INFLUENCE OF TEMPERATURE AND HYDRATION ON PROTEIN DYNAMICS

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ABSTRACT

Protein dynamics play a crucial role in protein function, since a protein needs to be protean in its conformations to fulfill its role as a biological machine (e.g. to act as an enzyme). A dynamic transition is believed to be closely related to the onset of protein activity that becomes measurable at temperatures above the dynamic transition temperature, $T_D$. Hydration and temperature are two important parameters for both the dynamic transition and protein activity. However, the correlation between protein dynamics and protein function has not been clearly established and the microscopic mechanism of the dynamics activated above $T_D$ is still a subject of discussion.

In our research, we used neutron and light scattering measurements to study the temperature and hydration dependence of protein (lysozyme) dynamics in the picosecond and nanosecond time window. We identified three main dynamic processes in protein molecules: i) methyl group rotation, ii) a fast process, and iii) a slow relaxation process. We demonstrated that the methyl group rotation is activated at $T \sim 100$ K regardless of hydration. Only wet proteins at hydration levels higher than 0.2 $h$ ($h = g$ of water per 1 g protein) exhibit the dynamic transition and the slow relaxation process, whereas the fast process is present even in proteins at hydration levels lower than 0.2 $h$. We showed that the slow relaxation process is responsible for the dynamic transition and is closely related to enzymatic activity. The temperature dependence of the slow relaxation process
exhibits an Arrhenius-like behavior at $T > T_D$. This result suggests that the dynamic transition is just the result of the slow relaxation process entering the experimentally accessible time window. An analysis of the influence of solvents on protein dynamics suggests that glycerol suppresses the fast process of protein more strongly than do other solvents (e.g. water and trehalose) at $T < T_D$. Conversely, trehalose suppresses protein dynamics better than other solvents at higher temperatures.
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CHAPTER I

INTRODUCTION

Proteins are the biological machinery of an organism. They perform their own specific biological processes essential for organic metabolism. For instance, the function of myoglobin (Mb) is to bind O$_2$ from blood and deliver it to mitochondria in the cells. Lysozyme, the model protein in this research, acts as an enzyme that destroys bacterial cell walls.$^1$

The efficiency of protein function depends strongly on the circumstances of the environments, such as solvent, temperature, and pH. Proteins are most biologically active under unique optimum conditions that preserve the landscape of their tertiary structures intact. The loss of their folded structure means loss of their bioactivity.

The structures of proteins are typically studied by time-averaged measurements like X-ray diffraction. They are produced from static results averaged over many possible conformational selections. As a matter of fact, proteins can experience dynamic fluctuations among their different conformational states.$^{2-4}$

Protein motions are also a function of environmental parameters, such as solvent, temperature and pH. All the parts of protein molecules can explore their conformational
energy minima. From this point of view, protein molecules exhibit liquid-like behavior, i.e. they keep fluctuating among different conformational states.

Some specific conformational motions of proteins are necessary for the activation of protein functions, since no permanent path by which protein molecules respond to their substrates has been found. For example, lysozyme is believed to need “hinge-bending” motions to bind bacterial cell walls\(^5\). Therefore, these dynamics of proteins are essential for their functions.

A complete understanding of protein dynamics is crucial to clarifying the mechanisms of bioactivity and the stabilization processes. The motions of the proteins from vibration to the folding process span in a time scale from \(10^{-15}\) seconds to a few seconds (THz to a few Hz) and in length scale from 0.1 to 10 Å\(^6\). Among these, the dynamical processes activated on a pico- to nano-second time scale involve conformational and structural relaxation modes. They are believed to be directly related to protein activity\(^2-4\). However, it is unclear to what extent the motions of proteins describe their functionally important dynamics. A major challenge is to understand the nature of these motions and how they contribute to protein functioning.

Despite significant efforts in experimental and computational biophysics, the details of molecular motions remain poorly understood, even on a qualitative level. Many relaxation processes (stochastic fluctuations between conformational states and sub-states) in biopolymers have been identified using NMR, Mössbauer spectroscopy, neutron scattering, and simulations of molecular dynamics\(^7-12\). However, their roles in protein function are not yet clear and the most important processes that control the bioactivities of proteins have not yet been identified.
We will present the experimental results obtained from neutron and light scattering measurements of dry and hydrated lysozyme at different temperatures, spanning a broad frequency range from 1 THz to 0.1 GHz. Neutron scattering measurement is one technique that is well established to study atomic-level protein dynamics.

Light scattering measurements were used to support the neutron scattering data. Using this broad frequency range allows one to successfully monitor various types of dynamical processes in protein molecules, including the slow relaxation process, the fast process and collective vibrations.

In accordance with academic conventions, this thesis is organized as follows.

In Chapter II, a concise literature review of the underlying background and current status of the relevant research are presented.

In Chapter III, methods of sample preparation used in our research are explained in detail and the fundamentals of neutron and light scattering measurements are presented.

In Chapter IV, unknown low-temperature anharmonicity that contradicts the traditional interpretation of protein dynamics is discussed.

In Chapter V, the hydration dependence of slow and fast relaxation processes is investigated. The relationship of these dynamic processes to the bioactivity of protein is discussed, making a parallel comparison to their hydration dependence.

In Chapter VI, temperature dependencies of the slow relaxation process and of the methyl group dynamics are analyzed. Activation energy of the slow relaxation process is estimated, assuming that it follows an Arrhenius-like behavior.
In Chapter VII the influence of glycerol and trehalose on protein dynamics are discussed and compared with the effects of hydration.
CHAPTER II

HISTORICAL BACKGROUND

2.1 Proteins

Biophysicists studying protein dynamics have chosen particular proteins for their samples, having considered structure, activity, stability and price. Lysozyme and myoglobin have been the most extensively used because they are much easier to obtain than other proteins and have relatively simple structures and specific functions that are easily exploited. In this section, some general aspects of proteins are discussed with a focus on lysozyme, myoglobin and other proteins that have been generally used for studies of protein dynamics.

2.1.1 General overview of proteins

Proteins are composed of the 20 different amino acids represented in Figure 2.1. They are polymerized via a dehydration condensation reaction. In terms of polymer architecture, protein molecules are linear polypeptide chains in the form of random copolymers. However, they are not randomly polymerized, since the sequences of protein molecules are genetically determined by their DNA codes. This well-defined
sequence is the reason why each protein molecule has a single molecular weight. Each specific function of the protein also originates from its sequence, leading to its particular three-dimensional structure.

![Amino acids found in proteins](image)

Figure 2.1 Amino acids found in proteins. Group (i) Non-polar: A, V, L, I, P, M, F, W; Group (ii) Polar: G, S, T, C, N, Q, Y; Group (iii) Ionizable acid: D, E; Group (iv) Ionizable base: K, R, H.

Protein structures can be categorized by their spatial arrangements. The primary structure is the linear amino-acid sequence of the polypeptide chain(s), without regard to spatial arrangement. This definition does not include the positions of disulphide bonds, and is, therefore, not identical with "covalent structure" (IUPAC-IUB, 1970).
The secondary structure of a segment is the local spatial arrangement of its main-chain atoms without regard to the conformation of its side chains or to its relationship with other segments (IUPAC-IUB, 1970). The main interaction driving secondary structures are hydrogen bonds formed by neighboring amino acids. There are three common secondary structures in protein molecules, namely \( \alpha \)-helices, \( \beta \)-sheets and turns.

The tertiary structure of a protein molecule is the arrangement of all its atoms in space, without regard to its relationship with neighboring molecules or subunits (IUPAC-IUB, 1970). The tertiary structure is called a “Fold” when it is perfectly shaped in accordance with nature’s demand without any errors. As an example of tertiary structure, lysozyme is shown in Figure 2.2, obtained from the X-ray diffraction measurement of a crystal state. The \( \alpha \)-helixes are represented by cylinders and \( \beta \)-sheets by arrows. As a consequence of the folding-mechanism of a protein molecule, most of the non-polar side chains are placed in the interior of the structure.

Figure 2.2 Diagrams of the crystal structure of native hen lysozyme. \( \alpha \)-helices and \( \beta \)-sheets are represented as cylinders and thick arrows, respectively.\textsuperscript{14}
The time-averaged picture from X-ray diffraction measurements reflects the most probable locations of the atoms of a protein molecule in space. A permanent conformation for a protein molecule does not exist because all parts of the protein molecules change their positions in three-dimensional space on a pico- to nano-second time scale. Therefore, a protein does not reflect a rigid but rather a liquid-like system; i.e. the conformation of a protein molecule keeps changing under appropriate environmental conditions. The most important fact is that proteins can function biologically only under the right three-dimensional folded tertiary structure.

The quaternary structure of a protein molecule is the arrangement of its subunits in space and the assembly of its inter-subunit contacts and interactions, without regard to the internal geometry of the subunits (IUPAC-IUB, 1970). The subunits in a quaternary structure must be in non-covalent association. Hemoglobin (Hb) contains four polypeptide chains held together non-covalently in a specific conformation as required for its specific functioning.

Structurally well-folded proteins can undergo biological tasks. Some proteins transport small chemical molecules like O₂, H⁺, and CO₂ which are important for maintaining the metabolism of living organisms. Other proteins catalyze biochemical reactions, like cleaving chemical bonds in bacterial cell walls. All biological processes experience complex multiple steps.

Lysozyme and myoglobin are among the proteins that have been most widely used for structure, dynamics, and bioactivity studies. The following sections describe their basic structural and functional properties in detail.
2.1.2 Lysozyme

Lysozyme is an enzyme that destroys a cell wall component of most fungi, as well as the major component of the exoskeletons of insects and crustaceans formed from polycarbohydrate chains. The bacteria burst out by the pressure difference between the interior and exterior of the cell. This phenomenon is accomplished by hydrolyzing the $\beta(1\rightarrow4)$ glycosidic linkages from N-acetylmuramic acid (NAM or MurNac) to N-acetylglucosamine (NAG or GlcNAc) in the bacterial cell peptidoglycans (Figure 2.3).

![Figure 2.3 $\beta(1\rightarrow4)$ glycosidic linkages from NAG to NAM.](image)

Lysozyme exists widely in the cells and secretions of vertebrates, where it may function as a bacterial agent or help dispose of bacteria after they have been destroyed by other means. Lysozyme catalyzes the hydrolysis of its substrate at a rate that is $10^8$ fold faster than it would have been without the catalyzed reaction.

Hen-egg white lysozyme is the most widely studied species of lysozyme and the microscopic mechanism of its function is probably the best understood among many proteins. Its molecular weight is about 14.6 kD. The single polypeptide chain consists of 129 amino-acid residues and four internal cross-linkages by four disulfide bonds.
The X-ray structure of hen-egg white lysozyme was obtained by David Phillips in 1965, showing that the lysozyme molecule is ellipsoidal with dimensions $45 \times 30 \times 30$ Å. Most of the non-polar side chains in the protein molecule are placed in the inner part of its structure. Its secondary structure is comprised of five $\alpha$-helices and two $\beta$-sheets. The most interesting feature of its structure is a prominent cleft known as an active site along one face of the lysozyme molecule. We will discuss the catalytic mechanism of lysozyme in more detail in section 2.3.2.

2.1.3. Myoglobin

Myoglobin is one of the most widely studied proteins, its structure having been the first determined by X-ray crystallography carried out by John Kendrew in 1959 (Figure 2.4)\(^1\). The major role of myoglobin is to facilitate oxygen transport in muscle. Myoglobin is a single polypeptide chain with one heme group, an oxygen-binding site. It is a useful model for other ligand-binding proteins.

Myoglobin is an intracellular protein as small as lysozyme, composed of 153 amino-acid residues. Eight $\alpha$-helices are arranged to form its globular shape with approximate dimensions of $44 \times 44 \times 25$ Å. A single heme group with a catalytically active iron atom at its center is tightly wedged in a hydrophobic pocket between two $\alpha$-helices.
2.1.4. Miscellaneous

A variety of proteins other than lysozyme and myoglobin has been regarded as model proteins for biophysical/biochemical studies. A brief description of some important characteristics of the proteins, including hemoglobin, cytochrome \( c \), bacteriorhodopsin, xylanase, ribonuclease A (RNase A) will be made in this section ¹.

Hemoglobin is an intracellular protein that provides the proper amount of oxygen to the tissues. It is a tetrameric protein that has four subunits structurally and evolutionarily related to each other and to myoglobin. Each subunit contains a single heme group.

Cytochrome \( c \) found in horse heart is a small heme protein loosely associated with the inner membrane of the mitochondrion. It is an essential component of the electron transfer chain.

Figure 2.4 Three-dimensional structure of sperm whale myoglobin obtained from X-ray crystallography ¹⁶.
transfer chain. It is capable of undergoing oxidation and reduction, but does not bind oxygen.

Bacteriorhodopsin is a kind of membrane protein containing multiple transmembrane \(\alpha\)-helices. It consists of 247 amino acid residues. Its biological function is to pump light-driven protons into the membrane.

Xylanase is an example of the digestive enzymes of herbivorous micro-organisms that degrade \(\beta(1\rightarrow4)\) linked polysaccharides into xylose. It breaks down hemicellulose, which is a major component of plant cell walls. Its presence in fungi makes possible the degradation of plant walls into usable nutrients.

Bovine pancreatic RNase A is also commonly used in biological research. However, it is one of the hardest enzymes to obtain with laboratory resources due to the difficulty in isolating it. It is a digestive enzyme that hydrolyzes RNA into its component nucleotides. X-ray studies show that four \(\beta\)-sheets are arranged in its structure.

2.2. Protein dynamics and protein functions

Structural pictures of proteins may lead to the false impression that they are fixed and rigid, while in reality proteins are rapidly fluctuating and very flexible. The mobility of proteins is functionally important, since the structures of proteins obtained by X-ray diffraction measurements makes clear that there is no permanent path or channel available for their substrates. The functional power of proteins is proportional to their ability to take their substrates into an active site in favorable orientations. This
observation strongly supports that proteins need to explore molecular-level dynamics to fulfill their roles as biological machinery.

2.2.1 Induced fit model

More than a century ago in 1890, when the concept of protein dynamics was not common among biophysicists, Fischer first suggested a lock-and-key model according to which a substrate fits into a perfectly shaped active site permanently present in a protein. However, it turned out that this model was not adequate for a number of proteins. Instead, in 1958, Koshland proposed an induced-fit mechanism of substrate binding. This model explains that a substrate-binding protein is accompanied by a conformational change that leads to the correct orientation of active groups in the protein and the substrate. The binding process of the substrate has been reasonably well represented in many proteins by the induced-fit mechanism.

As a result of X-ray diffraction measurements it has been concluded that proteins exist in different conformations depending on the presence and absence of substrates. This is hard evidence supporting the assertion that protein dynamics is essential for protein functioning. However, the relationship between protein dynamics and function has not been clearly understood. In addition, the extent to which protein dynamics is directly correlated with protein function needs to be estimated accurately.
2.3 Biological activities of proteins affected by hydration and temperature

The bioactivities of proteins change depending on hydration and temperature. Most proteins need a critical amount of hydration to undergo their biological functions \(^1\), even though some exceptions have been reported \(^7,19\). Generally, the bioactivities of proteins increase with increasing temperature in accordance with Arrhenius behavior. This section mainly describes the hydration and temperature dependence of biological activities of proteins, and main attention will be paid to lysozyme.

2.3.1 Hydration process of protein lysozyme

Hydration of protein molecules is usually achieved by exposing proteins to humid air. Hydration process of proteins is the step-like addition of water to dry proteins, until the hydration equilibrium is completely reached. A hydration level can be controlled, changing the relative humidity, i.e. isopiestic hydration. Different relative humidity is obtained in several ways, such as use of different acid concentration or different concentrated salt solution. Figure 2.5 shows how hydration levels of lysozyme depend on the H\(_2\)O and D\(_2\)O pressure.
Figure 2.5 Isothermal absorption of H$_2$O and D$_2$O on lysozyme at 300 K $^{20}$.

Time-average physical properties of hydrated proteins obtained from thermodynamic and IR measurements provide details of hydration mechanism on atomic level. They help better understand which parts of a protein molecule are influenced stronger or weaker by hydration. Figure 2.6 exhibits variation of heat capacity and spectroscopic properties of lysozyme with the hydration level.
Figure 2.6  

(a): carboxylate absorbance (1580 cm\(^{-1}\));  
(b): amide I shift (1660 cm\(^{-1}\));  
(c): OD stretching frequency (2570 cm\(^{-1}\));  
(d): apparent specific heat capacity;  
(e): diamagnetic susceptibility\(^{11,21}\).

Curve (d) presents hydration dependence of apparent heat capacity measurements of lysozyme. The result provides the extent to which thermal response of the lysozyme-water interface deviates from its ideal behavior. Step-like development in three regions was observed as non-ideality of the system.

Below 0.07 \(h\) (\(h = \text{g of water/1 g of dry protein}\)), water is absorbed to local charged groups in lysozyme. Hydration process is initialized even by the low water activities since water vapor can condense on soluble elements such as ionizable residues present on the protein surface. The rise and fall of heat capacity between 0 and 0.07 \(h\) is related to the transfer of protons from carboxylic acid to basic protein groups.
The discontinuity of the heat capacity at \( h \sim 0.07 \) is a result of a two-dimensional condensation process of formation of mobile water clusters from dispersed water with the charged sites in protein surface. The transition of hydration at \( h \sim 0.07 \) is also observed in IR measurements: carboxylate absorbance (curve \( a \)), amide I shift (curve \( b \)), and OD stretching frequency (curve \( c \)). The mobile water clusters grow at hydration levels between 0.07 and 0.25 \( h \). Based on the fact that there is no transition in heat capacity, no qualitative change in the chemistry of the interface is expected.

Looking at another transition of the apparent heat capacity, it is believed that at hydration \( \sim 0.25 \ h \), water molecules completes covering all polar groups and starts condensing on the weakest interacting regions of the surface. The IR measurements also exhibit the saturation of hydrogen bonds at interface before hydration level \( \sim 0.25 \ h \). This result supports that network of hydrogen bonds of the interfacial water occurs at hydration between 0.15 \( \sim 0.25 \ h \). The hydration dependence of the diamagnetic susceptibility, which is similar to the dependence of the heat transfer of water into the interface, parallels the saturation of hydrogen-bond sites.

It was understood that the fall of apparent heat capacity at hydration \( > 0.25 \ h \) results from the condensation of water on non-polar sites. The condensation of water completes the hydration shell at 0.40 \( h \) above which heat capacity is not dependent on the hydration level any more. This hydration level \( \sim 0.40 \ h \) is called first shell of hydration water. After this first hydration shell, the extra water added into the system is more like bulk water.

All the above pictures should be general for at least another globular proteins like myoglobin and RNase A. They show very similar dependence of their sorption isotherms
on hydration. Similar observation (Figure 2.6) has been reported for other globular proteins

2.3.2 Biological activities of protein lysozyme

The biological activities of various proteins have been investigated not only in solution state but also in powder state as a function of hydration level or water activity. Measurements of biological activities of hydrated protein powders can be achieved by preparation of protein-substrate assay at low-temperatures. Solutions of proteins and their substrates are mixed quickly to produce protein-substrate complexes, and the mixture is immediately quenched and lyophilized. The dry protein-substrate complexes are rehydrated under specific relative humidity until a final desired hydration level is reached, and then the rate of biological reaction between the protein and the substrate is measured.

As discussed in section 2.1.2, lysozyme cleaves the ether bond in polysaccharide into glucosamine by hydrolysis. Its catalytic mechanism has been well established. Because lysozyme is the model protein used in this research, more details about the catalytic reaction of lysozyme are introduced in this section.

Lysozyme’s catalytic site was identified through model building. Philips who analyzed structure of lysozyme by X-ray diffraction measurement used model building to investigate how a larger substrate could bind to the lysozyme. The active site cleft of a lysozyme molecule is big enough to accommodate six residues (-NAG-NAM-NAG-
NAM-NAG-NAM-) of an oligosaccharide that are labeled as A, B, C, D, E, and F.

Figure 2.7 shows the three-dimensional model of the lysozyme-substrate complex.

Only if the six residues of the substrate perfectly fit into the active site cleft of lysozyme, it is called productive complex, the catalytic reaction is able to take place. To do so, lysozyme needs to be flexible enough to change its conformation and substrate
should also distort its structure at D position due to steric hindrance. It turned out that the bond cleavage occurs in β(1→4) glycosidic linkage between D and E.

The catalytic reaction is involved with intermediate transition state that is resonance-stabilized carbocation (oxonium ion). The oxonium ion then adds water to yield a hemiacetal tetramer and dimer. The functional groups that directly participate in catalytic reaction are Glu 35 and Asp 52. They work under different environments. The Asp 52 is surrounded by several “polar” residues. It is unprotonated and hence charged negatively throughout 3 to 8 pH range over which lysozyme is catalytically active. On the contrary, the Glu 35 is placed in a predominantly “non-polar” region. It remains protonated at unusually high pH for carboxyl groups.

Phillips postulated catalytic mechanism as followings ¹:

1. Lysozyme approaches to a bacterial cell wall by binding to a hexasaccharide unit. Residue D is distorted toward half-chair conformation.

2. Proton of Glu 35 is transferred to the O1 of the D ring. The C1-O1 bond in the D ring is thereby broken, generating the resonance-stabilized oxonium ion.

3. The ionized carboxyl group of Asp 52 stabilizes the oxonium ion. The bond cleavage is facilitated by the strain applied to D ring in Figure 2.8.

4. Lysozyme first makes free the hydrolyzed E ring, yielding another oxonium ion stabilized by lysozyme. The enzyme finally releases the D ring with its attached saccharide.
Figure 2.8 Mechanism of lysozyme-catalyzed cleavage at active site.

Hydration influence on lysozyme’s bioactivity was comprehensively studied by Rupley et al. (1980). Hexasaccharide of N-acetylglucosamine \([(\text{GlcNAc})_6]\) was the substrate for the catalytic reaction. Figure 2.9 shows the enzymatic reaction rate \((V_0)\) of lysozyme as a function of water content at pH 8, 9, and 10 at \(T \sim 298 \text{ K}\). Since high pH was used to slow the enzyme reaction, the hydration process was not the rate-determining step.

