A SYNOPSIS OF NEUTRON SPECTROSCOPY OF PHOTOSYNTHETIC MEMBRANE PROTEINS.

A. Gall[1,2], S. Dellerue[1,2], B. Robert[2], M.-C. Bellissent-Funel[1]

[1]Laboratoire Léon Brillouin (CEA-CNRS)
[2]Section de Biophysique des Protéines et des Membranes and CNRS/URA 2096, CEA/DSV/Saclay, 91191 Gif-sur-Yvette

Introduction.

All cellular processes, whether in prokaryotes or eukaryotes, at some stage require proteins which may be located either in the cytosol or in membranes. As a result, many different laboratories now study the function and structure of proteins. This is mirrored in the Protein Databank (PDB) where over 8700 X-ray crystal structures of proteins have been deposited. It is currently agreed that about 30% of the total number of proteins in any organism are integrated into membranes. Unfortunately, less than 1% of all the PDB X-ray crystal structures are from membrane proteins. Therefore, any information pertaining to the function, dynamics and structure of a membrane protein is invaluable.
The aim of this project is to understand the detailed energetics of membrane protein folding, and to establish a precise relationship between the structure, function and dynamics of this class of biological macromolecule, including inter- and intra-protein interactions. Therefore, it is necessary not only to know their three-dimensional structures, but also to characterise, with the highest degree of accuracy possible, the internal motions of these proteins. Motions in proteins occur over a wide range of time scales from femtoseconds (10^{-15}s) to seconds or longer. Only a few experimental techniques permit one to study these motions. Among them, inelastic neutron scattering (INS) is capable of probing motions with characteristic times in the nanosecond (10^{-9}s) to picosecond (10^{-12}s) range. In fact neutrons interacting with molecular systems at normal temperature can exchange a significant proportion of their energy with thermal excitations and these energy changes can be readily measured by a variety of techniques. Moreover INS is a spectroscopic technique which allows to study internal motions on exactly the same time-scale that is now accessible by computer simulation.

Our membrane proteins: RC, LH1 and LH2.

We have chosen to study and compare the structural and dynamic properties of three membrane proteins involved in the primary steps of photosynthesis from purple bacteria. These proteins are called the photochemical reaction centre (RC), the core light-harvesting complex (LH1) and the peripheral light-harvesting complex (LH2). Solar energy is collected by the bacteriochlorophyll (BChl) pigments in the LH proteins and the captured excitation energy is then transferred to the RC. Subsequent electron transfer within this protein yields a chemical potential gradient across the membrane that is used to drive many cellular processes. The proteins were chosen for study because: 1) The X-ray crystal structures of the LH2[(1)] and RC[(2)] have been solved to resolutions better than 2.5 Å, and their accompanying detergent rings have been visualised using neutron diffraction experiments. 2) They are produced by the bacteria in large quantities making the biochemical purification and protein characterisation processes much easier. 3) The proteins contain BChl cofactors that serve as molecular markers which monitor protein structure and conformation. 4) Finally, they form a network of interconnecting proteins within the photosynthetic membrane (see figure 1). We can monitor the influence of inter-protein contacts on the structure-function-dynamics for each of our three proteins.

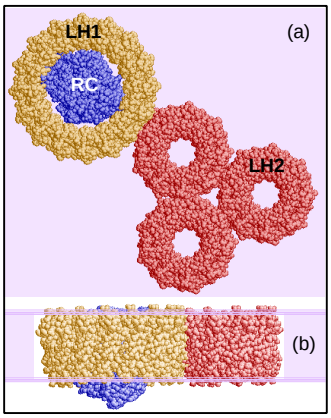

Figure 1 : A scheme of the photosynthetic integral membrane proteins from purple bacteria viewed (a) parallel and (b) perpendicular to the plane of the membrane which is coloured violet. Nearly all of the protein volumes are located within the lipid bilayer that makes up the membrane. Key to proteins : LH2, red; LH1, yellow; RC, Blue.

Principles of neutron scattering.

At this point we must remember that in INS experiment, the energy changes occurring when the incident neutrons interact with moving nuclei in the samples are measured such that an energy spectrum of scattered neutrons is obtained at each scattering angle. The fundamental quantity measured is the dynamic structure factor S(Q, ω) where Q is the momentum

transfer with $Q=(4\pi/\lambda)\sin q$ (λ is the neutron wavelength, $2q$ the scattering angle) and ω is the energy transfer. Neutron spectroscopy takes advantage of the fact that hydrogen and deuterium have different coherent and incoherent scattering cross-sections. By varying the H/D contents between the sample of interest (e.g. a membrane protein) and its environment (e.g. the membrane) we can highlight or eliminate parts of the system. This method of *contrast variation* is one reason why neutron spectroscopy may have significant advantages over similar X-ray based techniques. The benefits of neutron techniques, which may provide information on dynamics (e.g. time-of-flight and neutron spin-echo) or give structural information (e.g. small angle neutron scattering), are illustrated by presenting three examples, one from each protein, from the wide selection of experimental results obtained at the LLB (and complementary data collected at the Institute Laue-Langevin, Grenoble).

