6 - LIFE SCIENCES

The research activities in Life Sciences at the LLB are in full expansion. They focus on two main fields of interest: the conformation of proteins in solution and the relationship between structure, dynamics and function of biological systems.

Protein folding

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. The structure of proteins and their solvent interactions can be modified by temperature, pH or chemicals. The application of hydrostatic pressure to a protein solution also provides a controlled manner to alter these physical properties. Thus, characterisation of the denatured states of proteins is important for a complete understanding of the factors stabilising their folded conformation.

Whereas X-ray crystallography or NMR 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 neutrons or X-rays, is a very powerful tool giving structural information at low and medium resolution. Complementary information from circular dichroïsm, fluorescence and differential scanning calorimetry is used.

Dynamics and function of biological systems:

Biological macromolecules (e.g., proteins, lipids, DNA) share a structural complexity that is also reflected in a complex dynamic behaviour. 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. They involve groups of atoms undergoing a plethora of continuous or jump-like diffusion. Neutron spectroscopy permits the investigation of motions in a very broad time range from 10^{-14} to 10^{-7} seconds (time of flight, backscattering, spin echo techniques). Because of the large incoherent cross section of hydrogen nuclei (about 40 times larger than the cross section of other elements) and the fact that hydrogen atoms are distributed “quasi-homogeneously” in the biological macromolecule, this technique is a powerful tool for the study of all internal motions.

The strategy developed at the LLB is to combine the neutron results with that of light scattering and NMR and to compare them with that of Computer Molecular Dynamics (CMD) 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. CMD simulations are analysed with theoretical laws developed for polymeric systems, from which geometry of motions and distribution of relaxation times of various parts of the protein are obtained.

But first of all, let us present the work done at LLB on the structure and dynamics of biological membranes.

MEMBRANES: LIGNIN FILMS, PHOSPHOLIPID BILAYERS AND MEMBRANE PROTEINS

Neutron reflectivity studies of lignin films from plant cell wall

The cell wall of common plants is made of two interpenetrated polymer networks, lignins (phenolic polymers) and polysaccharides. Neutron reflectivity, combined with H/D contrast variation method, has been very powerful to study the first stage of lignin films on a solid surface. The distribution of the phenolic polymer within the polysaccharidic network has been determined (see “***highlight***”, B. Cathala et al).

Studies of phospholipid membranes

A recent activity concerns the collective dynamics of hydrated phospholipid bilayers of DLPC. The short wavelength density fluctuation of DLPC bilayers close to full hydration has been studied below and above the main transition temperature by inelastic X-ray scattering techniques at the ESRF. The dispersion relation of the high frequency sound mode has been constructed for the first time (Fig. 1). The marked softening of the excitation near k=14 nm^{-1}, corresponding to the lipid chain-chain correlation peak in the structure factor,

in the L_α phase implies prevalent occurrences of short-wavelength in plane motions of lipid chains that might be of importance for transportation of small molecules across membranes.
Collaboration M.-C.Bellissent-Funel, S.H. Chen, MIT

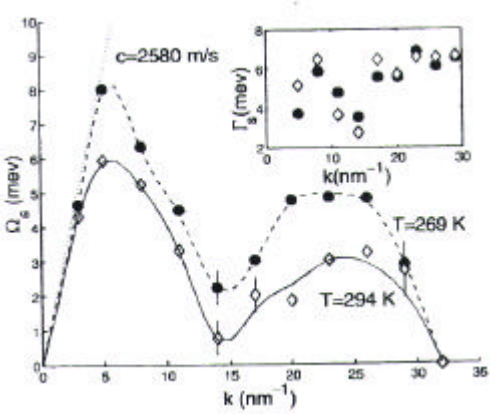

Figure 1 : Sound dispersion and damping (inset) for hydrated DLPC at two different temperatures.