Interestingly, the reported enzymatic activity of lysozyme has a step-like dependence on the hydration level as seen in Figure 2.9. The enzymatic activity is essentially non-measurable at hydration levels \(h < 0.2\). As the hydration level is increased, the onset of enzymatic activity of lysozyme occurs at \(h \sim 0.2\), then it increases sharply between \(h \sim 0.2\) and \(0.5\), and its increment strength becomes weak at \(h > 0.5\) asymptotically approaching the level of activity in a dilute solution.
Figure 2.9 Enzymatic reaction rate ($V_0$) of lysozyme in a logarithmic scale as a function of hydration, $h$ (g of water / 1 g dry protein), at pH 8, 9, and 10. Measurements on powders hydrated by isopiestic equilibration. Powder samples were 1:1 lysozyme-(GlcNAc)$_6$ complex, obtained by lyophilization \(^{11}\).

Rupley et al.\(^{23}\) studied temperature dependence of enzymatic activity of lysozyme to hexamer of N-acetylglucosamine. They measured the steady state rate of cleavage of the substrate as a function of temperature from 278 to 313 K and pH from 2 to 8 in aqueous solution.

In solution state, the lysozyme and the substrate form various types of complexes. Figure 2.10 exhibits schematic representation of the lysozyme-substrate complexes in many steps: nonproductive, productive, and reactive complexes. In this scheme, it is clear that in order to reach reactive complexes, nonproductive complexes should dissociate into unbound species and they need to orient correctly through productive complexes. Therefore, it is strongly suggested that the transformation from
nonproductive to productive complexes can be achieved only by dynamic conformational change of both lysozyme and the substrate.

Figure 2.10 Schematic representation of lysozyme-substrate forms important for the lysozyme-catalyzed cleavage of chitohexose. The non-productive complex is a result of incorrect binding of substrate to lysozyme involving ABC site with the reducing end unit (~). Correct binding of substrate to lysozyme at ABCDE with non-reducing unit (▱) forms productive complex. Cleavage of ether group in substrate is possible for partially and fully accommodated DEF sites of substrate.

The reaction rate was observed at pH ~ 6.3. Temperature dependence of reaction rate in each step shows Arrhenius behavior and the energy barrier of each process was estimated. Interestingly, formation of each complex shows similar energy barrier from 70 to 76 kcal/mol. This value is very close to activation energy required for the cleavage reaction. This observation suggests that all processes involved are important for the enzymatic reaction.
2.4. Energy landscape of conformational states in proteins

Native conformation of a protein molecule consists of a large number of slightly different substates. Each substate represents local minima in the potential energy surface of the protein system. Protein motions can be described as transitions between the conformational substates (CS). Not all of protein motions are directly coupled to protein function. Functionally important motions can be studied only if they can be selectively monitored. Different types of proteins have different sets of conformational substates, but it is believed that general concepts are likely to be universal.

The idea of conformational substates was proposed as a means of explaining dependence of ligand-binding rate to myoglobin. Frauenfelder et al. 4, Ansari et al. 2, and Elber & Karplus 3 have made active discussion of the conformational substates of a native protein, with particular reference to myoglobin binding CO (MbCO) or O₂ (MbO₂). Low-temperature flash photolysis measurements of MbCO and MbO₂ evidenced that the rebounding of the ligand to the heme center was non-exponential in time at temperatures below \( T \sim 200 \text{ K} \). This observation suggested that myoglobin did not have a single structure but could assume a large number of slightly different structures – conformational substates, each with a different rebounding rate.

Figure 2.11 presents the simplified sketch of the energy landscape of myoglobin as a function of conformational coordinates. In fact, the number of dimensions for possible conformational state is \( 3N \) (\( N \) is the number of atoms in a protein molecule). The author classified CS roughly into a hierarchy whereby CS\( i \) represents \( i \)th tier in the organization.
Figure 2.11 Structure and conformational energy landscape of MbCO. The top-row left is structure of MbCO and right is illustration of the conformational energy $E_c$ as a function of a conformational coordinate, $cc$. The second-row right displays three subsates present in tier 0, each of which reflects different orientation of the bound CO with respect to the heme. The third-row left is mean-square deviations for MbCO at 300 K (upper) and 80 K (lower). Tier 1 and 2 are oversimplified. In real, $E_c$ is a hypersurface in conformational space with a number of valleys. (Reprinted figure with permission from 4. Copyright (1991) Science.)
The top row shows the traditional picture of a unique energy valley. First tiers, A0, A1, and A3 were identified by the presence of multiple stretch bands obtained from the IR measurements of a CO-binding myoglobin. The first tiers for MbCO are functionally important, as each CS binds CO at different rates. The different rates suggest the presence of other substates that consist of a lower energy gap. The presence of the second tiers in the third row in Figure 2.11 was supported by the measurements of biological activity of Mb and the structural positions of its residues. The authors claimed that the second tiers lead to non-exponential time dependence of the CO re-binding. Their fluctuations are accurately estimated by using the difference of mean-squared atomic displacements, <x^2>, of the residues in Figure 2.11.

The general biological activities of proteins are determined by their reaction rate. Its temperature dependence is usually approximated by Arrhenius law with some characteristic energy barrier, E. The distribution function of energy barrier g(E)dE presents the probability of finding a protein whose energy barrier is between E and E+dE. The experimental result of the fraction of the re-binding of the CO to Mb shows that the non-exponential feature of re-binding processes of the first tiers is proportional to the number of second tiers in each first tier. The distribution of energy at the barrier was also found to be broad as a result of the large number of second tiers.

Friedrich et al. provided another scheme of energy landscape from hole-burning experiments. They confirmed that proteins in different conformational substates have spectral lines at slightly different wavelengths. Slow refilling of holes burned into the spectral lines by the used laser proved the presence of substates in the lower tiers.
2.5 Protein dynamics affected by hydration and temperature

The ultimate goal in studying protein dynamics is to bridge the gap among dynamics, structures, and functions. Structures (especially the three-dimensional folded structures of proteins) are actually the result of dynamic processes achieving the most favorable thermodynamic state. Therefore, protein dynamics are certainly of central importance to the study of the biochemical properties of proteins.

In a like manner to protein functions, protein dynamics involving substrate-binding and the folding processes change with the protein’s specific environment, such as solvent, temperature, pressure, salt, pH, etc. Among these water and temperature are known to be the most crucial for initiating both the flexibilities and bioactivities of proteins, as we reviewed in section 2.2. For instance, functional proteins in the human body have evolved to adapt to a physiology with a certain amount of water at a specific temperature. Therefore, one of the best ways to make parallel analysis between protein dynamics and function is to study their dependence on temperature and hydration.

Many spectroscopic experiments and computer simulations have been performed to study the influence of hydration and temperature on protein dynamics. It was shown that water acts as a plasticizer of protein conformational transitions, making protein molecules more flexible. For example, Figure 2.12 indicates that the glass transition temperature, $T_g$, decreases as water is added. Fully hydrated lysozyme exhibits $T_g$ of 170 K, whereas lysozyme at hydration ~ 0.1 $h$ shows $T_g$ of 300 K. Interestingly, dry protein rarely experiences any glass transition. Instead, it directly loses folded structure at melting or denaturation temperature, $T_m$. 
Figure 2.12 The hydration dependence of the glass transition temperature for lysozyme determined from positron annihilation lifetime spectroscopy (filled bars), together with results from thermally stimulated depolarization currents studies (open squares). The glass transition regions determined from various hydration studies at 298 K and from studies of fully hydrated proteins (the 200 K transition) are indicated by open bars. The hydration dependence of the denaturation temperature of lysozyme from calorimetric studies is also shown. The two curves separate the rigid, glassy native state from the flexible native state and the denatured state \(^{40}\).

Seen from the aspect of temperature, protein molecules can explore large-scale motions like conformational and structural relaxations at \(T > T_g\). The crucial influence of hydration and temperature on protein dynamics has been essentially discussed in the dependence of hydration on the dynamic transition of proteins observed in many experiments. However, the microscopic mechanism of the processes underlying the dynamic transition in proteins is still a subject for discussion and debate.
This section will first introduce the techniques used to investigate protein dynamics. Then a detailed review on the dynamic transition will be presented and its importance will be explained elucidating that nature-selected protein dynamics are essential for protein functions to be performed. Therefore, we will also discuss what kind of dynamic processes exist and which dynamic process is responsible for the dynamic transition.

2.5.1 Techniques used for studying protein dynamics

2.5.1.1 Neutron scattering spectroscopy

Many studies of protein dynamics were performed by neutron scattering technique. There are a few reviews. The neutron scattering technique is one of the useful methods for investigating protein dynamics due to its prominent technical advantages of time-space correlation and the high contrast between H and D atoms. The scattering happens by the direct interaction between the neutron and nuclei. The fact that the neutron wave-length (~ Å) and energy (~ µeV to meV) correspond to inter-atomic distances and the energy of thermal excitations makes it possible to monitor protein motions at atomic and molecular levels without any damage to the samples. Therefore, various dynamic processes from vibration to the structural relaxation of protein molecules can be characterized. Vibration modes appear as distinct inelastic peaks, whereas relaxation processes are represented by a broadened elastic part, the so called “quasielastic scattering”.

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Neutron scattering is comprised of coherent and incoherent scattering parts. Coherent neutron scattering results from the interference of waves scattered by different nuclei, whereas incoherent scattering occurs by the same nuclei. Incoherent neutron scattering can be likened to waves scattered by individual atoms and interfering only with the same atoms during a time course defined by instrumental resolution. In neutron scattering measurements of proteins, the incoherent scattering of H-atoms dominates. The motions of H-atoms reflect the globular motions of protein molecules, because H-atoms are homogeneously distributed over a protein molecule.

The contribution of proteins to neutron scattering also dominates, even in a protein-solvent mixture, if one uses deuterated solvent molecules (switching all H-atoms to D-atoms in solvent molecules). For instance, D$_2$O molecules need to be used instead of H$_2$O molecules for the hydration of proteins.

Another advantage of neutron scattering measurements in investigating protein dynamics is that their results can be directly compared with the results from Molecular Dynamics (MD) simulations, since both operate with similar variables. Therefore, a detailed microscopic mechanism and a picture of protein motions can be obtained by a combination of the neutron scattering measurements and the simulations. More details of the neutron scattering technique will be discussed in Chapter III and a concise review of previous results concerning protein dynamics obtained from neutron scattering measurements will be discussed in sections 2.5.2 and 2.5.3.
2.5.1.2 Infra-Red (IR) spectroscopy

Protein molecules have active vibration modes, the so-called Amide A, Amide I, Amide II, and Amide III modes, all of which are sensitive to the H-bonds of the amide groups. The Amide A band appears between 3225 and 3280 cm\(^{-1}\) due to the N-H stretching vibration, and the Amide I band, the most intense, between 1600 and 1700 cm\(^{-1}\) due to the stretching vibration of C=O and C-N groups.

Doster\(^9\) compared the inter-oxygen distance of the H-bond in hydrated myoglobin studied by IR spectroscopy with the mean-squared fluctuations of the protein. The results showed a big jump at a dynamic transition temperature. Increase in the inter-oxygen distance weakens the H-bonding. The latter increases the O-H force constant and consequently the stretching frequency.

The kinetics of transitions between different conformational substates can be studied by time-resolved IR spectroscopy. Frauenfelder \textit{et al.}\(^{46}\) used the technique in the form of flash-photolysis experiments to understand the biological processes of ligand-binding Mb. Recently, Frayer \textit{et al.}\(^{47}\) developed a spectrally resolved IR stimulated vibrational echo technique that can be used to study the dynamics of the ligand and heme proteins by measuring the vibrational dephasing of the CO stretching mode.

2.5.1.3 Raman spectroscopy and interferometry

Raman spectroscopy has traditionally been a useful technique for observing the vibrating motions of non-polar groups like C-H that are IR-inactive. The C-H bond has high optical polarizability that results in high Raman cross-sections. Consequently,
Raman spectroscopy appears to be more efficient for studies of C-H vibrations than is IR spectroscopy.

Low-frequency Raman scattering spectroscopy can reach a frequency down to ~90 GHz (3 cm\(^{-1}\)). This energy range provides good opportunities to monitor fast relaxation and even the high-frequency tail of slower-scale motions, as well as collective vibrations.

Rupprecht et al. \(^{48}\) tried to understand the dynamic behavior of hydrated water by observing the low-frequency (from 1 to 250 cm\(^{-1}\)) Raman spectra of lysozyme crystals and DNA films, with samples of varying water content. They found the spectra could be fitted by the sums of several damped harmonic oscillators and the relaxation modes in the samples. However, they made incorrect analyses of the spectra because of their oversimplified assumption of a simple feature of water layers in proteins.

The Tandem Fabry-Perot interferometer has a higher resolution than the Raman spectrometer, which can be used to study protein dynamics. Its accessible energy window is known to be between 100 MHz and 1 THz. In this energy window, one can detect relaxation modes in molecules.

Sokolov et al. \(^{31,41,49,50}\) have investigated the dynamic processes of hydrated protein and DNA molecules by combining Raman spectra at higher frequency and the interferometer spectra at lower frequency. In the combined spectra covering a broad frequency range, they observed evidence of complex dynamic processes, from vibration to slow relaxation modes of the biopolymers.
2.5.1.4 Mössbauer spectroscopy

Mössbauer spectroscopy on Fe-containing proteins like Mb and Hb has become one of the most valuable means of investigating protein dynamics. The dynamics of the Fe Mössbauer nucleus reflect the motions of the heme group in the proteins. The relaxation time of the dynamical process is close to the lifetime of the Mössbauer nucleus, broadening $\gamma$-absorption lines. The time window that is accessible for the Fe Mössbauer nucleus extends from $10^{-7}$ to $10^{-9}$ s. Any dynamic process having a relaxation time falling within this time window may broaden the absorption lines.

The Mössbauer spectra of proteins show three particular features: i) The intensity of the absorption lines decreases sharply above a characteristic temperature where the relevant relaxation occurs, ii) The width of the absorption lines increases above the characteristic temperature, and iii) the absorption line can be associated with an additional broad line above the characteristic temperature.

Parak and Frauenfelder mainly used Mössbauer spectroscopy to study the dynamics of the Mb binding CO molecule, obtaining similar results concerning the temperature dependence of mean-squared displacements as in other techniques. Lichtenegger et al. tried to make a correlation between the mean-squared displacements of MbCO obtained from Mössbauer spectroscopy and their biological activities. Debrunner and Keller estimated the conformational energy landscape of MbO by analyzing mean-square displacements and the relaxation time obtained from Mössbauer spectroscopy.
2.5.1.5 X-ray diffraction spectroscopy

In principle, X-ray diffraction spectroscopy is based on the interaction of the X-ray with electron density. These are time-averaged static measurements of protein structures rather than time-resolving measurements of the dynamics of proteins. A three-dimensional static structure describes the ground state of the given protein molecules. The structures of a number of proteins have been established by this technique. They are available in the Protein Data Bank (PDB, www.pdb.org).

The B-factor that is the same as the Debye-Waller factors provides information on the atomic mean-squared displacements of protein residues. They represent static positions of collective motions, understood as their definition. However, it is possible to study flexibility or the extent of the importance of these dynamics by observing the temperature dependence of the atomic mean-squared displacements.

One of the most important findings showing the correlation of protein dynamics with the biological activity of proteins resulted from an X-ray scattering study on mean-squared displacements in crystalline bovine pancreatic ribonuclease A. The result suggested that the temperature at which a sharp increase of the mean-squared displacements occurs is consistent with the temperature for onset of the protein functions. The biological activity shuts off below this temperature.

2.5.1.6 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectroscopy is one of the most widely used techniques to investigate the dynamic properties of protein molecules over a range of different time scales with atomic
resolution made possible by the isotopic labeling (e.g. $^2$H, $^{13}$C, $^{15}$N, $^{17}$O) of protein molecules. During the last decade, a significant number of new multidimensional NMR methods have been developed to study protein dynamics at a molecular level. They are monitored with a time reorientation of various bond vectors, like $^{15}$N-NH, $^{15}$N-$^{13}$C, and $^{13}$C-$^1$H to describe backbone dynamics, as well as the motions of the side groups on a broad timescale covered from millisecond to picosecond.

Gregory et al. investigated the influence of hydration on internal dynamics and the conformation of lysozyme and bovine serum albumin powder by performing solid-state $^{13}$C-NMR spectroscopy. Their results suggested that the binding of water to the polar groups in the protein allows conformational transition states.

Giraud et al. employed multidimensional solid-state NMR spectroscopy to obtain time resolved $^{15}$N-$^{13}$C correlation spectra. They observed high flexibility for residues that compose irregular secondary structures like loops. NMR spectroscopy gives detailed information on the local dynamics of specific nuclei, and thereby the dynamics of every residue that contains the labeled nuclei can be understood. However, NMR spectroscopy is too local and is not effective for studies of the global characteristics of the internal dynamics of protein.

2.5.1.7 Dielectric spectroscopy

Dielectric spectroscopy is one of the easiest techniques used to study dynamics, since its spectra can be recorded in a broad frequency range within a relatively short time. The resources of the spectrum originate from relaxation responses of dipole moment in
polar atomic groups or molecules to an alternating electric field. A protein molecule shows its own net charge based on polar groups of amino acids. For instance, the isoelectric point of lysozyme is pH 11.

However, there have been few dielectric studies for investigating the internal dynamics of protein molecules. One of the possible reasons is that protein molecules exhibit a huge complexity of dipole interaction among the polar groups coupled with the high dipole moment of the surrounding solvents like water molecules. In fact, dielectric spectroscopy is much more sensitive to small molecules that possess high dipole moments than to protein molecules. Therefore, dielectric spectroscopy has been known to be more useful for analysis of the dynamic behavior of water molecules mixed with protein molecules.

Dielectric studies of hydrated protein powder showed that the main dynamic mode came with the relaxation of protons migrating between ionized side chains rather than the internal motions of protein molecules. Interestingly, however, the hydration level needed for the onset of the proton migration is the same as for the onset of the biological activity of lysozyme.

Olenikova, et al. studied the dielectric relaxation of aqueous solutions of ribonuclease A as a function of protein concentration at 298.15 K. The obtained spectra were decomposed into five modes of Debye type diffusive behavior. Their interpretation of the complex dynamic modes includes, in order of increasing characteristic relaxation time: relaxation of bulk water, reorientation of hydration water, internal motions of the protein molecules, protein-water cross correlation, and protein tumbling.
Bonincontro et al. \textsuperscript{62} reported on the dielectric behavior of lysozyme and ferricytochrome-c in water and glassy solvent mixtures at different temperatures and pH values. The characteristic frequency of the rotational relaxation of the protein molecules was obtained, and the hydrodynamic radius of the proteins was estimated using the Stokes-Einstein approximation.

2.5.1.8 Simulations

Computer simulations including molecular dynamics (MD) simulations and normal mode analysis are most widely used to investigate protein dynamics. Owing to computational advances, now the simulations can be explored on a time scale as long as $10^{-8}$ s. This timescale corresponds to that of neutron scattering spectroscopy. The computer simulations help interpret experimental data at a microscopic level, especially the neutron scattering spectroscopy data.

Many simulation studies of the microscopic dynamics of hydrated proteins have been performed by Smith et al. They studied the dynamic transition in proteins using normal mode analysis \textsuperscript{63}. The results established the schematic idea that the rigid-body motions of secondary structures activate on a nanosecond timescale at the dynamic transition.

Advances in computer power will certainly improve accuracy and the timescale of simulations relative to those currently feasible. It will also help decouple the functionally important motions of protein molecules from thermal noise. We expect that the detailed
characterization of every single protein-type will be made possible by comprehensive simulation tools.

2.5.2 Dynamic transition in proteins

Dynamic transition in proteins is a physical phenomenon defined by onset of anharmonicity where atomic mean-squared displacement, $<x^2>$, rises sharply and nonlinearly with temperature. The dynamic transition has been widely observed in computer simulations, and neutron scattering, Mossbauer and X-ray scattering spectroscopy. Table 2.1 shows representative dynamic transition temperatures ($T_D$) observed in a range of experimental studies. Some of the variation seems to be because of the different sampling and the time scale of the tools used.

The dynamic transition has been considered important because it has been believed to directly correlate to the onset of biological activities of proteins, although there are some exceptions$^{64-66}$. The study by Rasmussen et al.$^{33}$ proved the direct relationship between dynamic transition and protein activity. They observed that ribonuclease A did not bind the substrate or inhibitor, cytidine 2'-monophosphate, below a broad transition centered around 220 K present in $<x^2>$ of the protein obtained from X-ray crystallography. But it bound rapidly to both of the molecules at 228 K. Interestingly, no significant structural change happened between the inhibitor-binding proteins and free proteins. They proposed that collective atomic fluctuations activate only above the dynamic transition temperature. According to their interpretation, the dynamic modes are essential for the rapid productive binding of large ligands.
The dynamic transition is sensitive to the kinds of solvents around protein molecules. Figure 2.13 shows $<x^2>$ obtained from neutron scattering measurements of dry lysozyme, wet lysozyme, and lysozyme in glycerol. It is traditionally accepted that rigid protein systems such as dry proteins and proteins in solvents with a high viscosity like trehalose do not exhibit the dynamic transition in the entire temperature range up to 300 K. On the contrary, sufficiently hydrated proteins and proteins in solvents with a low viscosity like glycerol show the pronounced dynamic transition.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Protein</th>
<th>Preparation</th>
<th>$T_D$ (K)</th>
<th>Activity transition (K)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutron scattering</td>
<td>Bacteriorhodosin</td>
<td>In membrane</td>
<td>~ 230</td>
<td>~ 230</td>
<td>36</td>
</tr>
<tr>
<td>Neutron scattering</td>
<td>Lysozyme</td>
<td>Hydrated powder</td>
<td>~ 200</td>
<td>~ 30</td>
<td>30</td>
</tr>
<tr>
<td>Neutron scattering</td>
<td>Myoglobin</td>
<td>Hydrated powder</td>
<td>~ 180</td>
<td>~ 67</td>
<td>67</td>
</tr>
<tr>
<td>Neutron scattering</td>
<td>Amylase</td>
<td>Hydrated powder</td>
<td>~ 200</td>
<td>~ 37</td>
<td>37</td>
</tr>
<tr>
<td>Mössbauer</td>
<td>Chromatophore membranes</td>
<td>In membrane</td>
<td>~ 170</td>
<td>~ 190</td>
<td>68</td>
</tr>
<tr>
<td>Mössbauer</td>
<td>Cytochrome C</td>
<td>Solution</td>
<td>~ 200</td>
<td></td>
<td>69</td>
</tr>
<tr>
<td>Mössbauer</td>
<td>Hemoglobin</td>
<td>Solution</td>
<td>~ 200</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>Mössbauer</td>
<td>Deoxymyoglobin</td>
<td>Crystal</td>
<td>~ 220</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>X-ray diffraction</td>
<td>Ribonuclease A</td>
<td>Crystal</td>
<td>~ 200</td>
<td>~ 210</td>
<td>33</td>
</tr>
<tr>
<td>X-ray diffraction</td>
<td>Metmyoglobin</td>
<td>Crystal</td>
<td>~ 200</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Infrared vibrational echo</td>
<td>Myoglobin</td>
<td>Solution</td>
<td>~ 200</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>Time-resolved transient hole burning</td>
<td>Cytochrome c</td>
<td>Solution</td>
<td>~ 200</td>
<td></td>
<td>74</td>
</tr>
</tbody>
</table>
According to traditional analyses, $<x^2>$ of dry proteins vary linearly with temperature up to 300 K: $<x^2> \propto kT$. This behavior has been interpreted as being the result of harmonic vibrations dominant in dry proteins. However, the previous interpretation missed details and failed to perform correct analyses of the motions of the protein. This subject will be discussed in more detail in Chapter IV.

