in BR was formulated following in-plane neutron diffraction experiments [40]. This work showed that hydration around lipid headgroups stays in place when PM is cooled to liquid nitrogen temperature from high relative humidity — cooling, therefore, does not dehydrate the lipid headgroups. In the hypothesis, BR within PM requires a 'fluid' environment in order to be active; hydration around lipid headgroups increases the projected area per molecule with a consequent increase in the fluidity of the chains. The relation between hydration and dynamics was verified by the first inelastic neutron scattering experiments on PM dynamics [4]. More recently, the role of the lipid environment in maintaining fluidity was characterised, when it was shown that removing lipids led to a stiffening of BR dynamics [10]. The previous results lead to an interesting speculation on the role of the lattice in PM. In fact, in the very high local protein concentration within PM, the alternative to a well-ordered lattice is very probably aggregated BR. The lattice provides a way to keep protein molecules apart, each surrounded as far as possible by hydrated lipids to maintain fluidity.

The water layer parallel to the membrane plane in PM stacks was examined by neutron diffraction as a function of temperature [11]. It was shown that under slow cooling conditions there is an enormous loss of water between the membranes so that the lamellar spacing drops to 53 Å. The fluctuation amplitudes of the lipids were not measured at this spacing. The authors concluded that cooling of PM results in dehydration. But the difference between the stack periodicities of the dehydrated and dry membranes indicated that inter-membrane water lost upon cooling does not include the hydration layer (the 'completely dry' membrane periodicity is close to 45 Å [49]; the 5 Å difference provides for at least one molecular water layer on each membrane). Zaccai [40] had already shown that lipid hydration stayed in place upon cooling. The question now is whether or not this 'minimum' hydration layer is sufficient for BR activity, as is the case, for example, for soluble proteins [41].

Neutron spectroscopy experiments on PM were essentially developed by two groups, at ILL and BENSC, respectively. Their approaches are complementary, with the ILL group concentrating on unoriented samples and elastic scans, and the BENSC group working mainly on oriented samples by quasielastic scattering. Both groups stud-

ied hydration-dynamics–function relations in PM in careful quantitative studies. Different populations of motion were identified in their response to hydration, by examining different windows in time (energy) [33] and space (scattering vector) [5], and also by hydrogen–deuterium labelling [6]. The populations include large amplitude, long correlation time and smaller amplitude, faster motions. Comparing the hydration dependence of the dynamics and functional aspects such as photocycle parameters, allowed to establish correlations between the dynamics of these populations and biological activity. The hydrogen–deuterium labeling experiments established that BR dynamics is heterogeneous and suggest a dynamics hypothesis for pump function. A stiffer core around the retinal binding site could control the stereospecific switch that ensures the valve function of the proton pump, while a softer global environment facilitates functional conformational changes and proton transfer in each half of the pathway through the protein [6]. A comparison of the high resolution structures between the ground and M state of the photocycle suggested 'structural' elements that determine Schiff base accessibility and the vectoriality of the pump (pK changes, hydrogen bond networks) [18]; the dynamics hypothesis refers to the local forces that enable these structural changes and maintain them. The elastic [5,30] and quasielastic [33] analyses converged to describe a dynamical transition in PM at 150–200K, for the large amplitude, long correlation time motions, in both wet and dry samples. The hydration dependence of these motions is triggered only at very high relative humidity and above 250 K, where a second dynamical transition appears. This behaviour is different from that of myoglobin, for example, in which a single dynamical transition at 180 K was revealed in wet samples [31]; in very dry, trehalose-coated samples, on the other hand, there are no transitions observed and myoglobin displays fully harmonic dynamics to above room temperature [42]. In dry PM, a population of small amplitude motions also does not undergo dynamical transitions, but, above 60% RH, a transition appears at approximately 230 K [5,30]. This dynamical behaviour was found to be correlated with a large conformational change occurring in BR during the photocycle, [5,43–46], contributing further to the dynamics–activity relations. In a recent review of neutron scattering experiments to study protein dynamics, a force constant model was introduced to characterise the 'softness' of structures; at physiological temperatures, the 'stiffer' active core of BR is described by a mean pseudo-force constant of 0.3 N/m, compared to 0.1 N/m for PM globally [47].

4. Conclusion and future perspectives

PM is so far the only natural biological membrane whose structure is known to submolecular resolution. This and the relative ease of its manipulation, its obtention in large quantities, the availability of mutants, etc., make it a unique model for the study of various important aspects of membrane structure and dynamics. Furthermore, *H. salinarum* belongs to the life domain of the Archeae, which have been shown to have similarities with the Eukarya. Archaea provide good models to study aspects that are much more difficult to study in eukaryotic cells. BR itself has striking structural similarities with the seven helix G-protein coupled receptors that are found in nerve cells of higher animals. Apart from the potential for general membrane studies on PM, there still remain important questions concerning structure-dynamics–function relations that should be addressed by neutron diffraction and spectroscopy: how do the different populations of motion identified in the elastic and quasielastic experiments relate to the BR structure? What is the role of the forces acting on the retinal during the photocycle and especially how does it 'turn over' in its valve function during vectorial proton translocation? What is the role of BR extra-membrane loop dynamics in guiding protons on the PM surface to the channel? What is the role of protein–lipid interactions? Of the three main hydration sites: the lipid headgroups, the extramembrane loops, the proton channel? Of the very high ionic concentrations on either side of the mem-

brane? *H. salinarum* is an extreme halophile; it lives in over 4 M NaCl and its cytoplasm is saturated KCl. When in vitro experiments are conducted on membranes, it is usually assumed that physiological conditions are best represented by 100% RH. But the water activity in saturated KCl corresponds to 86% humidity, in saturated NaCl to 75% relative humidity. In the detailed study of dynamics of Lehnert et al. [5], for example, which of the several relative humidity measurements presented is physiologically relevant?

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