Internal dynamics of the membrane mediated photosynthetic apparatus from purple photosynthetic bacteria.
The experimental work implied: (i) Large-scale preparation of H/D-exchanged RC, RC-LH1 and LH2 pigment-protein complexes. (ii) Quasi-elastic neutron scattering experiments 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. (iv) SANS measurements on LH1 subunit called B820 established that the structure is a dimer of two single membrane-spanning polypeptides with non-covalently attached bacteriochlorin molecules.
Compared to previous studies of detergent-isolated RC-LH1 in full solution (Thesis of S. Dellerue, 2000), in a lipid environment, the internal dynamics is more restrained. In collaboration with the University of Tartu, Estonia, electronic absorption and pre-resonance FT-Raman spectra for the RC, LH1 and LH2 proteins show that the proteins are still intact at pressures where globular proteins, such as lysozyme, are generally denatured. Thus, we have established the pressure parameters that will enable us to develop inelastic neutron scattering of membrane proteins. 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.
Postdoctoral work of A. Gall, Collaboration B. Robert (DBJC), M.-C. Bellissent-Funel

CONFORMATION OF PROTEINS IN SOLUTION

Influence of molecular weight
The refolding of a high molecular weight protein, fibronectin, has been studied for the first time by SANS. The tentatives to refold fibronectin have led to collapsed but still badly refolded conformations. This shows that efficient folding of high molecular weight nascent proteins depends on assistance of molecular chaperones. The study of *in vitro* refolding assisted by such molecular chaperones should be of great interest but has not been considered up to now (see "***highlight***", D. Lairez and J. Pelta).

Influence of high hydrostatic pressure on hydrated myoglobin
Pressures up to 300 MPa have been applied to azidometmyoglobin. The structural change cannot be observed by SANS experiments. The isothermal compressibility has been obtained in a pressure range that is not accessible by commercial densimeter, limited to 100 Mpa (see "***highlight***", C. Loupiac et al).

Influence of secondary structure on denatured states obtained by heat or guanidinium chloride
Different proteins, such as yeast phosphoglycerate kinase (PGK), β-casein and apo-neocarzinostatin (NCS) 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 $Å^{-1}$.

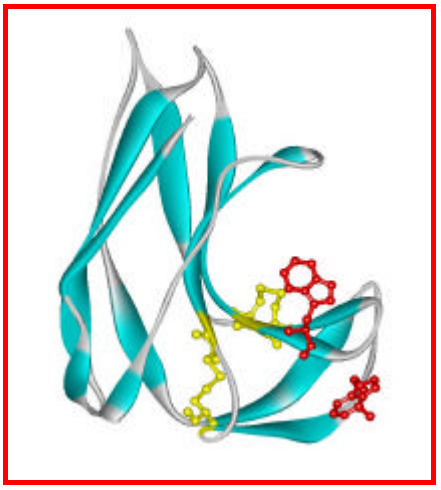

Figure 2. The Neocarzinostatin (NCS) displays a folding pattern typical of a large family, the immunoglobulin fold. NCS belongs to a family of antitumour proteins of bacterial origin that contain a labile chromophore. The protein component, apo-neocarzinostatin is a 113 amino acids protein with two short disulfide bridges, located within different loops. Its structure consists of a seven-stranded antiparallel β-sandwich.

Among the previous proteins, NCS (Fig.2) is the only one that can be denatured by heat without aggregating. Scattering from thermally unfolded NCS was also measured.
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 large 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. Such a behaviour, 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 concentration. Using Pedersen's description of semi-flexible polymers chains important structural parameters can be inferred from the scattering profiles.

STRUCTURE-DYNAMICS-FUNCTION RELATIONSHIP IN BIOLOGICAL SYSTEMS

a) Dynamics of proteins in their native state

The proteins are representative of the following biological functions: Photosynthesis (soluble proteins, C-Phycocyanin, and membrane proteins, RC, LH2 and RC-LH1); Calcium/magnesium regulation (Parvalbumin); Oxygen-carrying (Myoglobin, Haemoglobin); Enzymatic catalysis (Aspartate transcarbamylase, ATCase); Antibiotic function (Neocarzinostatin, NCS)