Activation of the dynamic transition requires a flexibility of protein molecules sufficient to explore different conformational substates \(^4\). Proteins, like an adequately wet lysozyme, tend to be flexible at a molecular level, fluctuating at $T > T_D$. The large-scale motions are attributed to the origin of the anharmonicity. Below $T_D$, protein molecules are frozen and trapped into ground conformational states. Therefore,
according to traditional interpretation, harmonic vibration motions dominate the
dynamics of wet proteins at $T < T_D$.

The dynamic properties of the solvents on the protein surface play a crucial role in
the dynamic transition of protein, showing that $T_D$ shifts with different solvents $^{30}$. It is
well known that the dynamic transition of proteins occurs at a temperature much higher
than the $T_g$ of the solvents.

Doster et al. $^{9,26}$ tried to understand the driving force and origin of the dynamic
transition. The $\langle x^2 \rangle$ of wet ($\sim 0.32$ $h$) and dry ($\sim 0.1$ $h$) myoglobin was estimated from
elastic neutron scattering measurements allowing for the monitoring of any motions
faster than 80 ps. The authors identified the dynamic transition at $T \sim 180$ K in both dry
and wet proteins, suggesting it is an intrinsic mechanism. However, the onset of
anharmonicity in dry protein that they interpreted as a dynamic transition was probably
not the traditional one that originates from conformational fluctuations.

Computer simulation studies have helped to evidence the role of solvents in the
dynamic transition in proteins. Tobias et al. $^{39}$ proved that translational motions of water
molecules were coupled with protein atomic fluctuation at 300 K. When the translational
motions of water molecules were inhibited, the dynamic transition disappeared. This
result suggested that the dynamic transition is driven by the translational motions of the
solvent molecules.

Smith et al. $^{38}$ performed dual heat-bath MD simulations to investigate the effect
of water on the dynamic transition of proteins. First holding $T$ of water at 300 K and
changing $T$ of proteins, no dynamic transition was observed in temperature dependence
of $\langle x^2 \rangle$ of the wet protein. On the contrary, changing $T$ of water and fixing $T$ of proteins
at 300 K, a dynamic transition appeared in $\langle x^2 \rangle$ of the wet protein at ~200 K. A qualitative change in the temperature dependence of the water translational diffusion happened at the same temperature as the dynamic transition in proteins. Therefore, the simulation studies provided the important confirming evidence that the translational diffusion of water molecules is the potential force that promotes the dynamic transition of protein molecules.

However, details as to the dynamic processes that are responsible for the dynamic transition remain unclear. The following section contains a concise review of the research into the development of a better understanding of the fundamentals of these dynamic processes in order to disentangle the motions responsible for the dynamic transition from other processes.

2.5.3. Dynamic processes in proteins

As we discussed, dynamic transition in proteins is strongly related to onset of protein functions. Therefore, unraveling the microscopic mechanism of the dynamic process responsible for the dynamic transition is obviously important to obtain an understanding of the role of protein dynamics in protein functioning.

The internal motions of protein molecules span widely from vibration to a folding-unfolding process. Among them, the conformational fluctuation and structural relaxation on a timescale between micro- and pico-second are strong candidates for the origin of dynamic transition $^6$. However, the extent to which the related motions are biologically important is unclear and it could be different for different proteins.
Therefore, a better understanding of the fundamentals of protein dynamics is essential to the unraveling of the microscopic picture of the dynamic transition and dynamic process that is directly related to protein activity.

Protein molecules have a complex dynamic process between a micro- and pico-second timescale (kHz and THz) as synthetic polymers do. There are at least three main components in the internal dynamics of protein molecules observable at frequency < 3 THz\textsuperscript{75}.

(i) Low-frequency collective vibrations, at $h \nu \sim 2 - 6$ meV ($\sim 15 - 50$ cm$^{-1}$ or 0.5 – 1.5 THz), so called Boson peak

(ii) Fast conformational fluctuations at $\nu \sim 50 - 300$ GHz

(iii) Slow relaxation process at $\nu < 50$ GHz

These dynamic modes have been experimentally identified in the energy-resolved neutron and the light scattering spectra of various proteins. Figure 2.14 exhibits the distinct Boson peak and the fast process in the spectra of dry and hydrated myoglobin. Computer simulations performed on hydrated ribonuclease-A ascribed the Boson peak to collective vibrations that involve side groups, backbone, and whole protein molecules\textsuperscript{76}. However, the microscopic mechanism of the Boson peak is still a subject open to discussion.
The microscopic mechanism of fast and slow relaxation processes in complex systems can be explained by Mode Coupling Theory (MCT) \(^{77-79}\). According to the MCT, fast dynamics are localized dynamic modes reflecting the motions of atomic groups or small molecules trapped in cages formed by their neighbors. The characteristic time of fast process shows, in general, weak temperature dependence. However, its intensity increases with increasing temperature.

The fast process is found in both dry and hydrated proteins even at very low temperatures \(^{80}\). At \(T < T_D\) of hydrated proteins, dry proteins show a higher intensity of the fast process than hydrated proteins. The explanation of this observation is that hydration water molecules fill and reduce the space available for the localized motions of the atomic groups in the protein molecules. Hydrogen bonds between water molecules and atomic groups in hydrated proteins are able to fluctuate at a temperature such as \(T \sim\)
150 K. However, the hydrogen-bond fluctuation at \( T \) lower than \( T_g \) of the protein-solvent mixture is too restricted to overcome the confinement, leading to localized motions weaker than the motions in dry proteins.

A slow relaxation process involves large-scale motions of the trapped atomic groups or small molecules. Given adequate time, the trapped elements can move out of the cages. The relaxation time is strongly dependent on temperature. In synthetic polymers, the temperature dependence of the characteristic time is well known to be non-Arrhenius. The slow relaxation process emerges only in sufficiently hydrated proteins, not in dry proteins. However, the microscopic mechanism of the slow relaxation process is still unclear.

The development of a basic understanding of the nature of the dynamic transition in proteins has been made by introducing the fast and the slow relaxation processes. Doster et al. performed neutron scattering measurements of dry and hydrated myoglobin. It was reported from their analyses that both weakly and sufficiently hydrated proteins \( \approx 0.1 \, h \) and \( \approx 0.32 \, h \) experienced dynamic transition. The interpretation of the nature of the observed dynamic transition was made with a two-site jump model by which fast hydrogen-bond fluctuation opens and closes. According to their claim, the fast process activated by hydrogen-bond fluctuation is the origin of the dynamic transition, and the slow process is due to the extra mobility of a sufficiently hydrated protein.

Sokolov et al. tried to understand which dynamic mode in protein molecules is responsible for the dynamic transition. The results of light scattering measurements of lysozyme placed in glycerol and pure glycerol are shown in Figure 2.15. From the
spectra, it seems that slow relaxation modes appear in the ~ GHz frequency window at $T > T_D$. The integrated part of the spectrum between 1 and 5 GHz is dominated by the slow relaxation process. The temperature dependence of the estimated slow relaxation process presented in the protein-glycerol mixture shows a strong jump at $T \sim T_D$ of lysozyme embedded in a glycerol solvent. Consequently, the activation of the slow relaxation process was suggested to be the origin of the dynamic transition.

Figure 2.15 (a) Light scattering susceptibility, $\chi''(\nu)$, of lysozyme in glycerol (thick lines) and pure glycerol (dashed lines) at different temperatures. (b) Integrated $\chi''(\nu)$ of lysozyme in glycerol and pure glycerol at frequency range from 1 to 5 GHz. (Reprinted from 41. Copyright (2002), with permission from Elsevier)

Recent computer simulation studies on hydrated myoglobin supported the idea that large-scale motions which are involved with a slow relaxation process on a sub-nanosecond timescale are responsible for the dynamic transition 63. They qualitatively identified the relevant relaxation modes as rigid-body motions of secondary structures. In the case of the sampled myoglobin, the seven $\alpha$-helices can change their structural positions globally along translational and rotational modes as shown in Figure 2.16.
However, it is still not clear to what extent motions activated in the dynamic transition describe functionally important protein dynamics. To successfully unravel which part of protein dynamics is directly correlated with protein functions, more effective parallel analyses between protein dynamics and protein functions are required.

Figure 2.16. Rigid-body motions of myoglobin at $T \approx 200$ K. $T_D$ of hydrated myoglobin is $\sim 180$ K. The secondary structures of myoglobin are shown in two energy minima (black and white) of the slowest relaxation mode. The helices are represented as cylinders. The amplitude of the motion is multiplied by a factor of 6. (Reprinted figure with permission from 63. Copyright (2003) by the American Physical Society)
CHAPTER III

DETAILS OF EXPERIMENTS

3.1 Basic concepts of scattering

Scattering is the “deflection” of incident particles or waves. Particle physics describes scattering as a class of phenomena by which incoming particles like neutrons and photons are deflected by collisions with other particles, like nuclei, or with other waves, like sound waves. In acoustics, scattering is the deflection of sound waves by particles or medium heterogeneities.

A variety of scattering techniques have been developed in neutron scattering, light scattering, and x-ray scattering. In this section, general concepts of three types of scattering (elastic, quasielastic, and inelastic) will be introduced, and then details of neutron and light scattering spectroscopy will be discussed.

The two basic parameters to be measured in a scattering experiment are:

(i) the energy transfer, $\hbar \omega$, between the incident, $E_i$, and final, $E_s$, energies of probing particles or waves,

$$\hbar \omega = \Delta E = E_s - E_i = \hbar (\omega_s - \omega_i)$$  \hspace{1cm} (3.1)

where $\omega_s$ and $\omega_i$ are the scattered and incident frequency, and

(ii) the momentum transfer, $Q$ (scattering vector)
\[ Q = k_s - k_i \]  

(3.2)

where \( k_s \) and \( k_i \) are wavevectors of scattered and incident waves, respectively.

Figure 3.1 represents the scattering phenomena including elastic, quasielastic, and inelastic scattering with regard to energy (\( \hbar \omega \)) and momentum transfer (\( Q \)). These two quantities provide information on the dynamics and structures of sampled molecules. Energy transfer reflects the timescale of probing particles’ motions and scattering vectors show their lengthscale and direction, as compared to their initial positions.

![Figure 3.1 Representation of elastic, quasielastic, and inelastic scattering](image)

Inelastic scattering \( \Delta E > 0 \)

Elastic \( \Delta E = 0 \)

Quasielastic scattering \( \Delta E \sim \text{small} \)

Inelastic scattering \( \Delta E < 0 \)

Incident plane wave

Sample

Figure 3.2 represents a general feature of a scattering spectrum to be obtained from energy resolved experiments. Relative to the dynamics, the elastic scattering is related to immobile sources, quasielastic scattering to mobile sources involving relaxation (local conformational motions, diffusion, and rotation), and inelastic scattering to a vibration.
3.1.1 Elastic scattering

Elastic scattering conserves the energy of incident waves (neutrons and/or photons) when they are deflected by samples. However, the direction of the wave vector changes, as described in Figure 3.1. The scattering vector, $Q$, can be estimated by

$$|Q| = \frac{4\pi \sin \theta}{\lambda}$$  \hspace{1cm} (3.3)\]

where $\theta$ and $\lambda$ are half of the scattering angle and wavelength of the incident particles.

Combining the equation (3.3) with Bragg’s law, $n\lambda = 2d\sin(\theta)$, (Figure 3.3), the dimension of the scattering system can be expressed by the scattering vector:

$$|Q| = n\frac{2\pi}{d}.$$  \hspace{1cm} (3.4)

The $d$ is the distance between parallel, neighboring planes of scattering sources and $n$ represents an integer. The equation (3.4) indicates that the shorter the wavelength, the smaller the probing lengthscale that is possible. Therefore, neutrons whose wavelengths are $\sim 1$ Å are better to detect microscopic motions at an atomic scale than light photons whose wavelengths lie between 400 and 700 nm.
Figure 3.3 Bragg diffraction of constructive interference\textsuperscript{82}.

From a dynamic point of view, the intensity of elastic scattering is proportional to the probability that a particle does not move out of a length scale $2\pi/Q$ within a time interval given by the energy resolution of the instrument. Conversely, the decay of the intensity of the elastic scattering reflects how many mobile scattering sources are activated at the accessible lengthscale and direction. The feature of $Q$ dependence of the elastic scattering intensity provides information on geometry, such as mean-squared
atomic displacements and jump distances involving the motions that cause decay of the intensity of the elastic scattering. It will be discussed in detail in the section for data treatment (3.2.2).

3.1.2 Quasielastic scattering

Quasielastic scattering is defined as a broadening of an elastic line with close-to-zero energy-transfer (Figure 3.1 and 3.2). The quasielastic scattering results from relaxational motions, such as an atomic or molecular conformational change, rotation and translation that occur in a timescale smaller than energy resolution (Figure 3.4). The relaxation rate can be determined from the full width of the quasielastic scattering peak at half maximum.

Figure 3.4 Sketch of relaxation motions.

3.1.3 Inelastic scattering

Inelastic scattering is a phenomenon of energy transfer which comes about as a result of the scattering on vibrations. Figures 3.1 and 3.2 depict the two parts of inelastic scattering to be observed by means of scattering spectroscopy, since the energy of the
target particles can be gained or lost, and conversely for the energy of the incident radiation.

The quantized energy levels of target particles and transitions stimulated by the incident radiation are shown in Figure 3.5. Resultant particles with lower energy generate a Stokes line on the red side of the incident spectrum. Incident particles are shifted to the blue side of the spectrum by the gained energy, thus generating an anti-Stokes line.

\[
I_s \propto I_i(n(\nu) + 1)
\]  

(3.5)
where \( n(v) + 1 = \left( 1 - \exp\left( \frac{hv}{kT} \right) \right)^{-1} \), \hspace{1cm} (3.6)

and the intensity of anti-Stokes scattering by

\[ I_{AS} \propto I_s n(v) \] \hspace{1cm} (3.7)

where \( n(v) = \left( \exp\left( \frac{hv}{kT} \right) - 1 \right)^{-1} \). \hspace{1cm} (3.8)

The scattering probability of the Stokes region turned out to be higher than the anti-Stokes by

\[ \frac{I_s}{I_{AS}} \approx \frac{n(v) + 1}{n(v)} = \exp\left( \frac{hv}{kT} \right). \] \hspace{1cm} (3.9)

3.2 Neutron scattering

3.2.1 Fundamental theory

A free neutron can be considered an elementary particle liberated during the process of fission of a heavy nucleus \(^{81}\). Its subatomic properties are:

- mass: \( m = 1.660 \times 10^{-24} \) g
- electric charge: 0
- spin: \( \frac{1}{2} \)
- magnetic moment: \( \mu = -1.913 \) nuclear magnetons.

Cold neutrons are neutrons that are equilibrated at very low temperatures, like 20 K, by a moderator, after being generated from a nuclear reactor or spallation source. Their
A typical wavelength of ~ 1 Å and energy of ~ 2.5 meV (~ 600 GHz) is comparable with the atomic length scale and thermal energy.

Figure 3.6 illustrates the geometry of neutron-scattering in three dimensions. The scattered neutrons are recorded by a detector ($^3\text{He(n,p)^3H}$) as a function of the scattering angle. The probability of the scattered neutrons in the unit solid angle [in the angle of the solid unit] is represented by the differential scattering cross section

$$\frac{d\sigma}{d\Omega} = \frac{I'}{I} = b^2.$$  

(3.10)

![Figure 3.6 Geometry for neutron scattering from a single nucleus in a sample. Neutrons are scattered in solid angle dΩ = dA/r under the scattering angle 2θ and the azimuthal angle φ along three dimensions. The I' is the average number of neutrons per unit time that are scattered in the solid angle and I is incident neutron flux.](image)

The b is the scattering length of the target nuclei which describe their scattering quantities in the linear dimension. The scattering lengths of deuterium and the major
atoms of which protein molecules are composed are summarized in Table 3.1. The total scattering cross section is the integral of the differential scattering section over the solid angle, and thereby

$$\sigma = \int_{4\pi} d\sigma d\Omega = 4\pi b^2. \quad (3.11)$$

Table 3.1 Neutron scattering and absorption by nuclei in protein molecules

<table>
<thead>
<tr>
<th>Atom</th>
<th>Coherent scattering length ($10^{-15}$ cm)</th>
<th>Incoherent scattering length ($10^{-15}$ cm)</th>
<th>Cross sections (1 barn = $10^{-24}$ cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coherent scattering</td>
<td>Incoherent scattering</td>
<td>Total scattering</td>
</tr>
<tr>
<td>1H</td>
<td>- 3.742</td>
<td>25.217</td>
<td>1.7599</td>
</tr>
<tr>
<td>2H</td>
<td>6.674</td>
<td>4.033</td>
<td>5.597</td>
</tr>
<tr>
<td>12C</td>
<td>6.635</td>
<td>0</td>
<td>5.563</td>
</tr>
<tr>
<td>14N</td>
<td>9.370</td>
<td>1.98</td>
<td>11.03</td>
</tr>
<tr>
<td>16O</td>
<td>5.805</td>
<td>0</td>
<td>4.235</td>
</tr>
<tr>
<td>32S</td>
<td>2.804</td>
<td>0</td>
<td>0.9880</td>
</tr>
</tbody>
</table>

In view of the energy transfer by the interaction of incident neutrons with the target nuclei, the scattering probability can be extended to the double-differential cross section that includes both the solid angle and energy exchange,

$$\frac{\partial^2 \sigma^2}{\partial \Omega \partial \omega} = N \frac{k'}{k} b^2 S(Q, \omega). \quad (3.12)$$

The ratio of $k'$ to $k$ describes the conservation of neutron flux after the scattering. $N$ is the number of scattering target nuclei. The physical meaning of the dynamic structure factor, $S(Q, \omega)$, is the probability that the target nuclei will be found with a specific space
and time range corresponding to the $Q$ and $\omega$. Therefore, $S(Q,\omega)$ is a result of two Fourier transforms of space and time correlation functions $G(r,t)$ represented as:

$$G(r,t) = \frac{1}{N} \sum_{j<k} <\delta\{r + r_k(0) - r_j(t)\}>,$$

(3.13)

$$I(Q,t) = \int_{-\infty}^{\infty} G(r,t) \exp(iqr) dr = \frac{1}{N} \sum_{j<k} \exp\{iQr_j(t)\} \exp\{-iQr_k(0)\},$$

(3.14)

$$S(Q,\omega) = \frac{1}{2\pi} \int_{-\infty}^{\infty} I(Q,t) \exp(-i\omega t) dt.$$  \hspace{1cm} (3.15)

The scattering length, $b$, in equation (3.12) has different contributions for coherent and incoherent scattering due to the spin-dependence of the neutron-nucleus interaction. For example, the coherent scattering length of $^1H$ is $-3.7 \times 10^{-15}$ cm, but its incoherent scattering length is $25.2 \times 10^{-15}$ cm (Table 3.1). The coherent neutron scattering is associated with the constructive interference of waves scattered by nuclei. The incoherent neutron scattering is interference of waves scattered by the same atoms. Therefore, the total correlation function in the equation (3.13) is divided into two parts:

$$G(r,t) = G_d(r,t) + G_s(r,t)$$  \hspace{1cm} (3.16)

where $d$ and $s$ denote distinct and self correlation function, respectively.

Therefore, the total scattering is split into two parts:

$$\frac{\partial^2 \sigma}{\partial \Omega \partial \omega} = \frac{\partial^2 \sigma_{coh}}{\partial \Omega \partial \omega} + \frac{\partial^2 \sigma_{incoh}}{\partial \Omega \partial \omega}$$

$$= \frac{1}{2\pi N} k \int_{-\infty}^{\infty} \left[ \sum_{k,j} b_{coh,k} b_{coh,j} \exp\{iQr_k(t)\} \exp\{-iQr_j(0)\} \right] \exp(-i\omega t) dt.$$  \hspace{1cm} (3.17)
Consequently, the total $S(Q, \omega)$ can be expressed by

$$S(Q, \omega)_{\text{total}} = S(Q, \omega)_{\text{coh}} + S(Q, \omega)_{\text{inc}} = \frac{1}{N} \frac{k}{k'} \left[ \frac{1}{b_{\text{coh}}^2} \left( \frac{\partial^2 \sigma}{\partial \omega^2} \right)_{\text{coh}} + \frac{1}{b_{\text{inc}}^2} \left( \frac{\partial^2 \sigma}{\partial \omega^2} \right)_{\text{inc}} \right]. (3.18)$$

It is well understood that the neutron scattering of non-crystalline biological samples is dominated by incoherent scattering of the hydrogen nucleus, since the hydrogen nucleus possesses an exceptionally high incoherent scattering cross section in comparison to other atoms (Table 3.1). Therefore, the spectra of the neutron scattering of proteins are dominated by the incoherent scattering of hydrogen atoms. Dynamics of the hydrogen atoms reflect protein motions because the hydrogen atoms are uniformly distributed in protein molecules.

