

BIOLOGY

1. INTRODUCTION

The research activities in Biology at LLB focus on two main fields of interest that are in full development.

1.1. Conformation of proteins in solution.

The understanding of protein folding remains one of the major goals of Biology. This requires, at least, a detailed structural characterization of both the folded and the unfolded states.

If we raise the temperature sufficiently, or make the pH extremely acid or alkaline, or add guanidinium chloride to the buffer, the protein structure will unfold. Thus, characterisation of the denatured states of protein is important for a complete understanding of the factors stabilising their folded conformation. Whereas X-ray crystallography or nuclear magnetic resonance allows this for native proteins, only few techniques can provide precise information about the mean conformation of flexible denatured states. Among these techniques, small-angle scattering, of either X-rays or neutrons, is a very powerful tool giving structural information at low and medium resolution. Complementary information from circular dichroism, fluorescence and differential scanning calorimetry is used.

An emerging and very promising field concerns biofunctional assemblies at lipid interfaces studied by neutron reflectivity (See “highlight” by Tristl et al).

1.2. Protein dynamics and role of water in Biology.

It is now admitted that a protein is a flexible entity and that this flexibility is required for protein to function. At physiological temperatures internal motions in proteins are partly vibrational and partly diffusive. The description of internal diffusion in proteins is complicated by the variety of existing motions. These involve groups of atoms undergoing a plethora of continuous or jump-like diffusion. Because of the high incoherent scattering cross section of hydrogen (80 barn) with respect to that of other atoms in proteins (C, O, N) inelastic neutron scattering is the most direct probe of diffusive dynamics on the 10^{-12} - 10^{-9} s scale (time of flight, spin echo techniques). Our strategy is to combine the neutron results with that of light scattering and NMR and to compare them with that of Molecular Dynamics (MD) simulations. This strategy offers a unique opportunity to validate potential of MD simulations and get a detailed knowledge of protein dynamics. Dynamics of water that plays an essential role in Biology becomes also accessible on a detailed way.

Analysis of MD simulations is made using some theoretical results obtained for polymeric systems, from which geometry of motions and distribution of relaxation times of various parts of the protein are obtained. Mode Coupling Theory (MCT) that is well appropriate to describe supercooled liquids allowed us to provide with a model for confined or interfacial water.

The knowledge of structural and dynamic properties of bulk water as well as that of confined water is of primary concern. The hydrophilic and hydrophobic interactions have also been studied.

2. DYNAMICS OF WATER

2.1. Hydrogen-bond dynamics model

The problem of hydrogen bond lifetime in liquid water has been analysed in a new way. Even if several incoherent scattering experiments have shown in the past that it is possible to identify a breaking mechanism for hydrogen bond through librational motions, other analysis of the scattered intensity remains possible. The new experiment is based on the decomposition of the structure factor, $S(Q)$, of liquid heavy water in its components. Actually, $S(Q)$ is a weighted sum of three partials corresponding to the pairs OO, OD and DD. Selecting the values of the exchanged momentum Q , it is possible to analyse in a selective way the contribution of each pair. The experiment consists to measure the quasielastic scattered intensity at two different values of Q . For a value at which most of the intensity is due to the OO pairs, it is the dynamics of the whole molecule that is dominant and one finds the classical broadening due to diffusive motions. Instead, at a value of Q at which the intensity is dominated by the pairs DD, one finds the characteristic short time that corresponds to hydrogen bond breaking. This time is very short (1 ps) and depends weakly on temperature, in contrast with the strong and anomalous temperature dependence of transport properties. Such

a separation of the two times with an experimental technique emphasises the importance of hydrogen bond dynamics in liquid water¹.

2.2. Structural relaxation of water supercooled in a porous glass

We have carried out neutron-spin echo measurements of the intermediate scattering function $S(Q,t)$ of supercooled D_2O contained in a porous Vycor glass on the new spin echo spectrometer (MUSES). The results establish clearly the existence of an α -relaxation with the relaxational parameters, consistent with that of Computer Molecular Dynamics data of SPC/E model water. These observations suggest that the dynamics of supercooled water can be described in the general frame of the MCT scheme of glass forming liquids².