Influence of concentration on the diffusion process of myoglobin and haemoglobin

In biological systems such as cells, biological reactions become limited by protein diffusion. To understand oxygen assisted protein diffusion, a detailed study of myoglobin and haemoglobin diffusion in very concentrated 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* haemoglobin diffusion from red blood cells has been studied, using the new spin echo spectrometer MUSES. For myoglobin and haemoglobin, the diffusion coefficient is reduced by a factor of 25 when going from dilute solutions to physiological concentrations.
*(see "**highlight**", S. Longeville and W. Doster).*

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 dynamic 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. The same findings are obtained for hydrated powders of Parvalbumin**,** solutions of ATCase and haemoglobin (postdoctoral work of C. Loupiac), at the same 10 ps time scale. At higher resolution (IN13, 100 ps time range), backbone motions are observed (see "**highlight**", J. M. Zanotti et al). Analysis of MD simulation leads to similar results (Thesis of S. Dellerue, 2000) and see also the chapter on modelling.

Influence of trehalose on the internal dynamics of globular proteins

Anhydrobiosis is a well-known phenomenon in nature. Several organisms and plant have been found to be able to survive periods of extreme external stresses such as high or low temperatures or periods of extreme drought. 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 first model, called the "water replacement theory", suggests that trehalose molecules can replace hydration water, and prevents in this way denaturation of the biomolecule. The other model (Angell's theory) focus on the fact that trehalose has an especially elevated glass transition temperature and may form a glassy structure in which the biomaterial gets embedded. This would slow down or even suspend all dynamical processes that could lead to degradation.

We studied the dynamics of a C-phycocyanin protein (CPC) by neutron scattering techniques and investigated the influence of the presence of trehalose molecules on the protein dynamics, on a time-scale from some pico- to several nanoseconds. The C-phycocyanin protein, commercially available in protonated and deuterated forms, is particularly suited for these studies (Thesis of I. Köper, 2002).

The intermediate scattering function of hydrated CPC powder has been compared with that of a hydrated powder when deuterated trehalose is added to water. In the latter case, one observes a slowing down of 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 giving no evidence for direct interactions between the sugar and the protein [3], in agreement with Angell's theory.

(Collaboration I. Köper, M.-C. Bellissent-Funel, W. Pétry (Munich)

b) ***Dynamic transition associated with thermal denaturation of neocarzinostatin (NCS)***

A complete understanding of protein folding requires the physical characterization of both native and denaturated state and evaluation of the thermodynamic parameters of the system. This involves obtaining information concerning the structure and dynamics of proteins denatured under various conditions.

We have studied the picosecond dynamics of NCS in solution during heat-induced denaturation, by quasi-elastic neutron scattering (MIBEMOL, resolution 96 μeV). Figure 3 shows the Elastic Incoherent Structure Factor (EISF) obtained for each temperature. The EISF decreases at high Q as the temperature increases, which suggests that the fraction of immobile hydrogen atoms p decreases as temperature increases.

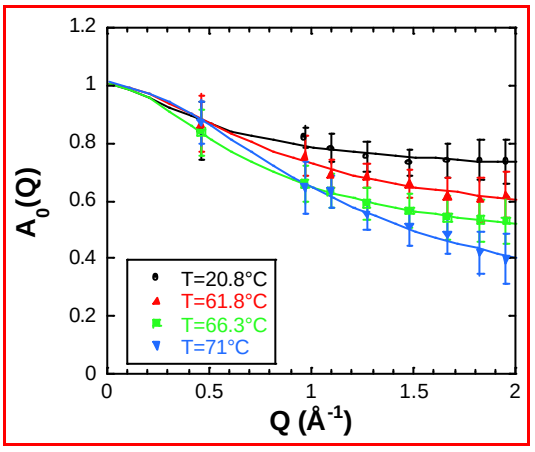

Figure 3. Elastic Incoherent Structure Factor (EISF) of NCS at all investigated temperatures. The lines are the fit, considering a fraction (1-p) of particles diffusing inside spherical confinement volumes with Gaussian distributions.

We found that *p* decreased by 30% between 20.8 and 61.9 °C, whereas the protein retained its native conformation, and then underwent a more abrupt decrease when the protein starts to unfold. The latter decrease in *p* to the small value of 0.09 at 71°C shows that almost all the protons in the protein were mobile at the half transition temperature.