3.2.2 Data treatment

Treatment of neutron scattering data can be conducted using specific software programs provided by the NIST center for neutron research (NCNR). They are open to and downloadable by the public. Among them, DAVE 84 is the software program that can be used to analyze data obtained from the High-Flux Backscattering Spectrometer (HFBS) and the Disk-Chopper Spectrometer (DCS). In the data-treatment process, the first step is to reduce the raw data obtained from many detectors (e.g. 913 detectors in the DCS spectrometer). The reduction process includes correcting for an empty sample can and detector efficiency. There are usually two kinds of scattering measurements: i) elastic scan, i.e. the total elastic scattering measurement and ii) energy-resolved spectroscopy, i.e. the quasielastic and inelastic scattering measurements.
3.2.2.1 Elastic scans

Elastic scans measure the intensity of the total elastic scattering, \( I(Q, t = \infty) \), as a function of \( Q \) and \( T \). Usually it is performed on the HFBS. Assuming that all scattering sources are dynamically equivalent, from the correlation function between initial positions of the target nuclei \( r_k(0) \) and their positions after a long time \( r_k(\infty) \), total elastic intensity is simply described by the intermediate scattering function when the instrumental energy resolution is infinitely close to zero:

\[
I_{inc}(Q, t = \infty) = \frac{1}{N} \sum_k |< \exp[iQ(r_k(0) - r_k(t))]>|^2. \tag{3.19}
\]

The \( I(Q, t = \infty) \) is proportional to the probability that hydrogen atoms are found in the initial equilibrium positions after infinite time intervals. Conversely, its decay from unit means dynamic motions have been activated in the sample.

The Fourier transform of the equation (3.19) is

\[
S_{inc}(Q, \omega = 0) = \exp[Q^2 <|r_k(0) - r_k(t)|^2>] = \exp[-\frac{1}{3}Q^2 <x^2>] \tag{3.20}
\]

where \(<x^2>\) is hydrogen mean-squared atomic displacement.

By a Gaussian approximation, the \(<x^2>\) can be obtained from:

\[
<x^2> = -3 \frac{d \ln[S(Q, \omega = 0)]}{d(Q)}|_{Q=0}. \tag{3.21}
\]

In practice, however, an instrument has an energy resolution limit. This means the measured total elastic scattering includes the original elastic parts and quasielastic parts unresolved by the HFBS. Therefore, \(<x^2>\) is a time-dependent quantity limited by the time that corresponds to the resolution function. The \(<x^2>\) represents an integrated
quantity including all possible vibrations, local conformational motions, diffusions, and rotations that occur on a time scale faster than the energy resolution (~ 1 ns). More details about time-dependent total elastic scattering and $<x^2>$ will be discussed in the sections including the experimental results and discussion.

3.2.2.2 Energy-resolved spectroscopy

Different dynamic processes can be distinguished in energy-resolved scattering measurements. The energy-resolved spectroscopy provides the information on quasielastic and inelastic scattering events. Intermediate scattering, $I_{total}(Q,t)$, is composed of time-independent (non-resolved) and time-dependent components:

$$I_{total}(Q,t) = I(Q,t = \infty) + I(Q,t).$$

(3.22)

Different kinds of motions will provide a different contribution to the time-dependent intermediate scattering function. For instance, solid samples usually show lattice phonons ($L$), molecular vibration ($V$), and reorientation ($R$) modes, and liquid samples that contain molecular vibration ($V$), translation ($T$) and rotation ($R$) modes. The intermediate scattering functions for solid and liquid samples can be written:

$$I(Q,t) = I^L(Q,t) \cdot I^R(Q,t) \cdot I^V(Q,t)$$

(3.23)

$$I(Q,t) = I^T(Q,t) \cdot I^R(Q,t) \cdot I^V(Q,t).$$

(3.24)

The Fourier transform of the equation (3.23) and (3.24) provides the dynamic structure factor:

$$S(Q,\omega) = S^L(Q,\omega) \otimes S^R(Q,\omega) \otimes S^V(Q,\omega)$$

(3.25)

$$S(Q,\omega) = S^T(Q,\omega) \otimes S^R(Q,\omega) \otimes S^V(Q,\omega)$$

(3.26)
where the symbol $\otimes$ is a convolution, i.e.

$$S^d(Q, \omega) \otimes S^b(Q, \omega) = \int d\omega' S^d(Q, \omega') S^b(Q, \omega - \omega').$$  \hfill (3.27)

The convolution results in:

$$S(Q, \omega) = \exp\left\{-\frac{1}{3} Q^2 < x^2_L > \right\} \left[ I(Q, t = \infty) \delta(\omega) + S^R(Q, \omega) + S^I(Q, \omega) \right].$$  \hfill (3.28)

$$S(Q, \omega) = \exp\left\{-\frac{1}{3} Q^2 < x^2_v > \right\} \left[ I(Q, t = \infty) \delta(\omega) + S^T(Q, \omega) \otimes S^R(Q, \omega) + S^I(Q, \omega) \right].$$  \hfill (3.29)

The first terms are Debye-Waller factors. The $< x^2_L >$ denotes the combined mean-squared atomic displacements including lattice $< x^2_L >$ and internal molecular vibrations $< x^2_v >$. The $S'(Q, \omega)$ is inelastic scattering as a result of the convolution of $S^i(Q, \omega)$ and $S^v(Q, \omega)$ with $S^g(Q, \omega)$ for solid samples, or of $S^T(Q, \omega)$ with $S^R(Q, \omega) \otimes S^T(Q, \omega)$ for liquid samples. The $S^R(Q, \omega)$ or $S^T(Q, \omega) \otimes S^R(Q, \omega)$ is the quasielastic scattering (QES) part of the spectrum. An experimentally measured dynamic structure factor, $S_M(Q, \omega)$, is a result of the convolution of $S(Q, \omega)$ with the resolution function, $R(Q, \omega)$:

$$S_M(Q, \omega) = S(Q, \omega) \otimes R(Q, \omega).$$  \hfill (3.30)

The characteristic time of each motion can be determined as the width of its quasielastic peak after deconvolution with the $R(Q, \omega)$.

Figure 3.7 shows $S_M(Q, \nu = \omega/(2\pi))$ of wet lysozyme at 0.50 h and vanadium (resolution function) at $T \approx 295$ K. The spectrum of wet lysozyme contains strong QES. Figure 3.8 presents $S_M(Q, \nu)$ of wet lysozyme at 0.50 h at $T \sim 150$ and 320 K in a broad frequency range. A big difference in QES intensity between 150 K and 320 K is observed.
Figure 3.7 $S(Q,\nu)$ of wet lysozyme ($\sim 0.50 \, \text{h}$) and vanadium (resolution function) at 295 K.

Figure 3.8. $S(Q,\nu)$ of wet lysozyme ($\sim 0.50 \, \text{h}$) at 150 K and 320 K.
Similar to the total elastic scattering, Elastic Incoherent Scattering Factor (EISF) is proportional to the probability that a target nucleus is found at its initial position \((1/Q)\) after some time \((t)\) corresponding to the resolution function. It is a function of momentum transfer, \(Q\). \(EISF(Q)\) represents the ratio of the pure elastic scattering component to total scattering:

\[
EISF(Q) = \frac{\Pi_{\text{Elastic}}}{\Pi_{\text{Elastic}} + \Pi_{\text{Quasielastic}}} 
\]

(3.31)

where \(\Pi_{\text{Elastic}}\) and \(\Pi_{\text{Quasielastic}}\) are the integrated intensities corresponding to the elastic and quasielastic parts of the spectra, respectively. An important advantage of analyzing the \(EISF(Q)\) is the possibility to estimate the geometry of the motions involved in the decay of the \(EISF(Q)\). For example, the \(EISF(Q)\) can be fitted by i) a three-site jump model, ii) a two-site jump model, or iii) the diffusive motions in a sphere model\(^{81,85}\):

i) \(EISF_{\text{HFRS}}(Q) = 1 - p_{\text{mobile}} + \frac{p_{\text{mobile}}}{3} \left[ 1 + 2 j_0(QR\sqrt{3}) \right]\)  

(3.32)

ii) \(EISF(Q) = 1 - p_{\text{mobile}} + \frac{p_{\text{mobile}}}{2} \left[ 1 + 2 j_0(Qd) \right]\)  

(3.33)

iii) \(EISF(Q) = 1 - p_{\text{mobile}} + p_{\text{mobile}} \left[ \frac{3 j_1(Qa)}{Qa} \right]^2\)  

(3.34)

The \(p_{\text{mobile}}\) is the mobile fraction involved with the relevant atomic motions. The \(R\), \(d\), and \(a\) are jump distances and a radius of the sphere. Figure 3.9 shows the \(Q\)-dependence behaviors of the \(EISF(Q)\) that follow different models for atomic motion.
Figure 3.9 Examples of $EISF(Q)$ for three-site jump model with $p_{mobile} = 1$ and $R = 1.03$, two-site jump model with $p_{mobile} = 1$ and $d = 3$, and diffusion in a sphere model with $p_{mobile} = 1$ and $a = 3$.

The features of the dynamic structure factors do not provide dynamically distinct relaxation modes in QES regions unless they are fitted to decay functions. Presentation of dynamic susceptibility, $\chi''(\nu)$, helps distinguish the relaxation modes as separate peaks (Figure 3.10). The definition of $\chi''(\nu)$ for the neutron scattering spectrum measured in the anti-Stokes region is

$$\chi''_{NS}(Q,\nu) \propto S(Q,\nu)/n_{B}(\nu)^{75}. \quad (3.35)$$

A few of the advantages of $\chi''(\nu)$ are well known: (i) relaxation modes appear in the $\chi''(\nu)$ presentation as distinct maxima at corresponding characteristic frequencies,
\( v = \frac{1}{2\pi \tau} \), where \( \tau \) is the characteristic relaxation time; (ii) results of scattering measurements can be directly compared with the spectra of dielectric or mechanical loss; (iii) the frequency dependence of the relaxation modes can be characterized by the power law, \( \chi''(\nu) \propto \nu^a \) for the low-frequency tail of the relaxation, and \( \chi''(\nu) \propto \nu^{-b} \) for high-frequency tail \((0 < a \text{ and } b \leq 1)\), thus showing their stretching. When \( a = b = 1 \), the relaxation mode is a single exponential (Lorentzian) process \(^7\).

Figure 3.10 Dynamic susceptibility, \( \chi''(\nu) \), of wet lysozyme (~0.50 h) at 295 K.
3.2.2.3 Multiple scattering

In order to obtain reasonable signals, samples with total neutron scattering \( \sim 10\% \) are usually utilized. This gives multiple scattering \( \sim 10\% \) of the total neutron scattering. More than 10\% multiple scattering significantly affects the quasi-elastic scattering (QES) spectra at low \( Q \). The first order incoherent scattering increases with \( Q \), while multiple scattering is essentially \( Q \)-independent. As a result, a contribution from multiple scattering is usually only significant at low \( Q \) and is negligible at higher \( Q \). Multiple scattering corrections are not a trivial task and complicate the analysis. No correction for multiple scattering has been made in the treatment of the data. To eliminate contributions due to possible multiple scattering in the analysis of QES spectra, we only considered intensities for \( Q > 0.5 \text{ Å}^{-1} \). This was not necessary in the analysis of the elastic intensities and thus a broader \( Q \)-range was utilized.

3.2.3 Spectrometers

There are a few types of instruments used for the measurements of the neutron scattering spectra. Protein dynamics is mostly studied with back scattering and time-of-flight spectrometers. NCNR has one backscattering and two time-of-flight spectrometers. For our purposes the main spectrometers are HFBS and DCS, since they provide a length scale \( (1/Q) \) and time window \( (1/\omega) \) suitable for studying vibration, conformational fluctuation and the structural relaxation of protein molecules.
3.2.3.1 High-Flux Backscattering Spectrometer (HFBS)

The basic principle of backscattering spectroscopy is that the wavelength spread, $\delta \lambda$, of a Bragg-diffracted neutron beam shortens as the scattering angle, $2\theta$, approaches $180^\circ$. Consequently, the best energy resolution is attainable, modulating the spread of the spacing, $d$ according to

$$\frac{\delta \lambda}{\lambda} \propto \frac{\delta d}{d} = \frac{\delta \theta}{\tan \theta}.$$  \hspace{1cm} (3.36)

The neutron beam used for HFBS has

- Neutron wavelength, $\lambda = 6.271 \, \text{Å}$,
- Wavevector, $k = 1.00 \, \text{Å}^{-1}$,
- Neutron speed, $v = 630.8 \, \text{m/s}$, and
- Neutron energy, $E = 2.08 \, \text{meV}$.

Figure 3.11 is a schematic view of HFBS at NCNR. First, an incident neutron beam passing through a converging guide is reflected by a phase space transform (PST) chopper and then led to the monochromator mounted on the Doppler drive. Interestingly, the energy (velocity) of the incident neutrons coming to a sample changes by the modulation of the Doppler motions of the monochromator to probe motions of the sample. The modulated neutrons interact with nuclei in the samples. The scattered neutrons are reflected by analyzer array. The analyzer is composed of groups of sharp resolution monochromators in concave geometry. Only neutrons with a particular final $E$ (2.08 meV) are reflected by the analyzer positioned at a few scattering angles, i.e. $Q (0.25 \, \text{Å}^{-1} < Q < 1.75 \, \text{Å}^{-1})$, and then focused into detectors.
The HFBS facilitates a dynamic range up to $\Delta E \sim \pm 36 \, \text{µeV} \sim \pm 8.64 \, \text{GHz}$ which is determined by the speed of the Doppler drive. It provides an energy resolution of $\delta E \sim 1 \, \text{µeV} \sim 0.24 \, \text{GHz}$. The corresponding accessible time scale lies between 1 ns and 20 ps. The energy resolution is usually measured by a vanadium spectrum that consists of 100% incoherent elastic scattering. Figure 3.12 presents the vanadium spectrum in the energy range, $\Delta E \sim \pm 36 \, \text{µeV}$ at $Q \sim 0.87 \, \text{Å}^{-1}$. The resolution is a Gaussian-like function.
3.2.3.2 Disk-Chopper Spectrometer (DCS)

The principle of the DCS is based on measuring the time \( t_{SD} \) that it takes the scattered neutrons to fly from sample to detectors. Figure 3.13 describes the geometrical principle of the DCS. The incident neutrons are pulsed and monochromated. The neutrons with well-defined energy, \( E_i \) or velocity \( v_i \), interact with the samples at time, \( t_s \). The number of scattered neutrons at the scattering angle is measured as a function of the time, \( t_D \), that it takes in its flight from the sample to a detector:

\[
I(2\theta, t_D) = N \Phi \frac{\partial^2 \sigma}{\partial \Omega \partial t} \Delta \Omega \Delta t
\]  

(3.37)

Figure 3.12 HFBS spectrum of vanadium with \( \Delta E \sim \pm 36 \mu \text{eV} \) at \( Q \sim 0.87 \text{ Å}^{-1} \). Symbols and solid line represent experimental data and the Gaussian fit to them.
where \( N \) is the number of scatterers and \( \Phi \) is the number of incident neutrons per unit area. The measured \( I(2\theta, t_D) \) can be converted to the \( S(Q, \omega) \) since the exact energy transfer can be estimated from the path distance \( D_{SD} \) and time-of-flight, \( t_{SD} \).

The energy resolution improves with an increase of the wavelength of the incident neutron waves. The usual range of wavelengths is from 2 Å to 9 Å. The energy resolution varies from \(~ 1500\) to \( 15 \mu\text{eV} \). The DCS with \( \lambda = 8 \text{ Å} \) gives an energy resolution, \( \delta E \sim 25 \mu\text{eV} (\sim 6 \text{GHz}) \) and elastic \( Q \)-range, \( 0.08 \text{ Å}^{-1} < Q < 1.46 \text{ Å}^{-1} \).\(^{89}\)

![Schematic diagram of the scattering geometry in the DCS](image)

Figure 3.13 Schematic diagram of the scattering geometry in the DCS.\(^{90}\)

A sketch of the DCS at NCNR is depicted in Figure 3.14. An optical filter eliminates most of the undesired beam components, like fast neutrons and gamma rays. The remaining fast neutrons are discarded using an oriented pyrolytic crystal filter. This filter is cooled down to \( \sim 77 \text{ K} \), facilitating a high transmission of the cold neutrons. Seven disk choppers placed in a series are used to obtain the monochromatic beam.
Scattered neutrons fly into 4 m long argon-filled path to 913 $^3$He detectors at different angles.

Figure 3.14 Plan view of DCS at NCNR $^{91}$.

3.3 Light scattering

3.3.1 Fundamental theory

Light scattering appears due to the interaction of the electromagnetic field of incident light with matter. According to theory $^{83}$, the electric component of the scattered light can be represented by

$$E_s = \frac{E_0}{4\pi R \epsilon_0} \exp(ik_f R) \int d^3r \exp[i(Q \cdot r - \omega t)] [n_f \cdot [k_f \times (k_f \times (\delta \epsilon(r,t) \cdot n_f))]]$$ (3.38)

where $E_0$ is the electric field of incident light, $\epsilon_0$ is the dielectric constant of the medium, $R$ is the distance from the scattering point, $Q$ is the scattering vector, $k_i$ and $k_f$ are the
wave vectors of the incident and scattered light, and \( n_i \) and \( n_f \) are the corresponding polarization.

Using the spatial Fourier transform of the dielectric constant, the equation (3.38) is now represented as

\[
E_s = \frac{-k_f^2 E_0}{4\pi R \varepsilon_0} \exp[i(k_f R - \omega_f t)] \delta\varepsilon_{if}(Q,t). \tag{3.39}
\]

Since the fluctuation of the dielectric constant happens along the initial and final polarization directions, it can be defined as

\[
\delta\varepsilon_{if}(Q,t) \equiv n_f \bullet \delta\varepsilon(Q,t) \bullet n_i. \tag{3.40}
\]

Consequently, time correlation function of the induced electric field between the initial and final state of the medium can be written as

\[
I(Q,R,t) = \langle E_s(R,0)E_s(R,t) \rangle = \frac{k_f^4 |E_0|^2}{16\pi^2 R^2 \varepsilon_0^2} < \delta\varepsilon_{if}(Q,0)\delta\varepsilon_{if}(Q,t) > \exp(-i\omega_f t). \tag{3.41}
\]

Apparent spectral density is represented as the time Fourier transform of the correlation function:

\[
I_\omega(Q,R,\omega_f) = \frac{k_f^4 |E_0|^2}{16\pi^2 R^2 \varepsilon_0^2} \int_{-\infty}^{+\infty} dt < \delta\varepsilon_{if}(Q,0)\delta\varepsilon_{if}(Q,t) > \exp[-i(\omega_f - \omega) t] \tag{3.42}
\]

According to the molecular approach, the scattering is caused by a fluctuation of the molecular dipole moment, \( \mu(t) \), that varies with time.

\[
\mu(t) = \alpha \bullet E(t) \tag{3.43}
\]

where the \( \alpha \) is a polarizability tensor. The scattered light with polarization of the molecule shows a proportional relationship to \( \alpha_i(t)\exp[iq \bullet r(t)] \). The \( \alpha_i(t) \) is the polarizability tensor of the molecule along the incident and final polarization, \( n_i \) and \( n_f \):
\[
\alpha_f(t) = n_f \cdot \alpha(t) \cdot n_i
\] (3.44)

Vibrational and rotational motions in the molecule modulate \(\alpha_f(t)\), while translational motions produce the phase factor, \(\exp[iq \cdot \mathbf{r}(t)]\).

The directional heterogeneity of the fluctuation of the dielectric constant and polarization in the isotropic sample medium leads to many optical components of the scattered light. Using polarizers and analyzers, one can selectively obtain specific polarization of the scattered light. Generally, polarized and depolarized components are used for understanding the dynamics in the medium. Polarized is defined as the same polarization component of the scattered light as that of the incoming light. The polarized components of scattered light reflect symmetric density fluctuations and translations. In contrast, depolarized scattering is defined as optical components with polarization perpendicular to that of the incoming light. The depolarized component provides information on shear deformations and rotations. For the completely isotropic system, the maximum ratio of the intensity of depolarized scattering to that of polarized scattering is 0.75. Real value depends on the type of system.

According to the equation (3.3), the probing length scale of light \(\sim 1/Q\) is much larger than the atomic and molecular levels due to a relatively long wavelength of the visible light. Therefore, larger scale motions, such as diffusion and phonon modes, can be studied by light scattering spectroscopy.
3.3.1.1 Raman scattering

Vibration modes that make a change in the molecular dipole moment lead to Raman scattering. The induced dipole moment can be represented by

\[
\mu = \alpha_0 E_0 \cos(2\pi \nu_0 t) + \frac{1}{2} \left( \frac{d\alpha}{dx} \right)_0 \nu_0 \left[ \cos(2\pi (\nu_0 + \nu_{\text{vib}}) t) + \cos(2\pi (\nu_0 - \nu_{\text{vib}}) t) \right] \quad (3.45)
\]

where \( \alpha_0 \) is the polarizability of the molecule, \( \nu_0 \) and \( \nu_{\text{vib}} \) are the frequency of incident light and of the relevant vibration, and \( \left( \frac{d\alpha}{dx} \right)_0 \) is the variation of polarizability along the displacement led by the vibration.

As discussed in section 3.1.3, according to quantum theory the inelastic Raman scattering caused by vibration motions is split into two regions due to energy loss and energy gain, i.e. Stokes and anti-Stokes. By comparing their intensities, the real temperature of a measured sample can be determined by equation (3.9). Advances in Raman spectrometers makes it possible to measure the low-frequency region down to \( \sim 100 \text{ GHz} \) (\( \sim 3 \text{ cm}^{-1} \)) where fast dynamics and collective vibration motions are observable. Details on the Raman spectrometer will be introduced in section 3.3.2.1.

3.3.1.2 Interferometry

Interference is the superposition of waves. It provides a new wave pattern. The principle of superposition of waves states that the resultant amplitude at a point is equal to the sum of the amplitudes of different waves at that point. It is possible to explore a lower-frequency range in the interferometer due to its high energy-resolution than in
Raman scattering. Combining the interferometer with the Raman scattering spectrometer covers a broad-frequency range from 100 MHz (~ 0.003 cm\(^{-1}\)) to 1 THz (~ 33 cm\(^{-1}\)).

3.3.2 Data treatment

3.3.2.1 Raman

Two corrections were made: i) the subtraction of dark counts and ii) the subtraction of the fluorescence background. Figure 3.15 shows the procedure used to correct the raw spectrum and obtain the spectral density of the sample. Dark counts are independent of samples. The contribution of fluorescence to the spectrum can be caused by impurities and the intrinsic fluorescence of some amino acid groups (e.g. Trp) in protein molecules. Generally, it is a smooth spectrum and we approximated it as a linear function of frequency in the low-frequency range of our interest.