Figure 1 displays the structure factor $S(Q)$ of D_2O and gives the values of the three fitted parameters: the Debye-Waller factor $A(Q)$, the stretch exponent β and the relaxation time τ , at 258 K.

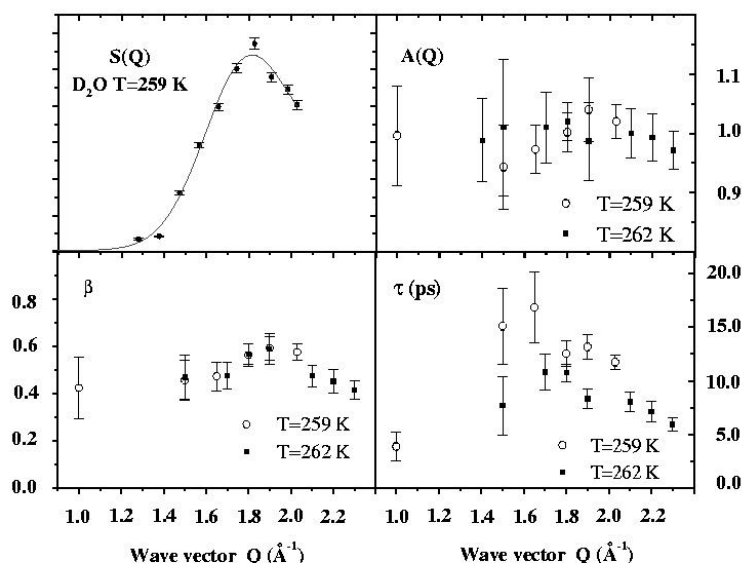


Figure 1. The values of the three fitted parameters, $A(Q)$, $\beta(Q)$, and $\tau(Q)$ according to the α -relaxation model. Also shown (upper left panel) is the experimental structure factor obtained from the elastic scan of the sample (with the signal due to the Vycor subtracted) at 259 K using the neutron spin-echo spectrometer MUSES. The peak position is in agreement with an independent neutron diffraction measurement performed on the 7C2 spectrometer of LLB.

2.3 Aqueous solutions

The effect of hydrophilic and hydrophobic solutes on dynamics of water has been studied in case of water/DMSO³ (Stage of J. Tavarès-Cabral, 1999), water/trehalose (Thesis of I. Koper), water/pyridine (Thesis of L. Almasy) solutions.

There is evidence of some slowing down of the translational diffusive motion of water. At the opposite, the effect on hydrogen bonding is not significant.

3. CONFORMATIONS OF PROTEINS IN SOLUTION

The effect of secondary structure on protein folding has been studied. Different proteins, such as yeast phosphoglycerate kinase (PGK), β -casein (Thesis of A. Aschi) and apo-neocarzinostatin (NCS) (Thesis of D. Russo, 2000) were completely unfolded by guanidinium chloride (GdmCl). Small angle neutron scattering (SANS) spectra from these denatured proteins were recorded at wave number transfers, Q , ranging from 0.006 to 0.4 \AA^{-1} . Among the previous proteins, NCS is the only one that can be denatured by heat without aggregating. Scattering from thermally unfolded NCS was also measured (Figure 2).

¹ J. Teixeira, to be submitted

² M.C. Bellissent-Funel, S. Longeville, J. M. Zanotti and S. H. Chen, Phys. Rev. Lett., 85, (2000) 3644

³ J.T. Cabral, A. Luzar, J. Teixeira and M.-C. Bellissent-Funel, J. Chem. Phys., 113, (2000) 8736-8745

The randomness of the final protein states depends on the denaturing conditions. In very concentrated GdmCl solutions, excluded volume interactions are generally present. This means that all the amino acids are fully solvated and repel each other at short distances. As a result, the configurational space available to the polypeptide chain is restricted. On the contrary, at 78°C NCS behaves as an ideal chain (Figure 2). Such a behavior, which can also be observed at moderate GdmCl concentrations, is prerequisite to folding. These properties of unfolded proteins can be directly inferred from the variation of the forward scattered intensity as function of protein concentration⁴.