The internal dynamics of the native fold at 21°C is consistent with diffusive motions arising from the side chains of the polypeptide loops external to the protein core. These side chains are free to move and to explore a large space. If the temperature increases to 61.8°C, just below the heat denaturation transition, the backbone of NCS become more flexible and the β-sandwich residues less constrained. Evidence for this change is provided in particular by the increasing number of protons with detectable diffusive motions. If the

temperature is the increased to the half-transition temperature, almost all the protons in the protein acquire the ability to diffuse locally. These results show an important aspect of the relationship between structure and dynamics in protein folding (Thesis of D. Russo, 2000).

c) Role of hydration water

The dynamics of hydration water has been studied in assemblies of identical small peptides. A chain of five alanins has been hydrated, at various levels of hydration, providing with 1 to 25 water molecules. The neutron quasi-elastic experiments allowed us to accessing the dynamics of water molecules that is limited to rotational motions acting to breaking hydrogen bonds. However, the H-bond lifetime remains 3 to 4 times longer than that between molecules in bulk water. The diffusion becomes less confined when the level of hydration increases. This study must be extended to chains containing about twelve monomers and giving rise to two turns of alpha helix.

Collaboration D. Russo (postdoctorant, Berkeley), P. Baglioni (Florence), J. Teixeira.

AN OPENING TO MEDECINE AND MEDICAL APPLICATIONS

Cancer Borotherapy:

First experiments have been carried out in order to elucidate the mechanisms at the molecular level of the radiolysis of ADN during the Boron Neutron Capture Therapy, used for the reduction of cancerous tumours. The results are very encouraging and promising (see "***highlight***", M. Charlier and E. Sèche).

Enzymatic degradation of gelatine gels as a model of tumour dissemination mechanism:

In vivo, proteins are often organized in a gel state, i.e. an elastic solid of macromolecules swollen by a large amount of solvent. On one side, gelation is involved in many biological processes such as blood coagulation or wound healing. Conversely, the transition from a solid gel to a liquid is also important mainly for the extracellular matrix (ECM) behaviour that constitutes a physical barrier isolating organs and regulates cell behaviour.

In tumour dissemination, invasive cells must solubilise the ECM and express for that up to 15 different proteolytic enzymes, especially metalloproteinases (MMP). These proteinases differ by their specificities and reaction mechanisms; moreover, the ECM composition and organisation are tissue-dependent. In spite of these peculiarities, in all cases cell invasion implies a similar process that requires the enzyme-catalysed degradation of the ECM. Beyond the complex biochemical processes involved at a molecular level, the understanding of the degradation mechanisms is crucial to inhibit or slow down the cell invasion.