Figure 3.15 (c) represents spectral density, \(I_n(\nu)\), after taking into account Bose statistics\(^{92}\). As opposed to neutron scattering measurements, light scattering measurements provide the Stokes region of the spectrum, and then

\[
I_n(\nu) = I(\nu)/\{\nu[n(\nu)+1]\}
\]

(3.46)

where \(I(\nu)\) is Raman scattering intensity after all corrections, \(\nu\) is the frequency shift. Therefore, the light scattering susceptibility is

\[
\chi''(\nu) = \nu I_n(\nu) = I(\nu)/[n(\nu)+1].
\]

(3.47)
3.3.2.2 Tandem Fabry-Perot Interferometer (TFPI)

Three corrections were for TFPI spectra: i) dark count subtraction, ii) normalization by a transmission spectrum of the system, and iii) fluorescence subtraction. The dark counts were measured with no signal and were subtracted from the $I(\nu)$. The transmission spectrum of the system was measured with white light from a lamp. The spectra were corrected for dark counts and normalized by the intensity of the light spectrum:

Figure 3.15 Example of data treatment of Raman spectrum for dry lysozyme ($\sim 0.03 \ h$) at 295 K. (a) Solid line: Raman scattering intensity after the subtraction of dark counts; Dash line: fluorescence contribution. (b) Raman scattering intensity after the subtraction of the contribution of fluorescence. (c) Spectral density, $I_n(\nu)$.
\[ I(\nu) = \frac{(I_{\text{measured}}(\nu) - I_{\text{dark}})}{(I_{\text{lamp}}(\nu) - I_{\text{dark}})}. \]  

(3.48)

Fluorescence was subtracted in the same way as in the Raman spectrum, even though its contribution is not crucial for the TFPI spectrum.

Figure 3.16 shows the procedure of joining the TFPI spectrum with a mirror distance of 0.4 mm (FSR ~ 360 GHz) and the Raman spectrum of a hydrated protein sample (~ 0.18 \( \mu \)). In principle, longer mirror distances like 3 mm (~ 50 GHz) and 15 mm (~ 10 GHz) are available for our TFPI, but the light scattering spectra of our protein samples were spoiled at the frequency range due to multiple scattering and a strong contribution of Brillouin scattering.

Figure 3.16 Raman and TFPI (FSR 360 GHz)\( \chi''(\nu) \) spectra of hydrated lysozyme (~ 0.18 \( \mu \)). (a) Before joint. (b) Joining spectrum, the dash line shows the joining point.
3.3.3 Spectrometers

A usual optical scheme for light scattering measurements is depicted in Figure 3.17. So-called back scattering geometry was used for both Raman and TFPI scattering measurements because it offers a much stronger intensity due to its large scattering volume.

![Diagram of light scattering setup](image)

Figure 3.17 Basic scheme of the fundamental optics used for light scattering measurements. The dashed line represents a reference beam used only for the TFPI.

The polarization rotator and polarizer were used to produce the desired incident light with specific polarization. The analyzer was set to select scattered light with specific polarization. Two lenses served to get the scattered beam lined up parallel and focused before and after the analyzer.
3.3.3.1 Laser

Light scattering spectroscopy needs a light source with high intensity, a well-selected energy level, and high polarization. Laser (Light Amplification by Stimulated Emission of Radiation) is the light source with such properties. In our measurements, green Ar$^{++}$ laser with wavelength $\sim 514.5$ nm was used as a light source. Its resolution is $\sim 5$ GHz ($\sim 0.17$ cm$^{-1}$) which is sharp enough for Raman spectroscopy. However, a narrow interference filter is needed to suppress plasma lines of spontaneous radiation coming from the laser.

A much higher resolution can be realized by selecting a single mode using etalon inside the laser cavity stabilized by an external iodine cell. In practice, a laser used for TFPI measurements was equipped with the iodine cell frequency loc right in front of the laser. The iodine cell (AC3504) has iodine vapor. Its absorption band is extremely narrow at 514.5 nm. A small part of the laser beam is sampled and its feedback is sent to the temperature controller of the laser cavity to stabilize the laser against any drift in frequency. The temperature controller changes the laser cavity by heating and tuning the drift back. By this means, a stable $\lambda$ can be achieved.

3.3.3.2 Raman spectrometer

The Raman spectrometer used for our study is Jobin-Yvon T64000 consisting of three monochromators. Figure 3.18 shows the simple scheme of the so-called subtractive mode that is effective for low-frequency Raman scattering measurements because it allows for the use of a multi-channel detector.
The gratings serve to disperse different wavelengths and the slits block undesired wavelengths. In this subtractive mode, elastic scattering is strongly suppressed and a low-frequency range down to 150 GHz is possible to measure. One can go down to ~ 30 to 50 GHz (~ 1 to 1.5 cm\(^{-1}\)) by using single channel detectors in additive mode. But it takes a much longer time to measure.

The type of detector used is a CCD camera (1024 × 256 pixels). The efficiency of the CCD camera in terms of signal to noise ratio can be improved by cooling it to \(T \sim 140\) K using liquid nitrogen. The advantage of a CCD camera over the photo-multiplier is that it can give the spectrum over a broad-frequency range at the same time.
3.3.3.3 TFPI

Sandercock TFPI was employed for our measurements in a low-frequency range down to 100 MHz. Classic Fabry-Perot interferometers consist of one set of two mirrors facing each other, so-called etalon. Some portion of light can transmit the mirrors or be reflected, depending on the transmission efficiency of the mirrors. The transmission intensity of the light as a result of interference can be represented by

\[ I_T = \frac{I_0}{1 + \Gamma \sin^2 (2\pi L / \lambda_0)^{95}}. \]  

(3.49)

where \( I_0 \) and \( \lambda_0 \) are the intensity and wavelength of the incident light, respectively, and \( L \) is the distance between two mirrors. The \( \Gamma \) stands for the finesse of the two mirrors, depending on their reflectivity, \( R \):

\[ \Gamma = \frac{4R}{(1 - R)^2}. \]  

(3.50)

According to the equation (3.49), the maximum possible transmission is obtained when \( L = \frac{1}{2} p\lambda_0 \) (\( p \) is an integer number) according to the interference principle.

Figure 3.19 Transmission function of a single interferometer.\(^{96}\)
In Figure 3.19, the $\Delta \lambda$ is the spacing distance between successive wavelengths, called the free spectral range (FSR). The $\delta \lambda$ is full width at half maximum intensity, describing the instrumental resolution. The phase shift of $2p \pi \pm \frac{\delta \lambda}{2}$ corresponds to the half maximum intensity:

$$\frac{1}{2} = \frac{1}{1 + \Gamma \sin^2 (2p \pi \pm \frac{\delta \lambda}{2})}.$$  \hspace{1cm} (3.51)

As $\Gamma$ increases, the transmission peak is getting sharper since $\delta \lambda \rightarrow 0$. The equation (3.51) turns into

$$\frac{1}{2} = \frac{1}{1 + \Gamma \left(\frac{\delta \lambda}{4}\right)^2}.$$  \hspace{1cm} (3.52)

The experimental finesse of the etalon, $F$, is defined by the ratio of the $\Delta \lambda$ to $\delta \lambda$ and is related to the $\Gamma$ by

$$\Gamma = \left(\frac{2F}{\pi}\right)^2.$$  \hspace{1cm} (3.53)

According to the relationship, $L = \frac{1}{2} p \lambda_0$, the maximum transmission of the different wavelengths can be obtained by changing the distance between mirrors ($L$) and they appear repeatedly due to interference. This is the problem of a single Fabry-Perot etalon: the measured intensity is a superposition of signals coming from different transmission orders. Using two sets of the etalons in tandem solves the problem. The schematic diagram of tandem etalons (FP1 and FP2) is shown in Figure 3.20.
Figure 3.20 Top view of tandem Fabry-Perot interferometer (Sandercock model). The $L_1$ and $L_2$ are mirror distances of the FP1 and FP2 ($L_1 \neq L_2$). Only the wavelength that satisfies the two conditions at the same time can transmit both etalons:

$$\lambda = \frac{2L_1}{p} = \frac{2L_2}{q}$$  \hspace{1cm} (3.54)

where the $p$ and $q$ are integers.

Figure 3.20 shows that $L_1$ and $L_2$ are always different because of the angle $\theta$ between two etalons:

$$L_2 = L_1 \cos \theta.$$  \hspace{1cm} (3.55)

The variations of the distances of the etalons are correlated with each other according to the geometry:

$$\delta L_2 = \delta L_1 \cos \theta.$$  \hspace{1cm} (3.56)

From the equations (3.54), (3.55) and (3.56), the successive positions of the etalons should satisfy that
\[
\frac{L_1 + \Delta L_1}{L_2 + \Delta L_2} = \frac{L_1}{L_2} = \frac{1}{\cos \theta} = \frac{p}{q}
\]  

(3.57)

to get the maximum transmission intensity. Therefore, the appearance of a multiple peak in right successive positions is now avoided (Figure 3.21). The experimental ratio of \( p \) to \( q \) is 5:4. This value means that the maximum intensities of 19 consecutive neighbor transmission peaks are suppressed. Therefore, the use of TFPI provides a much broader window to monitor the desired wavelengths (or frequency shifts) than a single FPI.

In our optical systems, narrow interference filters with bandwidth ~ 1.2 THz and ~ 120 GHz were used to suppress the contribution of multiple maximum peaks at 20\(^{th}\) transmission peak. The first broader one was used for larger mirror distances like 0.4 (~ 360 GHz) and 3 mm (~ 48 GHz) and the second narrower one for smaller mirror distances like 15 mm (~ 9 GHz).

![Figure 3.21 Transmission intensity of the incident light passing through two etalons in tandem mode](image)

Figure 3.21 Transmission intensity of the incident light passing through two etalons in tandem mode.
3.4 Sample preparation

Hen-egg white (HEW) lysozyme was used as the model protein in this research. Three times dialyzed and lyophilized HEW lysozyme (E.C. 3.2.1.17, L6876) with 14.3 k Dal ton was purchased from Sigma-Aldrich. The basic manipulation of the lysozyme for neutron scattering measurements was done in Dr. Gregory’s labs in the Department of Chemistry at Kent State University, Kent, Ohio. No special treatment of lysozyme was undertaken for the light scattering measurements. The following sections describe the details of sample preparation for the neutron and light scattering measurements.

3.4.1 Samples for neutron scattering measurements

As-received dry HEW lysozyme was dissolved in Millipore distilled water at a concentration of 20 mg/ml. The protein solution was dialyzed to remove salts (sodium acetate and sodium chloride) for 48 hours. The dialyzed lysozyme was lyophilized in vacuum at $5 \times 10^{-6}$ mHg. The obtained dry protein powder was dissolved in D$_2$O (Sigma-Aldrich) at 25 mg/ml, to replace exchangeable hydrogen atoms in the lysozyme molecules with deuterium atoms. In principle, the number of exchangeable protons was ~ 265 among total hydrogen atoms ~ 960. The exchange reaction proceeded for 36 hours at $T \sim 318$ K at pD ~ 8.4 using NaOD and DCl. The solution was cooled to room temperature and its pD was adjusted to ~ 3.8. The solution was filtered with a glass filter (pore size of the filter 0.2 µm) to remove possible aggregates. It was known from mass spectroscopy that the total number of exchanged protons in a lysozyme molecule is between 260 and 265 $^{93}$. After lyophilization of the solution in vacuum at $5 \times 10^{-6}$ mHg,
deuterium-exchanged lysozyme powder was obtained and stored in a refrigerator. In practice, the lyophilized powder is not completely dry, but rather it has a hydration level \( \sim 0.10 \ h \).

The desired hydration level of the deuterium-exchanged lysozyme powder was achieved by using isopiestic equilibration made possible by saturated solutions of LiCl, NaCl, and K₂SO₄ in D₂O. The lysozyme was placed into a dessicator containing one of the saturated salt-solutions. Their relative humidity resulted in hydration levels of \( h \sim 0.05, 0.18, \) and 0.30, respectively. Samples with 0.42, 0.45, 0.50 and 0.80 \( h \) were prepared by adding D₂O to the sample with 0.30 \( h \) and equilibrating the powders for at least 12 hours.

The mass of the samples was measured before and after the neutron scattering experiments and no loss of water was detected. The hydration levels were determined after performing neutron scattering measurements from the observed mass change on drying samples by thermogravimetric analysis (TGA).

Lysozyme in completely deuterated glycerol and lysozyme in partially deuterated trehalose were produced at a 1 to 1 ratio by Dr. Marcus Cicerone at NIST. Figure 3.22 shows the chemical structures of the glycerol and trehalose molecules. They made the mixtures directly in aluminum foil. It has extremely low scattering cross-sections for the neutron. The trehalose was partially deuterated due to non-exchangeable hydrogen atoms among 14 hydrogen atoms in the C-H groups. According to maximum conversion, the exchange reaction of a trehalose molecule can lead to deuteration of 10 hydrogen atoms in C-H groups and 8 hydrogen atoms in O-H groups. Consequently, deuteration conversion is 81.8 %, i.e. 18/22.
In practice, however, only 5.32 hydrogen atoms out of 14 in the C-H groups were found to be deuterated. $^1$H NMR measurement of the partially deuterated trehalose determined the level of deuteration as ~ 63 %. The 1 to 1 weight ratio used between lysozyme and trehalose molecules resulted in a ~ 30 % neutron scattering contribution from the non-exchangeable hydrogen atoms of the trehalose molecules.

Samples were wrapped in aluminum foil. One of the most important steps in sample preparation was the proper sample thickness so that total neutron scattering should be less than 10 %. Otherwise, multiple-scattering contribution becomes significant. The process of calculating the sample thickness is described on the NCNR web page $^97$.

![Chemical structures](image)

Figure 3.22 Chemical structures of (a) glycerol and (b) trehalose.
3.4.2 Samples for light scattering measurements

The as-received HEW lysozyme was used as purchased without further purification. Eight different hydration levels were prepared. LiCl, MgCl$_2$$\cdot$6H$_2$O, Mg(NO$_3$)$_2$$\cdot$6H$_2$O, NaCl, and K$_2$SO$_4$ saturated solutions with H$_2$O were used to produce lysozyme samples with 0.03, 0.10, 0.15, 0.20, and 0.35 $h$, respectively. Samples with 0.50, 0.75, and 0.85 $h$ were obtained by the addition of the appropriate amounts of water into the samples with 0.35 $h$. The hydration levels were determined by TGA as described above. Samples were prepared with a thickness ~ 0.5 - 1 mm by sealing the powder between sapphire windows which contributed a negligible intensity to the light scattering spectra in the frequency range of our interest ($\nu < 3$ THz).
CHAPTER IV

ONSETS OF ANHARMONICITY IN PROTEIN DYNAMICS

4.1 Introduction

As discussed in section 2.5.2, it has been observed that the $\langle x^2 \rangle$ varies linearly with temperature, $\langle x^2 \rangle \propto T$, up to $\sim 300 - 350$ K in dry proteins and up to $T_D \sim 180 - 230$ K in sufficiently hydrated proteins $^7,8,30,49$. The dynamic modes represented by the linear variations of $\langle x^2 \rangle$ with temperature were generally interpreted as harmonic vibrations of atoms and residues in protein molecules.

Strong anharmonic behavior of $\langle x^2 \rangle$ has been observed in the sufficiently hydrated proteins DNA and RNA above the $T_D \sim 180 - 230$ K $^{26,28,33,35,37,53,54,98}$. These variations of $\langle x^2 \rangle$ have been observed in neutron scattering, X-ray diffraction, and Mössbauer spectroscopic experiments and predicted by computer simulations for all hydrated proteins, DNA and RNA studied to date.

The onset of anharmonicity is believed to be important, since the onset of a number of protein functions coincides with the $T_D$ $^{26,33,36,53,54}$, although some exceptions have been reported $^{64-66}$. The dynamic transition has been ascribed to a slow relaxation process that appears in the nanosecond time range at $T > T_D$ and is suppressed in dry samples where no dynamical transition has been observed $^{30,49,80,93}$. Therefore, according
to the conventional view, harmonic vibrations dominate dynamics over the entire temperature range in dry proteins and below the $T_D$ in the hydrated proteins.

In this chapter, we discuss the existence of two temperature regions where the onset of anharmonicity in the dynamics occurs: i) one at low temperature that appears in all samples regardless of hydration and is primarily ascribed to methyl group rotation, and ii) the well-known dynamical transition at $T_D \sim 200 - 220$ K that appears only in the lysozyme samples with $h > 0.2$.

4.2 Samples and techniques

Samples of dry (0.05 $h$) and D$_2$O-hydrated lysozyme (0.18, 0.30, and 0.45 $h$) for neutron scattering measurements were prepared as explained in the section 3.4.1. Elastic scans were conducted upon cooling from 300 K down to 10 K at a 0.7 K/min using HFBS with energy resolution $\delta E \sim 1$ μeV, and wave vector range $0.25\text{Å}^{-1} < Q < 1.75\text{Å}^{-1}$. Energy-resolved spectra were obtained using HFBS with an energy range ±36 μeV, and the same energy resolution and $Q$ range.

4.3 Results

The $\langle x^2(T) \rangle$ of protein molecules was estimated using the Gaussian approximation based on the equation (3.21):

$$\langle x^2(T) \rangle = -3Q^{-2} \ln \left[ I_{el}(Q, T) / I_{el}(Q, 10K) \right] \quad (4.1)$$

where $I_{el}(Q, T)$ is an elastic incoherent neutron scattering intensity obtained at a particular $Q$ and $T$. This approximation works in the limit $Q \rightarrow 0$, thus the lowest $Q$- range is $0.35 \text{ Å}^{-1}$
\(< Q < 1.00 \text{ Å}^{-1} (0.1 \text{ Å}^{-2} < Q^2 < 1 \text{ Å}^{-2})\) was used. The \(< x^2(T) >\) reflects a variety of hydrogen atomic motions (vibrations, rotations, diffusive motions, etc.) in lysozyme on a time scale faster than ~ 1 ns (defined by the resolution of HFBS).

In Figure 4.1, the \(< x^2(T) >\) shows the characteristic dynamic transition at \(T_D \sim 200 - 220\) K, but only in samples with \(h > 0.2\). More interesting is the existence of a low-temperature onset of anharmonicity at \(T \sim 80 – 100\) K that appears in \(< x^2(T) >\) of all the samples regardless of the hydration level (inset in Figure 4.1). This observation clearly contradicts the conventional view (See e.g. \(26,30,34,36,37,42,43,64,65,99,)\) that \(< x^2(T) >\) in proteins exhibits only harmonic dynamics below \(T_D\).

![Figure 4.1](image)

Figure 4.1 \(< x^2(T) >\) vs \(T\) at different hydration levels (\(h\): ■ 0.05; ○ 0.18; ▲ 0.30; ▽ 0.45). Inset shows low temperature onset of anharmonicity at \(T \sim 80 – 100\) K with a line to guide the eyes.

Analysis of the quasielastic scattering (QES) spectra obtained from the HFBS measurements in the frequency range \(\sim 0.2\) GHz \(< \nu < 8.5\) GHz did not reveal any significant dependence of the width, \(\Gamma\), on \(Q\) in the measured \(Q\)-range \(100\). Figure 4.2
presents the QES spectra summed over all $Q$. The most surprising result is that a strong QES intensity is observed in the dry sample at $T \sim 295$ K and even at $T \sim 150$ K. According to the traditional view of harmonic dynamics, dry proteins are supposed to have negligible QES because their relaxation motions are assumed to be totally suppressed. However, our result indicates that a significant relaxation process is present in the dry protein at $T \sim 295$ and 150 K. This unknown relaxation mode is directly related to the motions involved in the low-temperature onset of anharmonicity. The QES intensity remains essentially unchanged when the hydration level is raised to $h \sim 0.18$. However, it increases strongly in hydrated proteins of $0.30 \, h$.

Figure 4.2 $S(Q, \nu)$ summed over all $Q$ and normalized by the mass of lysozyme. Main figure presents the $S(Q, \nu)$ of dry lysozyme of $0.05 \, h$ ($\bigcirc$), wet lysozyme of $0.18 \, h$ ($\cdots$) and $0.30 \, h$ ($\blacktriangle$), and vanadium($\bigtriangleup$) at $T \sim 295$ K. Inset shows the $S(Q, \nu)$ of lysozyme of $0.05 \, h$ ($\bigcirc$) at 150 K and resolution function presented by the spectrum of wet lysozyme of $0.42 \, h$ ($\bigtriangleup$) at 10 K.
4.4 Discussion

4.4.1 Low-temperature onset of anharmonicity and methyl group dynamics

In Figure 4.1, the most intriguing observation is of the existence of the low-temperature onset of anharmonicity that appears in all the samples irrespective of the hydration level. A survey of neutron scattering data from the literature shows that the changes in \(<x^2(T)>\) at \(T \sim 100\ \text{K}\) is apparent in the data of a number of proteins \(^{26,99-101}\), but its microscopic nature has not been discussed in detail. These observations support the conclusion that different anharmonic motions possibly exist in dry and wet proteins even at low temperatures. In most cases, the non-linear behaviors of \(<x^2(T)>\) at low temperatures have not been identified, because most measurements did not extend below 50 K and the statistics at low temperatures are poor. However, coming to an understanding of its nature is very important in finding the functionally important protein motions among many others. To the best of our knowledge, the clear characteristics of the non-linear behavior of \(<x^2(T)>\) found at low temperatures have not been presented.