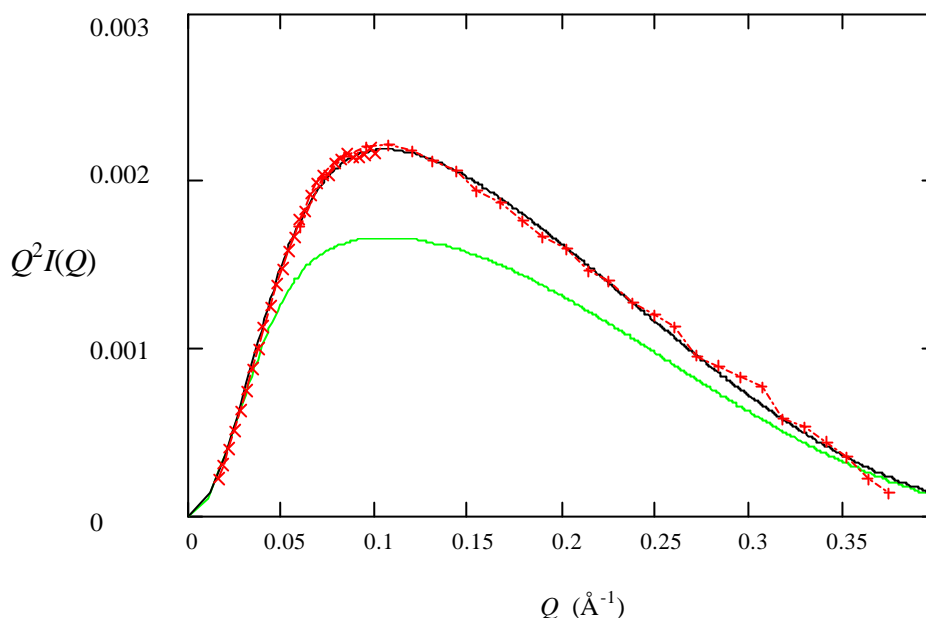


Figure 2. Kratky plot of the neutron intensity, $I(Q)$, scattered from apo-neocarzinostatin unfolded at 78°C. Q is the wave number transfer. Crosses: Experimental data. Lines: Theoretical model of Pedersen and Schurtenberger for an ideal chain (black) and an excluded volume one (green). This clearly shows that neocarzinostatin behaves as an ideal chain at 78°C.

Using Pedersen's description of semi-flexible polymers chains⁵, important structural parameters can be inferred from the scattering profiles. Values of the radius of gyration, of the fractal dimension, of the statistical length, and of the apparent radius of the chain cross-section were obtained in this way for the three proteins in various denaturing conditions. As far as the statistical length is concerned, its value depends on the protein and varies between 12 Å for NCS at 78°C and 32 Å for β -casein at room temperature. These values are the first correct experimental determinations of the statistical length of proteins. All the results previously published by other authors were actually based on incorrect theoretical predictions.

4. STRUCTURE-DYNAMICS-FUNCTION RELATIONSHIP

Our investigations have been performed on hydrated powders in order to limit the contribution of bulk water as well as on protein solutions. The protein is isolated from bacteria (cyanobacteria, purple bacteria), blood cells, pike muscles. When the protein is a commercial product, a special care is devoted to its purification (myoglobin). The studied proteins are representative of the following biological functions:

- Photosynthesis: soluble proteins (C-phycocyanin) and membrane proteins (reaction centre RC, light harvesting protein LH2 and complex RC-LH1).
- Calcium/magnesium regulation: parvalbumin
- Oxygen-carrying : hemoproteins (myoglobin, hemoglobin)
- Enzymatic catalysis : aspartate transcarbamylase (ATCase),
- Antibiotic function : neocarzinostatin (NCS)

⁴ V. Receveur, D. Durand, M. Desmadril and P. Calmettes, F.E.B.S. Lett. 426 (1998) 57.