Time-of-flight spectroscopy : LH2 in detergent micelles.

A rather unique tool used to gain experimental information on protein dynamics in the picosecond range (from 0.1 to a few hundred picosecond) is incoherent inelastic neutron scattering (IINS) which takes advantage of the large incoherent scattering cross-section of hydrogen atoms with respect to the relatively small cross-section of other constitutive atoms in proteins. A large fraction of the atoms (up to 50%) in globular proteins are hydrogen atoms, and since they are distributed nearly homogeneously within the protein molecule, IINS will thus allow protein dynamics to be characterized in a global manner by monitoring the individual dynamics (vibrations, translations, rotations) of hydrogen atoms. The fundamental quantity measured is the proton self dynamic structure factor $S_S^H(Q,\omega)$.

As an example, figure 2 shows the evolution of the $S_S^H(Q,\omega)$, as a function of ω for the LH2 protein in detergent micelles. The central part is analysed as a sum of elastic and quasi-elastic components[3]. The latter component is considered to be a Lorentzian line with a half-width at half-maximum Γ. In the top left insert, Γ is plotted as a function of Q^2. It follows a plateau from 0.10 to 0.85 $Å^{-2}$ and is linearly dependent on Q^2 at higher Q^2 values. This evolution is characteristic of a diffusion in a confined volume[4] whose shape is given by the elastic incoherent structure factor (EISF) (right top insert). From the high Q limit of the EISF, it appears that LH2 contains a large proportion of mobile protons (*ca.* 90%) compared to soluble proteins such as C-phycocyanin. This may be a consequence of the rather unique hollow ring-like structure of LH2 (see figure 1). The effect of different environments on the dynamics of the hydrophobic side chains is carried out by substituting deuterated detergent by deuterated lipids.

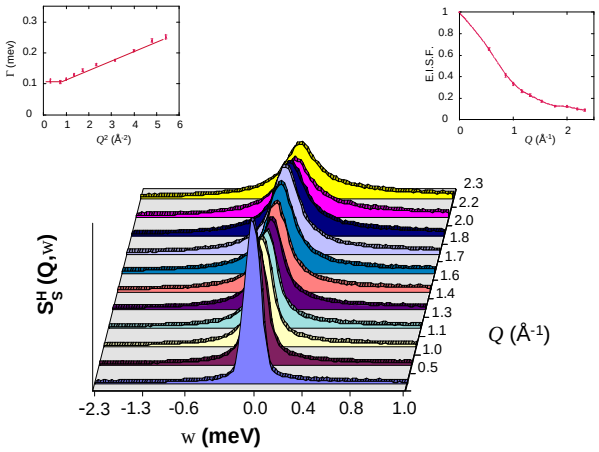

Figure 2 : $S_S^H(Q,\omega)$ of the LH2 protein in detergent micelles as a function of Q. T=277 K, l =5 Å. The spectra were collected with the time-of-flight spectrometer MIBEMOL. Top Left : Evolution of G as a function of Q^2 (see text). Top Right : Evolution of EISF as a function of Q.

Neutron spin-echo spectroscopy : Comparison between RC and LH2.

In order to study protein dynamics, *viz.* the nanosecond (10^{-9} s) time-scale, we use the neutron spin-echo [5] (NSE) spectroscopy. NSE allows precise measurement of velocity changes of neutrons via Larmor precession of the neutron's spin. If several spin-echo measurements have been made on polymers, few attempts have been made to use it to probe biological samples. Here we make use of the separation in reciprocal space of the coherent and incoherent scattering to demonstrate the detection of a neutron spin-echo signal both in RC and LH2 proteins. A further advantage of the spin-echo technique is that it gives direct access to the dynamics of the sample in the time domain t by measuring the *intermediate scattering function*, $I(Q,t)$. Figure 3 gives examples of $I(Q,t)$ for the RC and LH2 proteins for two values of Q. It is evident that the coherent motions of these complexes are somewhat different. It may be observed that at Q=0.197 $Å^{-1}$ when time, t, approaches zero, the normalised $I(Q,t)$ for LH2 is only $\approx$ 0.6 indicating that the LH2 contains significantly higher levels of sub-nanosecond motions than the RC. Although having near identical numbers of amino acids (*ca.* 850), the RC and LH2 proteins have widely differing tertiary and quaternary structures. The LH2 consists of 18 independent transmembrane spanning α-helices located within 9

identical α/β-heterodimer subunits that form a hollow ring-like structure in the membrane (see figures 1 and 3). However, the RC has three much larger subunits with less overall symmetry (blue colour in figures 1 and 3). Therefore, we conclude that the overall protein motion is dependent on the quaternary structure of the protein.