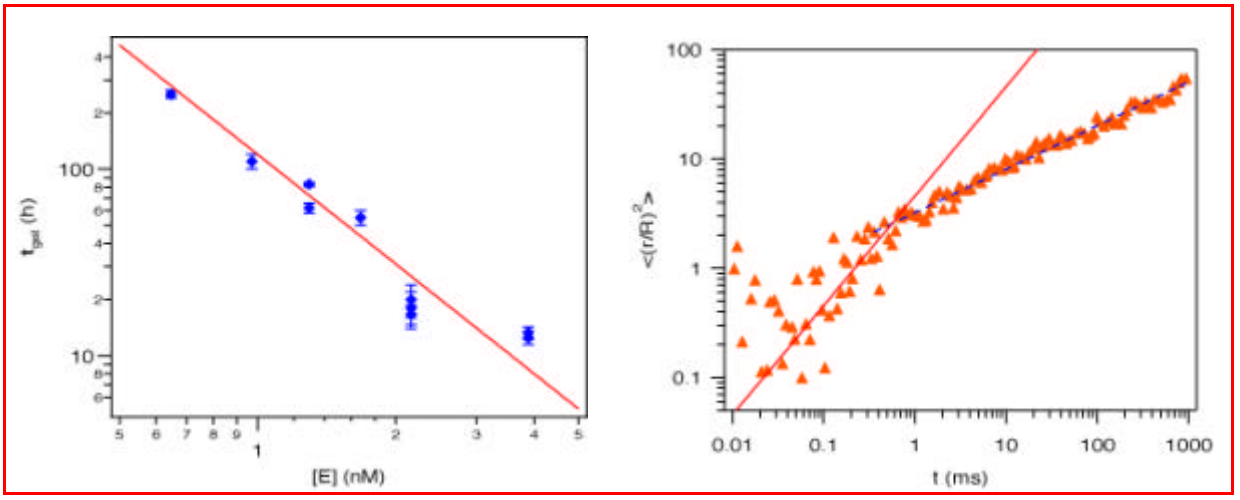

Figure 4. Variation of the gel proteolysis time, t_{gel}, on enzyme concentration, [E]. The straight line has a slope in Log-Log equals to -1.95±0.15.

Figure 5. Reduced mean square displacement of thermolysin in the gel deduced from FCS measurement. The full line accounts for the diffusion of thermolysin in water. The dotted line corresponds to the power law $<(r/L)^2>=(3.2\pm0.1)\ t^{0.40\pm0.01}$ with L the apparatus characteristic length (˜ 400 nm)

Thermolysin catalysed degradation of gelatine gels has been considered as a model system to study the ECM solubilisation. Gelatine is a denaturated collagen, and thermolysin is a Zn-metalloproteinase from Bacillus thermoproteolyticus, which is an analogue of MMP and displays the same basic mechanism.
A gel degradation rate varying as the square of the enzyme concentration has been observed (Fig. 4), whereas an ordinary Michaelis-Menten mechanism would lead to a linear behaviour. The thermally induced motion of enzymes in the gel has been measured by two-photon fluorescence correlation spectroscopy (FCS) and identified as being anomalously slow (Fig. 5). These experimental results have been interpreted from a theoretical point of view in terms of an anomalous diffusion-controlled mechanism for the gel degradation that should be inherent to the enzyme activity (Thesis G. Fadda, 2002).

IN13 CRG at the ILL and GDR 'Fonction et Dynamique des Macromolécules Biologiques'

In collaboration with IBS (Grenoble) and INFM (Italy), the LLB has participated to the creation of the IN13 CRG, at ILL. Because of its unique characteristics (resolution 8 μeV, Q_{max} = 5 $Å^{-1}$) this backscattering instrument allowed us to fill a gap between time of flight and spin echo instruments. However, a limiting factor of this instrument is the low flux, especially when one likes to do energy analysis of the spectra.
The Department of Life Sciences of CNRS has renewed on January 2003 (for four years) the GDR-1862 entitled 'Fonction et Dynamique des Macromolécules Biologiques' (Director: M.-C. Bellissent-Funel, Co-Director: J. Parello). In the frame of the GDR successful activities have been undertaken: opening workshop of the GDR, (February 2001, Saclay), workshop *"Catalyse Enzymatique, Dynamique Moléculaire et Réactivité"* (January 2002, ICSN) and thematic school *"RMN-Neutrons"* (November 2002, Saint-Rémy-lès-Chevreuse).
Recently, it has been decided to create the "DYNBIO" group, under the impulse of M.-C. Bellissent-Funel and G. Zaccai, the purpose of which is to inform biologists about the potential of neutrons to accessing dynamics (but also conformation) of biological systems. In the post genomic area, one is aware that proteomics will be central to the functional genomics efforts. In the field of proteomics, neutrons can be decisive to solving conformations of big biological assemblies.

B. PERSPECTIVES

During the coming years, the fruitful strategy applied to C-phycocyanin 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 also envisaged. The development of studies of conformation and internal motions of membrane proteins such as aquaporins, is foreseen, thanks to the new biologist researcher at LLB, S. Combet-Jancenel.
Combined with the proposed strategy, 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, etc). This will be done in close collaboration with G. Kneller and K. Hinsen (Orléans).
Finally, studies of conformations of big biomolecular assemblies (TAT membrane proteins) for which the crystallographic structure is not known are of interest and planed in collaboration with T. Granjon and B.C. Berks (Oxford).