⁵ J. S. Pedersen and P. Schurtenberger, Macromolecules 29 (1996) 7602.

4.1. Influence of hydration on the internal dynamics of globular proteins

Powders at different hydration levels, as well as solutions, were investigated by incoherent quasielastic neutron scattering. In particular, the quasielastic component of the spectrum reveals dynamical aspects related to diffusive motions that might be functionally important by participating to the general flexibility of the protein. From these components, one has inferred that diffusive motions of protein protons occur within a confined volume and that about 25% of the protons in the protein are involved in short-time (MIBEMOL, 10 ps time range) diffusive motions. These protons belong to the surface residues of the protein. For a hydrated C-phycocyanin protein ($h = 0.5$, h in gram of water per gram of protein), the volume of diffusion is close to that of a sphere of radius 2 Å (Thesis of S. Dellerue, 2000). The same findings are obtained for hydrated powders of parvalbumin⁶, and solutions of ATCase (see “highlight”, Hervé et al) and hemoglobin, (postdoctoral work of C. Loupiac), at the same 10 ps time scale. At higher resolution (IN13, 100 ps time range), backbone motions are observed. Analysis of MD simulation leads to similar results.

Nanosecond MD simulation and analytical theory have been used to model diffusive motions in hydrated C-phycocyanin ($h = 0.5$). The simulation-derived scattering function is in good agreement with experiment and is decomposed to find the essential contributions. It is found that the geometry of the atomic motions can be modelled as diffusion in spheres with a distribution of radii that is different for backbone (average radius = 1.1 Å) and side chains (average radius = 2.0 Å). The time dependence follows a stretched exponential behaviour, reflecting a distribution of relaxation times. With this description, the average side-chain and backbone dynamics are quantified and compared. The dynamical parameters are also shown to present a smooth variation with distance from the core of the protein. This is reflected in a progressive increase of the mean sphere size of diffusion and in the narrowing and shift to shorter times of the relaxation times distribution. This smooth, ‘depth-dependent’ dynamics may have important consequences for protein function. It may allow local reorganisation of the structure for efficient ligand binding without affecting the internal stability.

(Thesis of S. Dellerue, 2000).

4.2. Influence of ligand binding on the internal dynamics of globular proteins

(Postdoctoral work of C. Loupiac)

Previous studies of Benson et al⁷ using H-D or H-T exchange led to observe some modification of the kinetics exchange after ligation of oxygen to hemoglobin. On the basis of these previous observations, dynamics of human hemoglobin without O₂ (Hbdeox) and with O₂ ligand (HbO₂) in solution has been studied by quasi-elastic and inelastic neutron scattering. The results of experiments carried out, on spectrometer MIBEMOL (energy resolution of .96 µeV) show very small changes between the EISF's of Hbdeox and HbO₂. There is no variation of the proportion of mobile protons (close to 20 %) meaning that, at the picosecond time scale, the dynamics of Hb is not affected by O₂ ligand binding. The evolution of vibrational density of states as a function of temperature has been studied. In collaboration with D. Perahia (Orsay), some MD simulations are in development.

4.3. Influence of trehalose on the internal dynamics of globular proteins

(Thesis of I. Koper)

Among protecting agents, trehalose has the highest effectiveness to stabilise and protect biomaterials against denaturation caused by external stresses. Neither the protecting mechanisms of trehalose nor its highest effectiveness are clear. A strategy to elucidate this mechanism has been to study the interactions between trehalose and a protein, the C-phycocyanin (CPC), by quasi-elastic neutron scattering. Figure 3 compares the intermediate scattering function of hydrated CPC powder⁸ with that of a hydrated powder when deuterated trehalose is added to water. In the latter case, one observes a slowing down in the dynamics of the protein by one to two orders of magnitude. Addition of trehalose to the protein affects only slightly the geometry of movements, thus leaving no evidence for direct interactions between sugar and protein.