Cordone et al. \(^{101}\) attributed the observed increase in \(<x^2(T)>\) at \(T \sim 100\ \text{K}\) to the quantum effects for zero-point vibrations with characteristic energy \(\nu_{vib} \sim 210\ \text{cm}^{-1} (\sim 6\ \text{THz})\). It is possible to interpret our data with a similar value of \(\nu_{vib}\). However, there are no strong vibrational modes in this frequency range (See Figure 5.2.). The boson peak observed at \(\nu \sim 30\ \text{cm}^{-1} (\sim 1\ \text{THz})\) dominates the neutron scattering spectra of proteins and the corresponding quantum effects are only relevant at much lower temperatures. Thus the low-temperature onset of anharmonic dynamics cannot be ascribed to the quantum mechanical contribution of a vibration.
This conclusion agrees with the results of previously published MD-simulations of an isolated, essentially dehydrated, molecule of bovine pancreatic trypsin inhibitor\textsuperscript{102}. Using normal mode analysis to characterize the temperature dependence of the dynamics, the authors noted an onset of anharmonicity in $\langle x^2(T) \rangle$ at $T \sim 100$ K. They showed that it cannot be ascribed to quantum effects because no significant difference has been observed between classical and quantum mechanical calculations at $T > 100$ K. However, they did not discuss the microscopic nature of the observed anharmonicity and only indicated that not all of the hydrogen atoms are equally involved in the observed increase in $\langle x^2(T) \rangle$. A strong QES contribution observed in the spectra of the dry protein at $T \sim 295$ and $150$ K (Figure 4.2) means that at least some relaxation modes are activated even in a dry sample at low temperatures.

We propose that the activation of methyl group rotational dynamics is primarily responsible for the low-temperature onset of anharmonicity because 26\% of all non-exchangeable hydrogen atoms in lysozyme are on methyl groups. Earlier NMR studies have demonstrated that methyl group rotational dynamics in proteins and polypeptides are activated at low temperatures independent of the hydration level\textsuperscript{103-105}. Also, earlier neutron scattering studies of proteins and polypeptides indicate a significant contribution from methyl group dynamics\textsuperscript{44,106}. Moreover, detailed NMR studies suggest that methyl group rotations contribute $\sim 80$ \% of the total proton relaxation in dry lysozyme with the remaining proton relaxation occurring in flexible O-H and N-H groups\textsuperscript{103}. These particular hydrogen atoms do not exist in our study since they were exchanged with deuterium atoms in our samples. Thus, a higher relative contribution of methyl group dynamics should be expected in our measurements. A recent analysis of neutron
scattering data in dry myoglobin has also ascribed \( \approx 80 \% \) of the observed relaxation to methyl group rotational dynamics\(^{106}\).

We can test whether temperature, frequency, and \( Q \)-dependence of the observed QES contribution in dry lysozyme can be ascribed to methyl group rotational dynamics by analytical treatments of experimental data. \( S(Q,\nu) \) for methyl group rotation can be described by a three-site jump model that includes a distribution of relaxation time, \( \tau_i \), presented by a sum of Lorentzians \( L_i(\nu,\tau_i) \)\(^{107}\):

\[
S_{methyl}(Q,\nu) = \frac{1}{3} \left[ 1 + 2 j_0(QR\sqrt{3}) \right] \delta(\nu) + \frac{2}{3} \left[ 1 - j_0(QR\sqrt{3}) \right] \sum_i L_i(\nu,\tau_i) \tag{4.2}
\]

The \( j_0(x) \) is the zeroth order Bessel function and \( R \) is the radius of methyl group rotation. The \( \tau_i \) is residence time on each site: \( 1.5 \tau_i = \tau \) (mean residence time). The characteristic time (\( \tau \)) of methyl group rotation usually follows an Arrhenius temperature dependence,

\[
\tau_i = \tau_0 \exp(E_i/kT), \text{ with } \tau_0 = \frac{1}{2\pi} 1.8 \times 10^{-13} \text{ s} \tag{107}.
\]

The activation energy \( E_i \) depends on the type of amino acid residue and the local environment\(^{108-111}\).

The distribution of energy barriers \( g(E_i) \) that control methyl group rotation can be estimated from the temperature variations of the elastic intensity \( I_{el}(Q,T) \)\(^{107}\):

\[
I_{el}(Q,T,\nu \sim 0) \propto DW(Q,T) \left[ 1 - p_{methyl} + p_{methyl} \int_{-\infty}^{\infty} S_{methyl}(Q,\nu')R(\nu - \nu')d\nu' \right]^{v' = 0}
\]

\[
\propto DW(Q,T) \left[ \text{const}(Q) + \int_{-\infty}^{\infty} \left( \int_{0}^{\infty} R(\nu - \nu')g(E_i)\frac{1}{\pi} \frac{\tau_i}{1 + \nu'^2} dE_i dv' \right) \right]. \tag{4.3}
\]

Here \( DW(Q,T) = \exp(-Q^2<x_{vib}^2>/3) \) is the Debye-Waller factor, and \( <x_{vib}^2> \) is the vibrational mean-squared atomic displacement that was estimated from the slope of \( <x^2> \) versus \( T \) at \( T < 100 \text{ K} \) (solid line in inset of Figure 4.1). The \( p_{methyl} \) is the fraction of...
hydrogen atoms involved in the methyl group rotation and \( R(\nu) \) is the resolution function of the spectrometer. The latter was approximated by a Gaussian function with full width at half maximum \( \sim 1.2 \, \mu\text{eV} \) (290 MHz).

Our analysis shows that a single energy barrier cannot describe the experimental data for \( I_{el}(Q,T)/DW(Q,T) \) in the dry sample (Figure 4.3). Assuming a Gaussian distribution of energy barriers, \( g(E_i) \propto \exp[-(E_0-E_i)^2/2\Delta E^2] \), the equation (4.3) provides a good description of the data at different \( Q \) with \( E_0 \sim 16.6 \, \text{kJ/mol} \) and \( \Delta E \sim 5.8 \, \text{kJ/mol} \) (Figure 4.3). The estimated distribution of energy barriers \( g(E_i) \) agrees well with previously published NMR data for methyl group rotation in dry lysozyme \(^{103-105,111}\).

The distribution of energy barriers allows for the calculation of the relaxation spectrum:

\[
\sum_Q S(Q,\nu) \propto [\text{const} + S_{\text{methyl}}(\nu)] \otimes R(\nu) = \text{const} + \int_{-\infty}^{\infty} R(\nu - \nu') \int_0^{\infty} g(E_i) \frac{1}{\pi} \frac{\tau_i}{1 + \nu'^2 \tau_i^2} \nu d\nu', \quad (4.4)
\]

where \( \text{const} \) accounts for the contribution of faster processes. As shown in Figure 4.4, the equation (4.4) provides a good description of the QES spectrum of dry lysozyme at \( T \sim 295 \, \text{K} \), using only a single adjustable parameter, \( \text{const} \).
Figure 4.3 Temperature variations of elastic intensity, $I_{el}(Q,T)$, in a dry sample (0.05 h) corrected for the Debye-Waller factor (○). Solid lines are result of the fit using the equation (4.2) with a single barrier $E\sim 16$ kJ/mol (thin line) and a Gaussian distribution of the energy barriers with $E_0 \sim 16.6$ kJ/mol and $\Delta E \sim 5.8$ kJ/mol (thick line) for the methyl group rotation.

Figure 4.4 $S(Q,\nu)$ of the dry protein at $T \sim 295$ K (symbols) and the fit using the equation (4.4) (solid line).
The equation (4.2) also provides a description of the $Q$-dependence of the methyl group spectra. There is a contribution from an elastic scattering component (the first term) and a quasielastic component (the second term). One of the methods to analyze the data is to decompose the experimental spectra as EISF plus a QES component (See e.g. 25.). $EISF(Q)$ provides information on the geometry of the atomic motion and the fraction of hydrogen atoms involved. The usual method of approximation used to calculate the $EISF(Q)$ is to present the spectrum as a sum of a delta-function (elastic scattering) and a Lorentzian (quasielastic scattering), both convoluted with the resolution function of the spectrometer. The $EISF(Q)$ is calculated as a ratio of elastic scattering to the total elastic and quasielastic scattering. We applied this procedure to an analysis of the QES spectra of dry sample at each $Q$ and obtained $EISF(Q)$ (Figure 4.5). In the case of methyl group rotation, it should be described by the following equation involving a three-site jump model (equation (3.32)) (38,59):

$$EISF(Q) = 1 - P_{methyl} + \frac{P_{methyl}}{3} \left[ 1 + 2 j_0(QR\sqrt{3}) \right].$$  

Here the term $(1 - P_{methyl})$ represents the elastic intensity coming from non-methyl atoms and the remaining terms represent the elastic intensity due to methyl groups (the first term in the equation (4.2) weighted by $P_{methyl}$). The best fit of the data to the equation (4.5) was obtained with $P_{methyl} \sim 0.14 \pm 0.01$ and $R \sim 1.3 \pm 0.2$ Å (Figure 4.5). While the characteristic radius appears to be close to the radius of a methyl group ($R_{methyl} \sim 1$ Å), $P_{methyl} \sim 0.14$ is significantly lower than the fraction of hydrogen atoms on methyl groups in lysozyme, $\sim 0.26$. 
It is known that approximating the QES spectrum by a single Lorentzian is not accurate. Our earlier analysis (Figure 4.3) shows that use of a single energy barrier (or single \( \tau \)) does not describe the experimental data. Instead a distribution of \( \tau \) (distribution of Lorentzians) should be used. Thus, we used the spectra (Figure 4.4) instead of a Lorentzian in the calculations of \( EISF(Q) \). The resulting \( EISF(Q) \) appears to be much lower than the \( EISF(Q) \) obtained using the single Lorentzian approximation (Figure 4.5) because the relaxations that occur outside of our frequency window are also taken into account.

The best fit of the data to the equation (4.5) results in \( R \sim 1.3 \pm 0.2 \) Å and \( p_{\text{methyl}} \sim 0.25 \pm 0.03 \) which is in a very good agreement with the expectations for methyl group rotational dynamics in lysozyme (Figure 4.5). This analysis clearly shows that the traditionally used approximation of the QES spectra by a single Lorentzian (See e.g. \textsuperscript{25.}) is not accurate and can give misleading quantitative results.

Figure 4.5 \( EISF(Q) \) for the dry sample obtained using single Lorentzian approximation for QES (■) and using distribution of energy barriers (○). Solid lines are the fit to the equation (4.4).
The interpretation presented above is further supported by an analysis of simulation data performed by Dr. Curtis at NIST. In Figure 4.6, contributions to $\langle x^2 \rangle$ from methyl and non-methyl hydrogen atoms are easily separated in the simulation. Such an analysis clearly shows that the major contribution to the low-temperature anharmonicity comes from methyl group dynamics. Although methyl groups contain only about 25% of the hydrogen atoms, they contribute about 65% of anharmonicity even at $T \sim 200$ K. Note that the remaining anharmonicity arising from non-methyl hydrogen atoms is also apparent in the data at temperatures above 150 K.

![Graph showing contributions from methyl and non-methyl hydrogen atoms](image)

**Figure 4.6** Low-temperature behavior of $\langle x^2 \rangle$ from MD-simulations for wet lysozyme: The lines are an extrapolation of low-temperature harmonic behavior. The contributions of methyl and non-methyl atoms are presented separately. From Dr. Curtis at NIST.

MD-simulations for hydrated lysozyme at $T \sim 295$ K show that the methyl group dynamics can be described by a broad distribution of rotational correlation times with an average $\tau \sim 75$ ps (Figure 4.7(a)). This $\tau$ corresponds to an average energy barrier $\sim 16$
kJ/mol that agrees well with the value of $E_0$ obtained from the analysis of the neutron scattering data. Simulations, however, can provide a microscopic explanation for the broad distribution of relaxation times and corresponding energy barriers. The fastest methyl rotation ($\tau \sim 20 - 30$ ps) appears in methionine residues where there is an extremely low barrier to rotation in the thioether fragment. The longest $\tau (> 200$ ps) appears in some alanine and threonine residues (Figure 4.7 (a)).

![Figure 4.7 Distribution of effective rotational correlation times ($\tau$) for methyl groups in lysozyme obtained from molecular dynamics simulations for wet sample at $h \sim 0.43$: (a) presented as a histogram with residue names marked on top and (b) presented for each residue separately. From Dr. Curtis at NIST.](image)
However, the characteristic $\tau$ for most of the residues depends also on their positions in the protein (Figure 4.7(b)). For example, $\tau$ varies from ~ 25 - 40 ps (Ala-82, Ala-107) up to $\geq$ 200 ps (Ala-10, Ala-11) in Ala-residues and from ~ 35 ps (Thr-89) up to $\geq$ 200 ps (Thr-40) in Thr-residues. Thus, the broad distribution of relaxation times observed for methyl groups is dictated by the particular parent amino-acid residues containing the methyl groups and the local environment within the protein (Figure 4.7(b)). This conclusion agrees with earlier NMR studies of polypeptides $^{108-110}$.

Detailed analysis of the results of MD simulations shows that seven methyl groups (Met-12, Leu-17(both methyls), Ile-55 (both methyls), Leu-56 (C$\delta$1) and Met-105) exhibit anharmonic dynamics already at 125 K. They are buried near the active site of the enzyme $^{112}$ and therefore inherently they may experience a sterically less restricted local environment that is required for enzymatic activity.

Now we comment on why the low-temperature onset of methyl group dynamics was not identified in previous analyses of neutron scattering spectra of lysozyme $^{9,25,30}$. The data presented in the Figure 4.1 agree well with the results of Tsai et al. $^{30}$ obtained using the same experimental conditions (after taking into account the factor of 3 that the authors did not include in the definition of $<x^2>$). However, Tsai et al. interpreted the temperature variations of $<x^2>$ in dry and wet samples at $T < 200$ K as purely the contribution of harmonic vibrations, because they did not measure down to a low enough temperature.

The harmonic vibration contributions of $<x_{vib}^2(T)>$ for lysozyme at ambient $T$ have been estimated from an analysis of vibration spectra at $E > 1$ meV (240 GHz) $^{25}$. Using the range $Q < 2$ Å$^{-1}$, the authors estimated $<x_{vib}^2(T)>$ to be $\sim 0.17$ Å$^2$. This value is
significantly smaller than the total $<x^2(T)> \sim 0.4 \text{ Å}^2$ observed for dry lysozyme powder at $T \sim 295 \text{ K}$ (Figure 4.1). However, extrapolation of the estimated harmonic contribution (solid line in the inset of Figure 4.1) to 295 K gives $<x^2_{\text{vib}}(T)> \sim 0.15 \text{ Å}^2$ which is in good agreement with the estimated value of $\sim 0.17 \text{ Å}^2$.

The temperature dependence of $<x^2(T)>$ in wet lysozyme has also been analyzed by Doster and Settles using neutron scattering data measured with the IN13 spectrometer which has a broader energy resolution ($\sim 80 \text{ ps}$) and a broader $Q$-range (the relevant $Q^2$ range is up to $\sim 25 \text{ Å}^2$) than the spectrometer employed in this work $^9$. Harmonic behavior was observed up to 180 K, and extrapolation to 300 K gives $<x^2_{\text{vib}}(T)> \sim 0.1 \text{ Å}^2$ (taking into account the factor of 3 that the authors did not include in their definition of $<x^2(T)>$), which is smaller than the values obtained in our work and in other publications $^{25,30}$. It is known that the Gaussian approximation employed at higher values of $Q$ underestimates $<x^2(T)>$ due to the non-Gaussian behavior of the dynamic structure factor. Thus the difference might be related to the higher $Q$-range used by Doster and Settles $^9$ to estimate $<x^2(T)>$ (their paper does not describe the $Q$-range used, but it should be broader because of the higher energy neutrons used by IN13). Taking into account the broader resolution function used in $^9$, one would expect the onset of methyl group rotation to appear at $T \sim 150 \text{ K}$. However, this onset may be masked by the dynamical transition at $T \sim 180 \text{ K}$ in the data presented by Doster and Settles $^9$.

We also want to emphasize that our results are inconsistent with a recent NMR study $^{1113}$ of data for lysozyme, as well as an earlier simulation study of RNAse A $^{114}$, showing no evidence for a trimodal distribution of methyl dynamics; moreover, while the
low-$T$ anharmonic dynamics are dominated by methyl protons, they are not the sole contributor above $T_D$.

Thus the experimental data presented here agree well with earlier studies of lysozyme dynamics. However, the previous studies did not notice or describe in detail the low-temperature onset of anharmonicity. The present analysis shows that the temperature, spectral and $Q$-dependence of the low-frequency QES contribution in dry lysozyme can be quantitatively well described by methyl group rotational dynamics. Therefore, our analysis, including results from simulations (Figure 4.6), suggests that the major cause for the low-temperature onset of anharmonicity observed in all samples regardless of hydration is the onset of methyl group rotational dynamics. At least, methyl groups are fluctuating between different conformational states and provide a significant anharmonic contribution to the neutron scattering spectra below $T_D$. The analysis of MD simulations of appropriately hydrated lysozyme powder indicates a minor anharmonic contribution also by non-methyl non-exchangeable hydrogen atoms at $T > 125$ K (Figure 4.6).

4.4.2 High-temperature onset of anharmonicity

Figure 4.1 shows that sufficiently hydrated proteins (above $0.18 \, h$), have the well-known onset of anharmonic behavior at $T \sim 200 – 230$ K, as well as the low-temperature anharmonicity at $T \sim 100$ K. The results suggest that there is a critical concentration of water between 0.18 and 0.30 $h$ for the activation of additional motions that are responsible for the dynamic transition.
Unfortunately, the $<x^2(T)>$ are an integrated quantity including vibrations, fast dynamics, and a slow relaxation mode faster than the energy resolution $\sim 1$ ns. For the detailed analysis of the additional motions, we investigated the hydration and temperature dependence of protein motions over particular frequency ranges. The subjects will be discussed separately in Chapters V and VI. In Chapter V, parallel analysis between the additional motions and enzymatic activity of lysozyme will be also discussed.

4.5 Conclusions

The analyses of neutron scattering spectra demonstrate the existence of two onsets of anharmonicity in $<x^2(T)>$ of non-exchangeable hydrogen atoms on a nanosecond timescale: (i) the low-temperature onset that is observed in all samples regardless of their hydration levels and (ii) the well-known dynamical transition at $T \sim 200 – 220$ K that is observed only in samples at hydration levels higher than $\sim 0.2 \ h$. The dynamics of the dry protein include significant anharmonic contributions, and also the dynamics of the wet proteins are not completely harmonic below $T_D$. The latter conclusion agrees with the recent analysis of MD-simulations 102.

The analyses of the temperature, wave vector and, frequency dependencies of data for dry lysozyme show that the activation of methyl group dynamics is the major cause of the low-temperature onset of anharmonicity. These results suggest that an analysis of the incoherent neutron scattering spectra of proteins should explicitly include methyl group dynamics. They give the significant contribution to the QES spectra. Neglecting this
contribution might lead to misinterpretations. The MD-simulations that probed the contribution only from methyl groups support our interpretation.

Moreover, it might also be useful for the analysis of results of MD-simulations to exclude the contribution from methyl groups. The recent analyses of MD-simulations results for lysozyme placed in glycerol and in trehalose demonstrated that removing the contribution of methyl group hydrogen atoms in the calculation of the intermediate scattering function of lysozyme can provide a microscopic insight that would otherwise be missed\textsuperscript{115,116}. 
5.1 Introduction

One of the effective ways of investigating the role of protein dynamics in protein functions is to study their dependence on hydration levels, since this environmental parameter strongly controls both protein’s dynamics and protein’s functions. For example, the reported enzymatic activity of lysozyme has a step-like dependence on hydration level. The enzymatic activity is essentially non-measurable at hydration levels $h < 0.2$. As the hydration level is increased, the onset of lysozyme’s enzymatic activity occurs at $h \sim 0.2$. Then it increases sharply between $h \sim 0.2$ and $\sim 0.5$, and weakly at $h > 0.5$, approaching the level of activity in a dilute solution. This dependence of lysozyme’s enzymatic activity on hydration differs from the dependence of its hydrogen exchange rate on the hydration level. However, the increase in activity with the increase in the hydration level agrees with the electron paramagnetic resonance spectroscopy that characterizes the rotation of a probe molecule close to the protein surface.
Recently, lysozyme-water interactions have been studied by sorption calorimetry, where the requirement of water to induce a glass transition in the protein was re-verified and a phase-diagram of the water-lysozyme system over a broad temperature and hydration range was presented\textsuperscript{118}. Many neutron scattering and Mössbauer spectroscopic measurements of proteins, DNA, and RNA have shown a significant change in their dynamics with hydration levels\textsuperscript{12,25,26,28,37,49,98}. This change has been interpreted as a change in the number of atoms involved in the motions, rather than as a change in the type of motion, and also reflects a change in the flexibility of the protein structure.

A simple quantity to characterize protein dynamics is $\langle x^2(T) \rangle$. The analysis presented in Chapter IV shows that methyl group rotation is activated at $T \sim 100$ K regardless of hydration levels (Figure 4.1). We expect that this methyl group rotation does not affect enzymatic activity, at least not in our model protein, lysozyme. However, an understanding of its contribution to protein dynamics is critical in obtaining the correct information on protein motions and identifying functionally important dynamic modes.

In Chapter IV, the traditional dynamic transition that is believed to be closely related to the onset of enzymatic activity was observed only in wet proteins at $h > 0.2$. An understanding of the microscopic mechanism in the dynamic modes that activate the dynamic transition is essential for an understanding of the relationship between protein dynamics and protein functions. However, the microscopic nature of the dynamic transition in proteins remains unclear, although the importance of solvent translational motion for the dynamic transition seems to be well established\textsuperscript{38,39}.

Some authors have suggested that proteins are more flexible above $T_D$\textsuperscript{119}. However, not all regions of the protein undergo the dynamical transition\textsuperscript{40} and the idea
of a molten surface state has also been proposed \cite{120-122}. The activation of a slow process that appears during a nanosecond time window at a temperature above $T_D$ has been observed \cite{26,41,49,63}. Analysis of the molecular dynamics (MD) simulations of proteins under analogous conditions has provided microscopic information as to the origins of the dynamical changes activated above $T_D$. Tournier and Smith \cite{63}, using MD simulation of myoglobin and normal mode analysis, speculated that motions of secondary structures are activated above $T_D$. In previous experimental studies \cite{9,26}, the main contribution to $<x^2(T)>$ at temperatures above $T_D$ has also been ascribed to the motion of side chain groups and loop structures. Thus, we still have no clear picture of the dynamic transition and the qualitative change in dynamics at temperatures above $T_D$.

The main goal of the present chapter is to present a detailed study of the influence of hydration on the dynamics of the protein lysozyme. We employed neutron and light scattering spectroscopy to cover a broad energy range from $\sim 100$ MHz (0.5 $\mu$eV) to $\sim 5$ THz (20 meV), at the same time covering a time range of $\sim 1$ ns to $\sim 0.1$ ps.