The analysis of NSE data gave us access to the effective diffusion coefficient, D_{eff} of highly concentrated protein solutions which was not possible with dynamic light scattering (DLS) experiments. By comparing the results with those of dilute solutions of proteins we have demonstrated that in the case of RC the diffuse properties are invariant over a wide range of concentrations. $I(Q,t)$ is described by an exponential function, $\exp^{(-t/\tau)}$, and $1/t=D_{eff}Q^{2}$. For our membrane proteins, DLS is limited to a maximum protein concentration of 0.8 mg ml^{-1} for which we have obtained a value of D_{eff} for the RC in detergent micelles of 3.7×10^{-8} cm^2s^{-1}. Using the spin-echo spectrometer MESS we have measured $I(Q,t)$ over Q values between 0.055 $Å^{-1}$ and 0.197 $Å^{-1}$ and have obtained a very similar diffusion coefficient of 3.6×10^{-8} cm^2s^{-1} for higher concentrations of RC in the same detergent. We are presently investigating the role of the individual domains within these proteins by selectively deuterating different components. Other approaches include insertion within lipid bilayers and, in the case of the RC, selective subunit removal.

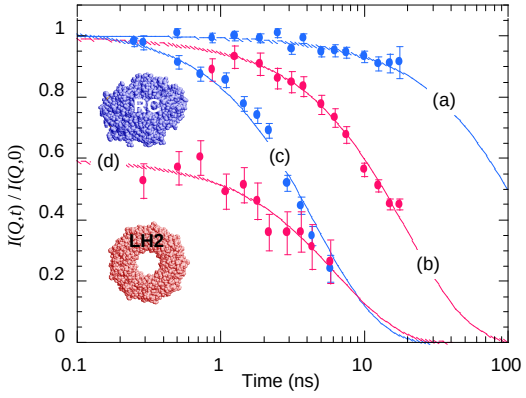

Figure 3 : The time dependence of normalised I(Q,t) for the RC (shown in blue) and LH2 (shown in red) proteins at 0.055$Å^{-1}$ (a,b) and 0.197 $Å^{-1}$ (c,d) measured at 288 K. The solid lines represent the best fits using an exponential function which may provide information on D_{eff} (see text). The spectra were collected with the spectrometer MESS. Key to proteins : LH2, red; RC, Blue.

Small angle neutron scattering : structural information on the B777 subform of LH1.

Unlike the previous two techniques which provide information on macromolecule dynamics, the *small angle neutron scattering* (SANS) technique allows us to obtain structural information. Combining SANS techniques and H/D *contrast variation* on dilute solutions, or micro-emulsions, of biological macromolecules may provide information on the shape of the molecule *via* the *form factor* and on the interactions between the molecules *via* the *structure factor*. Although we have carried out a number of different SANS experiments on the RC and LH2 proteins in different sample environments, we have chosen to present some new structural results on our third protein, LH1.

The LH1 protein is similar in overall structure and function to the LH2. However, the number of α/β-dimers increases from 9 to 16 per ring. Let us notice that this closed ring structure may only be valid for an isolated protein. *In vivo*, it is possible that the ring may be open. An important property of the LH1 protein (also called B873) is that it is possible with detergents to progressively dissociate the ring of 16 α/β-dimers into its individual α– and β-subunits (B777), *via* an intermediate form (B820). This process is shown in figure 4 and has the added advantage of being fully reversible. Since it is possible to isolate individual RC proteins, co-purify RC-LH1 complexes and the different structural units (B777 and B820) that aggregate to form LH1, one can imagine that it is possible to attribute the influence of protein-protein contacts on individual secondary and tertiary structures.

Based on biophysical data, a theory on the thermodynamics of membrane polypeptide oligomerisation in light-harvesting complexes proposed that each B777 consists of a single polypeptide sequence with a single non-covalently attached BChl molecule [6]. Recently, we decided to measure the SANS spectrum of the B777 complex in a fully deuterated detergent micro-emulsion. Figure 5 displays the data as compared with a fit using the *form factor* of an ellipsoid of length, L, of 60 Å, with a diameter, D, of 28 Å. The length of the ellipsoid is consistent with the distance required for the occupancy of a transmembrane-spanning α-helix. If the α-helix had unfolded it would not have given similar fitting parameters. Together with our Raman data [7], this is the first structural information that confirms the hypothesis that the B777 is made from one polypeptide containing a single membrane spanning α-helix with one bound BChl molecule. Further experiments with the B820 will allow to characterize the structural changes and the aggregation, that lead to the formation of the $(\alpha/\beta)_{16}$ membrane protein. This in turn will significantly help to improve our understanding of membrane protein thermodynamics and oligomerisation.