⁶ J.-M. Zanotti, M.-C. Bellissent-Funel and J. Parello, *Biophys. J.*, 76 (1999) 2390

⁷ Benson et al, *Biochem.*, 12 (1973) 2699

⁸ S. Dellerue, A. Petrescu, J.C. Smith, S. Longeville and M.-C. Bellissent-Funel, *Physica B*, 276-278 (2000) 514-515

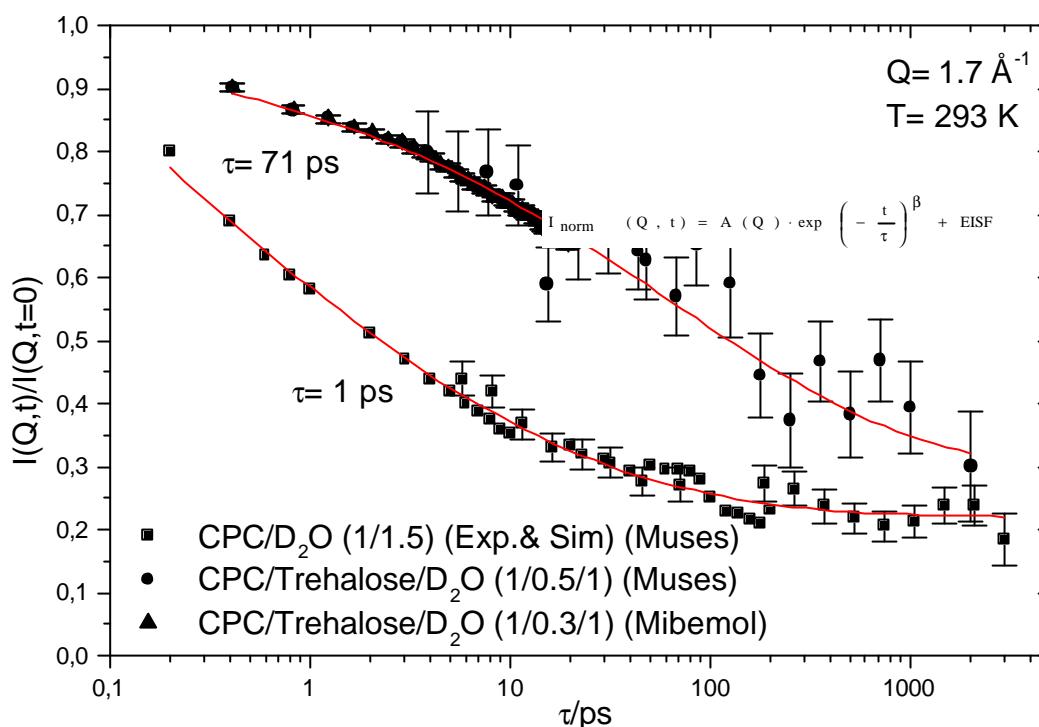


Figure 3. Intermediate scattering function combining different instruments and techniques. Results obtained at a scattering vector of 1.7 \AA^{-1} and at 293K for a sample containing hydrated powers of CPC. 1.) A sample of CPC protein, hydrated to 1.5 g D_2O per g protein, combined with results obtained by molecular dynamics simulation; 2.) A sample containing hydrated CPC/trehalose mixtures. Combination of results obtained on the time-of-flight spectrometer MIBEMOL and the neutron spin-echo spectrometer MUSES. Red lines are results of a fit of the combined curves using a stretched exponential function, taking into account an elastic incoherent structure factor (EISF) to describe the plateau value at longer times.