In addition to the methyl group rotation, there are two other relaxation processes: fast and slow. Our results support the idea that dynamic transition is related to activation of the slow relaxation process in the accessible experimental frequency window. It seems to be closely related to enzymatic activity, showing its consistent hydration dependence.

5.2 Samples and techniques

5.2.1 Neutron scattering measurements
Samples of dry (0.05 \(h\)) and D\(_2\)O-hydrated lysozyme (0.18, 0.30, 0.45, 0.50 and 0.80 \(h\)) were prepared for neutron scattering measurements, as explained in section 3.4.1. Two neutron scattering (NS) spectrometers, HFBS and DCS, were employed in order to cover a sufficiently broad energy range from \(\sim 1\ \mu\text{eV} (\sim 240 \text{MHz})\) to \(\sim 100 \text{meV} (\sim 24 \text{THz})\) for the analysis of the complex relaxation spectra of proteins. The quasi-elastic scattering spectra at lower energy range \(\Delta E = \pm 36 \mu\text{eV} (9 \text{GHz})\) and scattering wave vector range \(0.25 \text{Å}^{-1} < Q < 1.75 \text{Å}^{-1}\) were measured at \(T \sim 295\ \text{K}\) using a HFBS spectrometer. Neutron scattering spectra at higher energies were measured at \(T \sim 295\ \text{K}\) using DCS with \(\lambda = 8\ \text{Å}\) (corresponding energy resolution \(\delta E \sim 25 \mu\text{eV} (\sim 6 \text{GHz})\) and elastic \(Q\)-range \(0.08 \text{Å}^{-1} < Q < 1.46 \text{Å}^{-1}\)). Neutron scattering data were treated in the same way as described in section 3.2.2 and 4.2.

5.2.2 Light scattering measurements

Lyophilized lysozyme was used as purchased without further purification. Samples for eight different hydration levels (0.03, 0.10, 0.15, 0.20, 0.35, 0.50, 0.75, and 0.85 \(h\)) were prepared for light scattering measurements, as described in section 3.4.2.

Depolarized light scattering (LS) spectra were measured at \(T \sim 295\ \text{K}\) in back scattering geometry using a Raman spectrometer (triple-monochromator Jobin Yvon T64000) and a six-pass tandem Fabry-Perot interferometer (Sandercock model). A frequency range from \(\sim 25 \text{GHz} (\sim 0.83 \text{cm}^{-1})\) up to \(\sim 25 \text{THz} (\sim 833 \text{cm}^{-1})\) was covered by these spectrometers. An Ar\(^{++}\) laser with \(\lambda = 514.5\ \text{nm}\) and \(\sim 25\ \text{mW}\) power on the sample was used as the excitation source.
A small contribution to the spectra was due to fluorescence background, that was corrected as described previously in section 3.3.2. The spectra were normalized in the high frequency region above ~3 THz (~100 cm\(^{-1}\)), where vibrational modes do not show a significant dependence on hydration. The contribution of water to the light scattering spectra in the frequency range of interest was negligible at the hydration levels studied (See e.g. \(^{31}\)) due to the weak optical polarizability of water. Thus, the light scattering spectra at the hydration levels measured were dominated by the internal dynamics of the protein.

5.3 Results

Analysis of the quasielastic spectra obtained from the HFBS measurements in the frequency range 0.2 GHz < \(\nu\) < 8.5 GHz did not reveal any significant dependence of the width (\(\Gamma\)) on \(Q\) in the measured \(Q\)-range \(^{100}\). Figure 5.1 presents the QES spectra summed over all \(Q\) for protein samples of 0.05 (dry), 0.18, 0.30, 0.50 and 0.80 \(h\) at \(T\) ~ 295 K. As we discussed in Chapter IV, the strong QES contribution of the samples of 0.05 and 0.18 \(h\) results primarily from methyl group rotation. It is worth mentioning that the hydration dependence of the QES intensity shows a step-like behavior: i) no change at \(h\) lower than 0.18, ii) a strong increase at \(h\) between 0.18 and 0.5, and iii) small increase at \(h\) higher than 0.5.

Figure 5.2 shows neutron and light scattering spectra measured at higher frequencies. They are dominated by two contributions: (i) QES due to various relaxation processes at frequencies below \(\nu\) ~ 200 GHz, and (ii) the Boson peak at \(\nu\) ~ 1 THz that is
usually assigned to the collective vibrations of protein atoms\textsuperscript{27,28,50}. The QES contribution increases monotonically with hydration level and overshadows the Boson peak at higher frequencies. The QES intensity at higher frequencies increases with hydration level even at lower levels, \( h < 0.2 \). This differs from the QES behavior observed at lower frequency (Figure 5.1). Moreover, in Figure 5.2, the dependence of the QES intensity on the hydration level appears to be stronger in the light scattering spectra than in the neutron scattering spectra.

Figure 5.1 \( S(Q,\nu) \) of the protein samples of 0.05 (dry), 0.18, 0.30, 0.50, and 0.80 \( h \) at \( T \sim 295 \) K. \( S(Q,\nu) \) was summed over all \( Q \) and normalized by the mass of lysozyme. A vanadium spectrum presents the resolution function.
Figure 5.2 (a) $S(Q, \nu)$, obtained from DCS measurements for the protein samples of 0.05 (dry), 0.18, 0.30, 0.50, and 0.80 $h$. $S(Q, \nu)$ was summed over all $Q$. (b) Light scattering intensity, $I(\nu)$ for the protein samples of 0.03 (dry), 0.10, 0.15, 0.20, 0.35, 0.50, 0.75, and 0.85 $h$. All neutron and light scattering spectra were obtained at $T \sim 295$ K and normalized at a high frequency region $> 2.5$ THz that is not sensitive to hydration.
5.4 Discussion

5.4.1 The influence of hydration on the methyl group dynamics

In Figure 5.1, the QES spectrum of the dry protein (\( \sim 0.05 \ h \)) is mainly ascribed to methyl group rotation. The QES contribution of the sample at 0.18 \( h \) shows little difference from that of the dry protein. This means that the methyl group rotation is insensitive to hydration level. This interpretation is supported by the observation that the \(<x^2(T)>\) amplitudes for all the samples are very similar until the temperature reaches around 200 K where the activation of other dynamic modes starts in samples with higher hydration levels (Figure 4.1). A possible explanation is that most of the methyl groups are located in hydrophobic inner-sites of protein molecules and they barely interact with water molecules. Reat et al.\(^99\) reported that the inner sites of protein molecules were not dynamically affected by water molecules. Simulation results from Dr. Curtis showed that the relaxation time of methyl group rotation does not change significantly between dry and wet lysozyme.

5.4.2 Relaxation spectra over a broad frequency range

When analyzing relaxation processes, quasielastic neutron scattering spectra are often approximated by a sum of a few Lorentzians, where the \( Q \) and \( T \) dependence of the Lorentzian line width (\( \Gamma \)) is determined\(^{25,28}\). It is known, however, that relaxation in complex systems, including biological macromolecules, involves a number of processes, each of which is often better described by a strongly stretched exponential decay
The latter means that the decay can be approximated by the so-called Kolrausch-Williams-Watts (KWW) equation, \( I(Q,t) \propto \exp\left[-(t/\tau)^\beta\right] \) with \( \beta < 1 \), or by a Cole-Davidson distribution function for the dynamic susceptibility function in the frequency domain, \( \chi''(\nu) = (1-i\nu\tau)^{-b} \), \( b < 1 \). This function is a simple Lorentzian when \( b = 1 \), \( \chi''(\nu) = (1-i\nu\tau)^{-1} \). Thus approximation of a relaxation spectrum by a sum of a few Lorentzians (i.e. a sum of a few exponential decays) is not accurate and can provide misleading results. \( \chi''(\nu) \), for neutron and light scattering spectra can be understood by the equations (3.35): \( \chi_{NS}''(Q,\nu) \propto S(Q,\nu)/n_b(\nu) \) and (3.47): \( \chi_{LS}''(\nu) \propto I(\nu)/[n_b(\nu)+1] \).

The advantages of using the susceptibility presentation are summarized in section 3.2.2.2.

We start our analysis with the comparison of neutron and light scattering susceptibility spectra in a high-frequency range. Figure 5.3 shows that neutron and light scattering \( \chi''(\nu) \) spectra appear to be similar at high hydration levels \( h \geq 0.3 \) and show two well-separated relaxation processes, a fast process that dominates at frequencies, \( \nu > 100 \) GHz and a slow process that dominates at lower frequencies. The slow process has a strongly stretched high-frequency tail that can be approximated by a power law, \( \chi''(Q,\nu) \propto \nu^{-b} \), with a small exponent \( b \sim 0.2 \). The tails of the slow process disappear (or become negligible) at hydration levels lower than 0.2 \( h \). Apparently, it is the same process that appears in the low-frequency QES spectra of proteins at \( h > 0.2 \) (Figure 5.1).

Analysis of QES spectra shows that the slow relaxation process that is activated at higher hydration levels had an average \( \tau \sim 2.5 - 3 \) GHz \( (\tau \sim 50 \) ps) at \( T \sim 295 \) K independent of \( Q \). This observation strongly differentiates the slow process in proteins from the slow process (the main structural \( \alpha \)-relaxation) in glass-forming systems. The
characteristic $\tau_a$ in glass forming liquids has a strong $Q$-dependence indicating an underlying diffusion-like process. A $Q$-independent $\tau$ for the slow process in lysozyme suggests that this process is localized at a distance smaller than $r < 2\pi/Q_{\text{max}} \sim 3.5$ Å.

Figure 5.3 $\chi''(\nu)$ spectra of neutron (symbols) and light (solid lines) scattering for the protein samples at different hydration levels (neutron scattering measurements: $h \sim 0.05$ (dry), 0.18, 0.30, 0.50 and 0.80; light scattering measurements: $h \sim 0.03$ (dry), 0.20, 0.35, 0.50 and 0.85). The dashed line shows the slope of the high-frequency tail of the slow relaxation process, and the dotted line shows the slope of the low-frequency tail of the fast relaxation process.

The slow relaxation process seen in combined neutron scattering susceptibility spectra (Figure 5.4(a)) shows a broad relaxation maximum with $\nu_{\text{max}} \sim 2 - 6$ GHz (consistent with our estimate of the average $\Gamma$), corresponding to $\tau \sim 30 - 80$ ps. An increase in the hydration level seems to increase the amplitude of the process rather than
Extremely strong stretching (the exponent $b \sim 0.2$, Figure 5.3) seems to be a general property of the slow relaxation process in biological macromolecules. The strong stretching has been found for myoglobin ($b \sim 0.25$) and for DNA ($b \sim 0.3$). This type of spectrum corresponds to either a very complex relaxation process or to an extremely broad distribution of relaxation times. It is obvious that the fit of these spectra by a single Lorentzian will not provide reliable results. For example, the spectra of hydrated lysozyme measured with a resolution of 80 - 140 μeV (20 – 34 GHz) have previously been fit using a single Lorentzian function. The authors estimated the Lorentzian width to be $\Gamma \sim 70 - 150$ μeV ($\sim 17 - 35$ GHz), which differs from our estimates by a factor of 5 - 10 and, most probably, is limited by the resolution of the spectrometer employed. Our data (Figure 5.3 and 5.4(a)) do not show a relaxation maximum in the frequency range 15 – 40 GHz. Thus the results obtained with the single Lorentzian fit should be considered with particular care (this question has been discussed previously by Fitter et al. 28,37).

One of the interesting results in Figure 5.3 is that the neutron and light scattering spectra differ significantly at low hydration levels. It is known that methyl group rotation does not contribute to the light scattering spectra (See e.g. 123.). We can estimate the contribution of methyl group dynamics to the high frequency neutron scattering spectra using results from the previous section. Figure 5.4(b) shows the combined low- and high- frequency spectra of dry lysozyme. The dashed line marks the contribution from the methyl groups that is estimated at lower frequencies and extrapolated to higher frequencies using the distribution $g(E_i)$ obtained in Chapter IV. After subtracting this contribution, the high-frequency neutron scattering spectrum becomes similar to the light
scattering spectrum (Figure 5.4(b)). These results further confirm that methyl group
rotational dynamics contribute significantly to the neutron scattering spectra of proteins.
This contribution is especially important in the dry state where the slow process is
suppressed.

Figure 5.4 (a) Neutron scattering susceptibility, $\chi''(\nu)$, of the protein samples at 0.05 (dry),
0.3, and 0.8 $h$ in a broad frequency range. (b) Neutron (□) and light (thinner solid line)
scattering susceptibility spectra of dry samples ($h \sim 0.05$ and 0.03). The thicker solid line
shows the fit of the low-frequency spectrum using the equation (4.4) (Figure 4.4); the
dashed line shows the estimated contribution of the methyl group rotation. The high-
frequency neutron scattering spectrum after correction for the methyl group contribution
($\Delta$) agrees well with the light scattering spectrum.
It is difficult to analyze the spectral shape of the fast process because it overlaps with both the slow process and methyl group dynamics at lower frequencies and with the boson peak vibrations at higher frequencies. The contribution of the slow process is negligible in the case of the dry protein and we can analyze the spectral shape of the low-frequency tail of the fast process from the light scattering data (Figure 5.3) where the contribution of methyl group dynamics is also negligible. The low-frequency tail of the susceptibility spectrum is well described by a power law $\chi''(\nu) \propto \nu^{0.55}$. This is far from the expected linear dependence, $\chi''(\nu) \propto \nu$, for a single Lorentzian and suggests either a highly stretched process or a broad distribution of relaxation times in the sample.

Analysis of the spectra also suggest that the relaxation maximum of the fast process should be at a frequency higher than ~100 GHz, i.e. $\tau$ should be ~1 ps or shorter. Similar relaxation times and spectral shapes (with a stretched low-frequency tail) are also characteristic of fast relaxation in glass-forming and polymeric systems. It is usually interpreted as a “rattling” of molecular units in a cage formed by their neighbors. We speculate that the fast conformational fluctuations in proteins might also be related to a “rattling” of atoms of a particular residue in a cage formed by neighboring residues and water molecules.

Thus, the relaxation spectra of lysozyme have at least three major contributions in the frequency range studied: a slow process that appears only at hydration levels $h > 0.2$ (Figures 5.1, 5.3 and 5.4) and at $T > T_D^{41,49}$; hydration-independent methyl group rotational dynamics that are activated at $T > 100$ K and are not detectable in light scattering spectra; and finally, hydration-dependent fast processes (Figure 5.3). Both slow and fast processes are strongly stretched (Figures 5.3 and 5.4).
5.4.3 The influence of hydration on protein dynamics

An increase in the hydration level strongly increases the QES intensity of lysozyme (Figure 5.1). However, the change in relaxation behavior upon hydration appears to be different at low and high frequencies. A step-like increase in QES intensity is observed between \( h \approx 0.2 \) and \( h \approx 0.5 \) for the slow dynamics, while a monotonic increase of the QES intensity with hydration levels is observed at high frequencies (Figures 5.2 and 5.3). The temperature dependence of \( \langle x^2(T) \rangle \) shown in Figure 4.1 indicates that an additional relaxation process enters the available frequency window at \( T \) above \( T_D \approx 220 \) K, but only at hydration levels higher than \( 0.2 \) \( h \). This is consistent with an increase in the QES intensity at low frequencies that also appears only at \( h > 0.2 \) (Figures 5.1 and 5.4). Thus the sharp change in \( \langle x^2(T) \rangle \) is associated with the slow relaxation process. This conclusion agrees with earlier observations for DNA \(^{49}\) and the results of light scattering measurements for lysozyme \(^{41}\).

Analysis of the \( EISF(Q) \) of the slow relaxation process can provide information as to the type of the relevant motions. It is obvious from the previous section, 5.4.2, that the \( EISF(Q) \) of the hydrated sample has at least three components: (i) the slow process, (ii) methyl group rotational dynamics and (iii) the fast process. The possible fast process has a negligible contribution at lower frequencies \( (\nu < 10 \text{ GHz} \approx 50 \text{ } \mu \text{eV}) \). Thus only the first two components make a significant contribution to the low-frequency neutron scattering spectra (Figures 4.4 and 5.1). Assuming that the contribution from methyl group hydrogen atoms and the slow relaxation process are additive, we can write the \( EISF(Q) \) as:
According to our previous analysis, the contribution from methyl group hydrogen atoms dominates the spectrum of the dry sample. So, as a rough approximation we can rewrite the equation (4.5) as:

\[
EISF_{dry}(Q) = \left(1 - p_{methyl}\right) + p_{methyl} EISF_{methyl}(Q) .
\] (5.2)

Combining the equations 5.1 and 5.2 provides an expression that can be used to estimate the \( EISF(Q) \) of the slow relaxation process:

\[
EISF_{hydrated,slow}(Q) = (1 - p_{slow}) + p_{slow} EISF_{slow}(Q) = EISF_{hydrated}(Q) - EISF_{dry}(Q) + 1 .
\] (5.3)

This is a crude approximation, but it might be appropriate for a qualitative model-independent analysis of our experimental data. As we discussed in section 5.4.2, traditional calculations of \( EISF(Q) \) from the measured QES spectra usually assume a single Lorentzian function. This approximation does not provide an accurate estimate of the relaxation times, nor does it provide a correct representation of the mobile fraction of hydrogen atoms involved in the relevant process (For example, see our estimates of \( p_{methyl} \) given in section 4.4.1.). Due to the poor statistics of our neutron scattering spectra, a fit of the QES spectra measured at each \( Q \) without prior knowledge of the shape of the relaxation spectrum is not reliable. Thus, in many cases, an approximation by a single Lorentzian might be the only choice for qualitative analysis of \( EISF(Q) \). So, we use the single Lorentzian approximation for a qualitative analysis of the geometry of the slow process and its variation with hydration level. As a first step we fit the spectra of the dry sample to obtain \( EISF_{dry}(Q) \) and then we fit the spectra of hydrated samples to obtain
$EISF_{hydrated}(Q)$. $EISF_{hydrated,slow}(Q)$ is calculated at different hydration levels using the equation (5.3).

The resulting $EISF_{hydrated,slow}(Q)$ (Figure 5.5) at different hydration levels has been fit to the two-site jump model \(^{81}\):

$$EISF(Q) = 1 - p_{slow} + \frac{p_{slow}}{2} \left[ 1 + 2 j_0(Qd) \right], \quad (5.4)$$

and to a three-site jump model, the equation (4.5), as well as to a model describing freely diffusive motions in a sphere \(^{85}\):

$$EISF(Q) = 1 - p_{slow} + p_{slow} \left[ \frac{3 j_1(Qa)}{Qa} \right]^2. \quad (5.5)$$

Here $p_{slow}$ is the mobile fraction of hydrogen atoms involved in the slow relaxation process on our experimental time scale ~ 20 ps to 1 ns, the $d$ is a jump distance and the $a$ is the radius of the sphere in which the hydrogen atoms move. The results for two- and three-site jump models are indistinguishable over our limited $Q$-range and it seems that the diffusion in a sphere model provides a slightly better fit of the data at higher $Q$ (Figure 5.5). We note, however, that the $Q$-range studied is not sufficient to identify which model best describes the slow relaxation process.

The dependence of the fit parameters on hydration is presented in Figure 5.6. It appears that both jump distance $d$ and the sphere radius $a$ have a value of ~ 3 Å (Figure 5.6(a)); i.e. they differ significantly from the jump distance characteristic of methyl group hydrogens, ~ $R_{methyl} \sqrt{3} \sim 1.78$ Å \(^{44,107}\). The estimated value agrees with our earlier estimate based on the $Q$-independence of the characteristic $\tau$ of the slow relaxation process. The characteristic length scale of the slow relaxation process appears to be...
essentially independent of hydration. The mobile fraction of the hydrogen atoms involved in the slow process, \( p_{\text{slow}} \), shows a particular dependence on hydration: it remains low at \( h < 0.2 \), it increases strongly between \( h \sim 0.2 \) and \( h \sim 0.5 \) and then it slightly increases at \( h > 0.5 \) (Figure 5.6(b)).

![Graph](image_url)

**Figure 5.5** \( EISF_{\text{hydrated,slow}}(Q) \) of hydrated samples (For definition, see the equation (5.3).) at \( h \sim 0.18 \) (○); 0.29 (▲); 0.51 (▽); 0.80 (♦). The dashed lines present fit to the two-site (equation (5.4)) and three-site (equation (4.5)) jump models (fits are indistinguishable at this \( Q \)-range) and the solid lines present fits to freely diffusive motions in a sphere model (equation (5.5)).

The results suggest that an increase in the hydration level leads to an increase in the number of flexible regions, rather than to an increase in the amplitude of the particular motion. This conclusion is independent of the model used to fit the \( EISF(Q) \) data. Moreover, the variation of \( p_{\text{slow}} \) with hydration essentially reflects the variations in the model-independent parameter, the integrated QES intensity (Figure 5.6(b)).
Figure 5.6 (a) Amplitude of motions (jump distance $d$ and radius of sphere $a$), involved in the slow relaxation process at different hydration levels obtained from the fit to the two-site jump model (▲) and to freely diffusive motions in a sphere model ( ■). The dashed line shows the jump distance expected for methyl groups. (b) Variation of mobile fraction of hydrogen atoms involved in the slow relaxation process, $p_{slow}$, with hydration obtained from the fit to two-site jump model (△, outside labels) and to freely diffusive motions in a sphere model (□, inside labels). QES intensity integrated in the frequency range from 2 to 8 GHz (○) is presented for a comparison.

It is important to note that the value of $p_{slow}$ does not reflect the actual number of hydrogen atoms involved in the slow relaxation process. It is a significant underestimate of the number of hydrogen atoms because of the use of the single Lorentzian approximation in the calculations of $EISF_{slow}(Q)$ for the strongly stretched process (Figure 5.4). This is similar to the results of our analysis of the methyl group dynamics.
We emphasize that the analysis of the same data without correcting for methyl group rotational dynamics will result in a smooth increase in the length scale of the motion with an increase in hydration level. Thus, correction for the methyl group dynamics is crucial in the analysis of the neutron scattering spectra of proteins.

Now we turn to the analysis of the fast process and its dependence on hydration. We don’t expect that the analysis of the \( EISF(Q) \) of the fast process can provide any reliable information. Its spectral shape is not known and cannot be modeled as a single Lorentzian function. Moreover, it overlaps with both the methyl group dynamics and the slow relaxation process at lower frequency and with the Boson peak vibrations at higher frequencies. Therefore, it is difficult to obtain microscopic information on the fast process from the experimental data. Because of these limitations we decided to restrict our analysis to variations of the integrated intensity of the fast process with hydration levels.