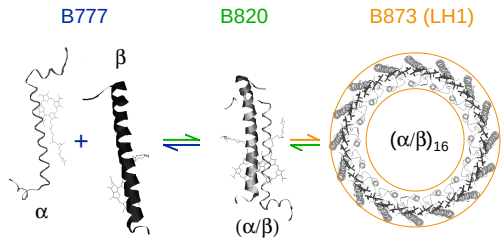

Figure 4 : The LH1 proteins are composed of a ring of 16 a/b dimers, each of which may be dissociated into individual a - and b-subunits. Each B777 polypeptide has a single non-covalently attached BChl that is responsible for capturing solar energy.

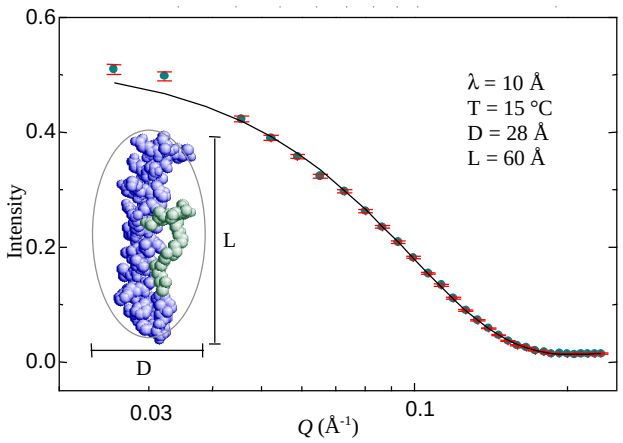

Figure 5 : SANS spectrum of the B777 complex in a deuterated detergent (D_{28}-n-octyl-ß-D-glucopyranoside) micro-emulsion. The solid line results from the fit of data with an ellipsoid (L=60 Å, D=28 Å). Therefore, each B777 subunit consists of a single protein polypeptide with a non-covalently attached BChl. The scheme of the B777 is for guidance only and is based on the X-ray crystal structure of LH2 where the polypeptide is coloured blue and the BChl blue-green. The spectra were collected with the spectrometer PAXE. T=15°C, l =10 Å.

Summary and prospectives

In summary, the photosynthetic proteins from purple bacteria provide an unique opportunity to compare the influence of tertiary structures on internal protein dynamics. Our family of interconnecting membrane proteins also allows us to measure the effect of inter-protein interaction on protein dynamics and structure. Using SANS we can obtain novel information on protein structures, as shown by our approximation of the *form factor* of the B777. Furthermore, based on experimental data from time-of-flight and NSE spectroscopies, improved pisosecond and new nanosecond molecular dynamics algorithms for membrane proteins are currently being developed.

Finally, our protein system provides an important contribution to the overall understanding of the inherent differences between integral membrane proteins and globular proteins, which are located in the cytosol. One such major physical difference is the pressure-sensitivity of proteins. In collaboration with the University of Tartu, Estonia, we have measured a series of electronic absorption and pre-resonance Fourier Transform - Raman spectra for the RC, LH1 and LH2 proteins which show that they are still intact at pressures where globular proteins, such as myoglobin, are denatured. In addition to our present series of experiments, we are presently planning Fourier Transform - Infra-Red and neutron measurements to establish the membrane protein structure-function-dynamic relationships as a function of applied hydrostatic pressure.

1. McDermott, G., Prince, S. M., Freer, A. A., Hawthornthwaite-Lawless, A. M., Papiz, M. Z., Cogdell, R. J., and Isaacs, N. W. (1995) *Nature (London) 374*, 517-21.
2. Roy, C., Lancaster, D., Ermler, U., and Michel, H. (1995) *Adv. Photosynth. 2*, 503-26.
3. Bee, M. (1988) *Quasielastic Neutron Scattering. Principles and Applications in Solid State Chemistry, Biology, and Materials Science*, Adam Hilger, Bristol, UK.
4. Volino, F., and Dianoux, A. J. (1980) *Mol. Phys. 41*, 271-9.
5. Mezei, F. (1980) *Lecture Notes in Physics, Vol. 128: Neutron Spin Echo, Springer-Verlag, Berlin.*
6. Sturgis, J. N., and Robert, B. (1994) *J. Mol. Biol. 238*, 445-54.