4.4. Influence of concentration. Oxygen transport in crowded protein solutions

(Postdoctoral work of S. Longeville, LLB, W. Doster, T.U. Munich)

When increasing the concentration of macromolecules in solutions, self-diffusion becomes hindered by interactions with neighbours. Above a certain concentration, molecules are trapped in transient cages formed by neighbours. In biological systems such as cells, biological reactions become limited by protein diffusion. Does myoglobin facilitate oxygen transport in muscle cells by macromolecular diffusion at high concentration? How does the kinetics of oxygen exchange in blood cells depend on hemoglobin mobility? To understand oxygen assisted protein diffusion, a detailed study of myoglobin and hemoglobin diffusion in very concentrated or *crowded* solutions has been undertaken. In this case, neutron spin-echo spectroscopy provided a very fruitful and unique tool to study diffusion of protein solution on length scales corresponding to the centre-of-mass distances. In fact, dynamic light scattering experiments are impeded by strong absorption by the heme and multiple scattering. For the first time, *in vivo* hemoglobin diffusion from blood cells has been studied using the new spin echo spectrometer MUSES. For myoglobin, the diffusion coefficient is reduced by a factor of 15 when going from dilute solutions to 32 mM solutions.

4.5. Internal dynamics of the membrane mediated photosynthetic apparatus from purple photosynthetic bacteria. (Postdoctoral work of A. Gall)

During 1999-2000, the experimental work was divided into four parts: (i) large scale preparation of H/D-exchanged RC-LH1 and LH2 pigment-protein complexes. The exogenous detergent was then replaced by a deuterated lipid (DMPC) environment. Protein integrity was verified by electronic absorption and infrared spectroscopies. (ii) Incoherent quasi-elastic neutron scattering (IQENS) experiments using the above proteins were undertaken. (iii) Structural studies of these same proteins (in detergent or native membrane) by

electronic absorption and Raman spectroscopies under applied hydrostatic pressure were made as a prelude to neutron scattering experiments under similar conditions. (iv) Small-angle neutron scattering measurements on the LH1 subunit called B820 established that the structure is a dimer of two single membrane-spanning polypeptides with non-covalently attached bacteriochlorin molecules⁹.

Compared to previous IQENS studies using MIBEMOL ($\lambda = 6\text{\AA}$, Resolution $96\text{ }\mu\text{eV}$, $Q_{\text{max}} = 2\text{ }\text{\AA}^{-1}$) of detergent-isolated RC-LH1 in full solution (Thesis of S. Dellerue, 2000), in a lipid environment the internal dynamics is more restrained. Since the RC is encircled by the LH1 protein, it is unclear to which extent each component is influenced by the hydrophobic region of the lipids. This work is continuing using a range of different resolutions such as those obtained at ILL with IN13 (Resolution $8\text{ }\mu\text{eV}$, $Q_{\text{max}} = 5\text{ }\text{\AA}^{-1}$) and IN16 (Resolution $1.2\text{ }\mu\text{eV}$, $Q_{\text{max}} = 2\text{ }\text{\AA}^{-1}$). This allows us to cover the largest temporal and Q-space windows with the aim of better characterising the dynamics of the system.

In collaboration with the University of Tartu, Estonia, electronic absorption and pre-resonance FT-Raman spectra for the RC, LH1 and LH2 proteins using their bacteriochlorins as internal molecular probes show that the proteins are still intact at pressures where globular proteins, such as lysozyme, are generally denatured. It should be noted that Raman spectroscopy is a vibrational technique complementary to inelastic neutron scattering.

It was established that the stability of LH2 is both strain and medium specific, and that under optimised conditions protein denaturation was not observed. In the case of the RC from *Rhodobacter sphaeroides*, two proteins were studied, one where large internal cavity is produced (mutant) and the second (wild type) where no such internal void is present. It was established that there are at least 3 pressure domains where different structure changes occur without denaturing the proteins between atmospheric and 0.8 GPa ¹⁰. Thus, we have established the pressure parameters that will enable us to develop inelastic neutron scattering of membrane proteins, something that has never before been achieved. To this end, this 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. Finally, in collaboration with R.J. Cogdell FRSe (University of Glasgow, Scotland) who elucidated the structure of the LH2 protein and a number of RC mutants, the biochemical aspects of the project have been optimised. More specifically, we have characterised numerous altered LH2 proteins by resonance Raman spectroscopy. These studies were preceded by more general Raman studies, into the structure and conformation of the molecular interactions assumed by the pigment cofactors with the polypeptide scaffold^{11 12}.

5. CONCLUSION

5.1. Perspectives

During the coming years, the fruitful strategy applied to C-phycocyanin protein will be extended to other systems. In order to get a full landscape of the dynamics of biological systems in relation with their function, it is necessary to do experiments at different energy or time resolutions. For this purpose, efforts will be devoted to get samples fully and specifically deuterated. Membrane proteins are good candidates for that, but some soluble proteins are envisaged. Combined with the proposed strategy, the MD simulations will be performed to access a detailed knowledge of the protein dynamics in terms of relaxation times and geometry of motions of various parts of proteins (domains, backbone, side chains,...). Up to now, the effect of temperature on conformational changes and dynamics of protein has been considered. Another interesting parameter is the pressure and its influence on protein folding. It is now well accepted that a protein strongly interacts with its environment. Hydrostatic pressure allows the properties of a solvent to be modified in a simple way and may lead to a better understanding of some of the solvent effects on the protein structure and function. Preliminary SANS measurements on horse myoglobin (Postdoctoral work of C. Loupiac) have shown that no significant structural changes occurs up to 3 kbar . Higher pressures may lead to protein denaturation. The conformation of the pressure denatured states is unknown and is one of the future research

⁹ A. Gall, S. Dellerue, K. Lapouge, B. Robert and M.-C. Bellissent-Funel, Biopolymers, 58 (2001) 231-234

¹⁰ A. Gall, A. Ellervee, M.-C. Bellissent-Funel, B. Robert and A. Freiberg, Biophys. J., 80, (2001) 1487-1497

¹¹ A. Gall, N.J. Fraser, M.-C. Bellissent-Funel, H. Scheer, B. Robert, R.J. Cogdell, FEBS Letters, 449 (1999) 269-272

¹² A. Gall and B. Robert, 1999, Biochemistry, 38, (1999), 5185-5190

ways. For such a study, it is interesting to use horse myoglobin as a model protein to take advantage of the experimental results previously obtained under pressure. In particular, results from infrared spectroscopy show an increase of the strength of hydrogen bond network with a reorganisation of the α -helices for pressures up to 12 kbar¹³. Finally, the studies of dynamics of protein solutions under pressure will be extended to membrane proteins. New pressure cells are being designed to sustain 12 kbar.

5.2. Responsibilities at the interface Physics-Biology and Chemistry-Biology

The expertise of LLB in the field of water is internationally recognised. Two LLB researchers (M.-C. Bellissent-Funel and J. Teixeira) have organised, in August 2000, the Research Gordon Conference on Water, held in Plymouth (USA).

LLB has an active participation at the Physics-Biology and Chemistry-Biology interfaces as demonstrated by its involvement to organising schools, congress, Hercules Practicals, LLB Fan etc.. Examples are the organisation of a -NATO School, entitled 'Hydration Processes in Biology', with the publication of the book in 1999, and of a minicolloquium at the interface Physics-Biology held during the Congress of Physics of Condensed Matter in 2000 in Poitiers. LLB has also participated at the session on Biology during the Table Ronde ESS Evaluation Stratégique (TRESSES) on January 2001 and contributed to the resulting report.

The Department of Life Sciences of CNRS created in 2000 the GDR- 1862 entitled 'Fonction et Dynamique des Macromolécules Biologiques' (Responsibles : M.-C. Bellissent-Funel and J. Parello), for which a congress took place at Saclay on January 2001.

In collaboration with the IBS (Grenoble) and INFM (Italy), LLB has participated to the creation of IN13 CRG, at ILL. Because of its unique characteristics (resolution 8 μeV associated to a wide Q range, $Q_{\text{max}} = 5 \text{ \AA}^{-1}$), this backscattering instrument allowed us to fill a gap between the time of flight (MIBEMOL) and spin echo (MUSES) instruments.

¹³ Le Tilly, Eur. J. Biochem. 205 (1992) 1061