We started analyzing the light scattering spectra because there is no contribution from methyl group dynamics. To analyze the relaxation spectra at higher hydration levels, the light scattering susceptibility spectra in the frequency range of interest can be represented by a sum of two contributions:

\[
\chi''(\nu) = \chi''_{\text{slow}}(\nu) + \chi''_{\text{fast}}(\nu) \approx CP_{\text{slow}}\nu^{-0.2} + \chi''_{\text{fast}}(\nu) .
\]  

Here, the high-frequency tail of the slow relaxation process was approximated using a power law with the intensity proportional to the mobile fraction of atoms involved in the slow relaxation process. The constant, \( C \), is assumed to be independent of hydration. In order to estimate the fast process, we subtract the contribution of the slow relaxation process from the light scattering spectra (Figure 5.7(a)). The resulting spectra of the fast
process show a strong increase in intensity with hydration levels at low $h$ and no significant dependence on hydration levels at $h > 0.15$. Figure 5.8 shows the hydration dependence of the fast process intensity integrated over the frequency range 50 GHz – 100 GHz.

Figure 5.7 High-frequency susceptibility spectra corrected for the contribution of the slow relaxation process: (a) light scattering and (b) neutron scattering measurements.
Figure 5.8 Light (□) and neutron (Δ) QES intensity corrected for the slow relaxation process (Figure 5.7) and integrated in the frequency range from 50 to 100 GHz as a function of hydration. It reflects essentially the dependence of the fast relaxation process on hydration.

A similar approximation has been used to correct for the contribution of the slow process to the high-frequency neutron scattering spectra (Figure 5.7(b)). However, the remaining spectra in this case include the fast process and a contribution from methyl group hydrogen atoms. Assuming that the contribution from methyl group dynamics does not vary much with hydration, we can estimate the variations of the fast process with hydration from the high-frequency neutron scattering spectra corrected for the slow relaxation process (Figure 5.7(b)). Both light and neutron scattering data show a similar hydration dependence of the fast process: It increases sharply at $h < 0.2$ and varies slower at higher hydration levels (Figure 5.8). This differs significantly from the hydration dependence of the slow relaxation process and suggests that the fast conformational
fluctuations in the picosecond time range are already active in the dry state and become stronger with an increase of hydration levels, even much below a monolayer coverage, $h \sim 0.38$ \textsuperscript{11}. Therefore, it seems that the fast process varies strongly with hydration level during the formation of the surface water H-bonded network (reached at $h \sim 0.15$) and is less sensitive to water that condenses on weakly interacting patches of the protein surface (starts at $h \sim 0.25$) \textsuperscript{11}. It does not change much at $h > 0.2$, where the contribution of the slow relaxation process starts to increase sharply (Figure 5.6(b)).

5.4.4 Correlations between protein dynamics and enzymatic activity

The influence of hydration on the enzymatic activity of lysozyme has been presented in \textsuperscript{11}. A solution of lysozyme was prepared with equimolar amounts of the hexasaccharide of N-acetylglucosamine at pH values between 8 and 9 to minimize catalysis during preparation. The solutions were lyophilized to produce the dry enzyme-substrate complex, which was subsequently rehydrated to the required hydration levels, and catalytic activity has been measured as a function of hydration. These enzymatic activity measurements done by \textsuperscript{11} were not hydration-controlled, since the high pH makes enzymatic reaction much slower than the hydration process.

The observed hydration dependence of the slow relaxation process (mobile fraction of hydrogen atoms $p_{slow}$) correlates well with the hydration dependence of catalytic activity (Figure 5.9(a)): (i) the mobile fraction remains very small up to $h \sim 0.18$; (ii) it increases strongly between $h \sim 0.18$ and $h \sim 0.50$ and (iii) a further weak
increase is observed above $h \sim 0.50$. These results suggest that the slow relaxation process includes motions important for the enzymatic activity of lysozyme.

![Graph showing hydration dependence](image)

Figure 5.9 (a) Parallel comparison of the hydration dependence of the mobile fraction of hydrogen atoms involved in the slow relaxation process (▲) and enzymatic reaction rate, $v_0$, of the lysozyme to hexasaccharide of N-acetylglucosamine [(GlcNAc)$_6$] (★) as estimated in $^{11}$. (b) Parallel comparison of hydration dependences of the integrated QES intensity of the fast process (■) and hydrogen exchange rate (▷) in units of mole of exchanged H atoms / 1 mole of lysozyme / 24 hours as estimated in $^{117}$.

The microscopic mechanism of this slow process remains unclear. Tournier and Smith $^{63}$ identified a mode involving the rigid body motion of two groups of helices that
activates above the dynamic transition in hydrated myoglobin. A hinge-bending motion
is a well-known mode in lysozyme\(^5\). These types of rigid-body motions involving
correlated motions of secondary structures are consistent with the estimated length scale
of the motion and the significant number of hydrogen atoms observed in the slow process.
A more detailed analysis of simulation results could help elucidate the nature of the slow
relaxation process in hydrated proteins.

The fast process and enzymatic activity have different dependencies on hydration
levels (Figure 5.8). The fast process is activated at much lower hydration levels than
enzymatic activity and is significant even in dry protein. It is interesting to note that the
dependence of fast conformational fluctuations on the hydration levels is similar to the
hydration dependence of hydrogen exchange (Figure 5.9(b)). It is possible that the
increase in flexibility promoted at the onset of hydrogen isotope exchange is associated
with the activation of the fast conformational fluctuations. Based on our analysis, we
speculate that the fast process is not directly related to enzymatic activity but might be an
important precursor for the slow relaxation process.

The following scenario emerges. There are two significant anharmonic
contributions to the dynamics of dry protein: methyl group rotational dynamics and fast
picosecond conformational fluctuations. Initially, the slow relaxation process is strongly
suppressed and the protein is rather rigid. With an increase in hydration up to \( h \sim 0.2 \), the
contribution of the fast process to protein dynamics increases sharply, while the slow
relaxation process remains suppressed. When the fast process (rattling of residues in
cages formed by their neighbors) reaches a particular level at \( h \sim 0.2 \), it creates a level of
protein flexibility sufficient to activate the slow relaxation process. The slow relaxation
process then increases sharply with $h > 0.2$. In other words, fast conformational fluctuations are a necessary precursor for the motions of secondary structures. The latter become active on our picosecond-nanosecond time scale only when the fast fluctuations reach a particular level. When the motion of secondary structures (the slow relaxation process) is activated, lysozyme can become catalytically active.

5.5 Conclusions

We have presented a detailed analysis of the influence of hydration on the pico- to nano-second internal dynamics of lysozyme. In addition to methyl group dynamics, as discussed in Chapter IV, two other relaxation processes have been identified: (i) fast conformational fluctuations and (ii) a slow relaxation process with larger scale motions, but still localized to ~ 3 Å. They are dependent on the hydration level differently. Methyl group dynamics seem to be rather insensitive to $h$. The fast fluctuations increase rapidly at $h < 0.2$ and then increase only slightly with further increase in $h$, while the slow relaxation process does not activate until $h \sim 0.2$. Then the latter increases sharply between $h \sim 0.2$ and $h \sim 0.5$ and only slightly at $h > 0.5$. Therefore, we speculate that the effect of an increase in the hydration level is first to increase the fast conformational fluctuations and then at $h \sim 0.2$ the hydration water activates the slower relaxation process.

The most important observation is the correlation between the slow relaxation process and enzymatic activity, suggesting that the activation of the slow relaxation process might be necessary for lysozyme’s enzymatic activity. The microscopic
mechanism of the slow relaxation process remains unclear, but it might be related to motions of secondary structures.
6.1 Introduction

Protein dynamics and functions are strongly influenced by temperature as well as hydration \(^7,^8\). As we discussed in Chapters II and IV, \(T_D\) is an important temperature where both onset of the anharmonic motions and onset of the enzymatic activity occur simultaneously \(^7,^30,^33,^36,^67-^74,^119\). In Chapter V, the slow relaxation process is ascribed to the mode that activates the dynamic transition and is closely related to enzymatic activity. Therefore, an understanding of the microscopic mechanism of the slow relaxation process is important to figure out the nature of the dynamic transition.

Frauenfelder et al. \(^55\) recently showed that protein molecules experience two types of functionally important motions: i) bulk solvent-slaved or \(\alpha\)-fluctuations that show non-Arrhenius behavior and ii) hydration-shell coupled or \(\beta\)-fluctuations that follow Arrhenius behavior. They suggest that there is no real dynamic transition and the \(\beta\)-fluctuations simply appear in the experimental frequency window. Carri et al. \(^115\) supported the idea by demonstrating that the fluctuations of hydrogen-bond networks at the protein-glycerol interface control the relaxation of the protein. The latter shows
Arrhenius-like behavior around the dynamic transition and is responsible for the sharp rise of $<x^2(T)>$ at $T > T_D$.

The main goal of this chapter is to analyze the temperature dependence of protein dynamics, especially the slow relaxation process. We performed neutron scattering measurements for dry and wet lysozyme at temperatures up to 300 K. Our detailed analyses of protein dynamics demonstrate that the slow relaxation process exhibits Arrhenius-like behavior at $T > T_D$. This observation suggests that the dynamic transition of protein seems to originate from the slow relaxation process moving into the accessible experimental time window, (pico- to nano-second), at $T > T_D$.

6.2 Samples and techniques

Samples of dry ($0.05 \, h$) and D$_2$O-hydrated wet lysozyme ($0.42$ and $0.50 \, h$) for neutron scattering measurements were prepared as explained in section 3.4.1. Neutron scattering measurements at various temperatures from 10 K to 320 K were done using HFBS and DCS spectrometers as described in section 5.2.1. The only difference was that HFBS covers the energy range $\sim \pm 17$ $\mu$eV (4 GHz) and the resolution $\sim 0.8$ $\mu$eV ($\sim 190$ MHz). Neutron scattering data were treated in the same way as described in sections 3.2.2 and 4.2.

6.3 Results

Figure 6.1 presents $<x^2(T)>$ of dry ($\sim 0.05 \, h$) and wet ($\sim 0.42 \, h$) lysozyme estimated using a Gaussian approximation (equations (3.21) and (4.1)). Similar to what
is seen in Figure 4.1, the dry lysozyme clearly exhibits low-temperature anharmonicity at 
\( T \approx 100 \text{ K} \) related to activation of the methyl group rotation. The wet lysozyme has the
dynamic transition at \( T \approx 200 \text{ K} \) as well as the low-temperature anharmonicity.

Figure 6.1 \( \langle x^2(T) \rangle \) of dry (~ 0.05 \( h \)) and wet (~ 0.42 \( h \)) lysozyme.

Figure 6.2 presents neutron scattering \( S(Q, \nu) \) of dry and wet proteins at various
temperatures. It is obvious that the QES intensity increases with increasing temperature.
It appears in spectra of both dry and wet lysozyme even at low temperatures ~ 150 K
(Figure 6.2(a) and (c)).

The \( S(Q, \nu) \) of dry lysozyme shows little difference from that of wet protein in
low-frequency range at temperatures lower than \( T_D \) (Figure 6.2(a)). The QES in the low-
frequency region at temperatures lower than \( T_D \) is mostly caused by methyl group
rotation that is clearly not sensitive to hydration. However, at higher frequencies, the QES in dry lysozyme is slightly higher than in wet protein at $T \sim 150$ K (Figure 6.2(c)), suggesting a stronger fast process in dry lysozyme.

A big difference between dry and wet proteins appears at high temperatures, above $T_D \sim 200$ K, for both the slow and fast processes (Figure 6.2(b) and (c)). Dry lysozyme still retains distinctive collective vibrations (Boson Peak) even at $T \sim 295$ K, whereas wet lysozyme exhibits a total merge between QES and Boson Peak due to the stronger relaxation processes.

![Graphs showing QES vs frequency for different temperatures]

Figure 6.2 $S(Q, \nu)$ of the protein samples at 0.05 (dry), 0.42 and 0.50 $h$ at various temperatures. $S(Q, \nu)$ was summed over all $Q$ and normalized by the mass of lysozyme. $S(Q, \nu)$ of wet lysozyme at 10 K presents the resolution of HFBS spectrometer. (a) Low-frequency $S(Q, \nu)$ at temperatures lower than $T_D$; (b) Low-frequency $S(Q, \nu)$ at temperatures higher than $T_D$; (c) High-frequency $S(Q, \nu)$ at 150 and 295 K.
6.4 Discussion

6.4.1 Temperature dependence of the methyl group dynamics

As shown in Chapter IV, the methyl group rotation in protein molecules has a broad activation energy distribution: $E_0 \sim 16.6$ kJ/mol and $\Delta E \sim 5.8$ kJ/mol. Temperature dependence of the methyl group rotation needs to be well described for a detailed analysis of the temperature dependence of other dynamic processes, especially the slow relaxation process.

Figure 6.3 presents $\chi''(\nu)$ of the methyl group rotation estimated at various temperatures using the equations (3.47) and (4.4). At $T \sim 150$ K, a small part of the high-frequency tail of the methyl group rotation appears in our frequency window. The methyl group contribution moves significantly into the accessible frequency window at $T > 200$ K.

Therefore, it is necessary to correct the total scattering spectra for the methyl group rotation in order to distinguish specific relaxation processes. At $T \sim 295$ K, the methyl group rotation spreads to a much higher frequency and significantly overlaps with the slow and fast processes.
6.4.2 Protein dynamics at temperatures below $T_D$

At $T \sim 150$ K, $\chi''(\nu)$ of dry and wet proteins in the low-frequency range (Figure 6.4(a)) exhibit only high-frequency tail of the methyl group rotation with the broad activation energy distribution, as shown in Figure 6.3. However, at high frequencies, the fast process of dry lysozyme is slightly stronger than that of wet lysozyme (Figure 6.4(d)). According to our estimate of temperature dependence of the methyl group rotation (Figure 6.3), the contribution to the high-frequency dynamics at $T \sim 150$ K is expected to be negligible. Therefore, at $T \sim 150$ K the protein motions that dominate at the frequency...
range between 50 and 500 GHz are mostly attributed to the fast process. This difference in the fast process between dry and wet proteins may be explained as follows: Water molecules are likely to act as stiffeners at temperatures lower than their $T_g$. In a way, water molecules form rigid solid structure and suppress the fast fluctuation of protein molecules.

6.4.3 Protein dynamics at temperatures above $T_D$

At $T \sim 200$ K, wet lysozyme has a slightly higher QES intensity at low frequencies than dry lysozyme (Figure 6.4(a)). The difference can be ascribed to the tail of the slow relaxation process in wet protein that reaches our frequency window at this temperature.

At $T \sim 225$ K, wet lysozyme shows a strong increase of QES intensity in comparison to 200 K. The relaxation peak of the slow process remains out of the accessible frequency window (Figure 6.4(b)). But its tail appears in our frequency window. The tail of the slow relaxation process leads to the sudden increase in the $<x^2(T)>$ of wet lysozyme at $T \sim 200 - 230$ K, and that is called the dynamic transition.

At $T \sim 250$ K, $\chi''(\nu)$ of wet lysozyme differs from that of dry lysozyme in terms of the position of the relaxation peak (Figure 6.4(b)). The relaxation peaks of both the slow process and methyl group rotation enter the accessible frequency window at this $T$. The dry sample has a broad peak with maximum at $\nu \sim 1 - 3$ GHz in the $\chi''(\nu)$ spectrum of dry lysozyme (Figure 6.4(b)). This frequency is consistent with the estimated temperature-dependent relaxation time of methyl group rotation (Figure 6.3). In the case of wet
lysozyme, there is a very distinct relaxation peak appearing in the vicinity of the resolution of the spectrometer, $\nu \sim 0.4$ GHz.

At $T \sim 275$ K, the relaxation peak of the slow process shifts to $\nu \sim 4$ to 5 GHz and it starts to overlap with the methyl group rotation (the relaxation peak in dry lysozyme lies between 3 and 5 GHz). At 295 K, the broad peak of wet lysozyme is in the same frequency range as that of dry lysozyme, as we discussed in Chapter V. At $T \sim 320$ K, the relaxation peak of the slow process shifts to frequency $\sim 30$ GHz (Figure 6.4(d)).

Figure 6.4 $\chi''(\nu)$ of dry ($\sim 0.05$ h) and wet (0.42 and 0.50 h) proteins at various temperatures. (a) Low-frequency $\chi''(\nu)$ at 10, 150 and 200 K; (b) Low-frequency $\chi''(\nu)$ at 10, 225 and 250 K; (c) Low-frequency $\chi''(\nu)$ at 10, 275 and 295 K; (d) High-frequency $\chi''(\nu)$ at 150, 295 and 320 K. The $\chi''(\nu)$ of wet lysozyme at 10 K presents the resolution of HBFS spectrometer.
6.4.4 Temperature dependence of the slow relaxation process

Figure 6.4 clearly shows that the slow relaxation process in wet lysozyme appears in our frequency window at $T \geq 225$ K. However, its contribution overlaps with the methyl group rotation and/or the possible fast process. In this section, we will discuss how to estimate contribution from the slow relaxation process itself.

Figure 6.5 presents $\chi''(\nu)$ of the slow relaxation process of wet lysozyme after the correction for the methyl group rotation and the fast process. We assume that the methyl group rotation is independent of hydration. The correction for the fast process is relatively significant at higher frequencies where the slow relaxation process strongly overlaps with the fast process. The total $\chi''(\nu)$ can be represented as a function of hydration ($h$) at a constant temperature ($T$):

$$\chi''_{total}(\nu, h)|_T = \chi''_{slow}(\nu, h)|_T + \chi''_{met}(\nu)|_T + \chi''_{fast}(\nu, h)|_T.$$  \hspace{1cm} (6.1)

One example of the correction procedure for an estimation of $\chi''(\nu)$ of the slow relaxation process is explained with wet lysozyme (~ 0.50 $h$) at $T \sim 295$ K. According to the equation (6.1), the slow relaxation process can be obtained simply by subtracting the methyl group rotation and fast process from the total dynamics. The best candidate for correction is the wet lysozyme at $h \sim 0.18$, because i) its slow relaxation process is negligible, ii) methyl group rotation is believed to be insensitive to hydration, and iii) its fast process is very similar to that of 0.50 $h$ (Fast process tends to increase at low hydration levels, but it is saturated at $h \sim 0.15 – 0.20$. See Figure 5.9):

i) $\chi''_{slow}(\nu, 0.18h)|_{295K} \sim$ negligible

ii) $\chi''_{met}(\nu, 0.18h)|_{295K} \approx \chi''_{met}(\nu, 0.50h)|_{295K}$
iii) \( \chi''_{\text{fast}}(\nu,0.18h)\big|_{295K} \sim \chi''_{\text{fast}}(\nu,0.50h)\big|_{295K} \)

Therefore, the pure slow relaxation process of wet lysozyme of 0.50 \( h \) can be obtained by

\[
\chi''_{\text{total}}(\nu,0.50h)\big|_{295K} - \chi''_{\text{total}}(\nu,0.18h)\big|_{295K} = \chi''_{\text{slow}}(\nu,0.50h)\big|_{295K} . \tag{6.2}
\]

Figure 6.5 \( \chi''(\nu) \) of the slow relaxation process of wet lysozyme corrected for methyl group rotation and fast process at \( T \sim 200, 250 \) and 295 K. (a) \( \chi''(\nu) \) of lysozyme at \( h \sim 0.50 \) and \( h \sim 0.18 \) and the difference of these spectra that we ascribe to the spectrum of the slow relaxation process (the equation (6.2)). (b) Estimated spectra of the slow relaxation process at \( T \sim 200 \) and 250 K.

We estimated the contribution of the slow process at \( T \sim 295 \) K by using equation (6.2) (Figure 6.5(a)). The maximum of the slow relaxation appears at \( \sim 6 \) GHz. The
spectral shape of the slow relaxation process shows a strongly stretched high-frequency tail that can be represented by a power law, $\chi''(\nu) \propto \nu^{-0.2}$. This spectrum shape is the same as we discussed in Chapter V, without the correction for methyl group rotation. This means the slow relaxation process dominates the dynamics in wet proteins at $T > T_D$.

The main structural relaxation process, like segmental relaxation in synthetic polymers, can be represented by the Cole-Davison distribution function, in which low- and high-frequency tails show $\chi''(\nu) \propto \nu$ and $\chi''(\nu) \propto \nu^{-b}$, respectively. However, the estimated relaxation process in lysozyme is strongly stretched from both low- and high-frequency sides, suggesting that the Cole-Cole or Havriliak-Negami distribution function will be more appropriate for the description of the process. The shape of the spectrum suggests a difference between the slow process in proteins and the segmental relaxation in synthetic polymers.

Figure 6.5(b) presents other examples for the estimation of $\chi''(\nu)$ of the slow relaxation process at $T \sim 200$ and 250 K. Corrections were made only for the spectra of dry protein because the contribution of the fast process at low frequencies is negligible. At $T \sim 200$ K, only a high frequency tail of the slow relaxation is visible in our time window, which indicates the slow relaxation process touches our frequency window already at $T \sim 200$ K. At 250 K, the relaxation peak enters the accessible frequency window and has a maximum at $\sim 0.6$ GHz.

The position of the slow relaxation peak changes very little after all corrections. It is not surprising, because the contribution of the methyl group rotation is much weaker than that of the slow relaxation process. This observation makes it possible to estimate the peak position of the slow relaxation process at different temperatures (at least $T > 250$ K).
K) without any corrections. The relaxation peaks corresponding to 275 and 320 K are 3 and 30 GHz, respectively (Figure 6.4(c) and (d)).

Assuming that the spectral shape of the slow relaxation process does not change significantly with temperature, we estimated the relaxation peak at $T \sim 200$ K from the intensity of its high frequency tail (Figure 6.5(b)). The relaxation time of the slow process seems to follow Arrhenius-like behavior (Figure 6.6). The estimated activation energy of the slow relaxation process is $\sim 43$ kJ/mol. However, the $\tau_0$ appears to be too short ($\sim 10^{-19}$ s), suggesting that the relaxation process does not really follow Arrhenius behavior. The observed temperature variation suggests the absence of any kind of true dynamic transition. The slow relaxation process simply enters the accessible frequency window at $T > T_D$, and the so-called dynamic transition is just a result of the shift of the slow relaxation process into this frequency window.

Figure 6.6 also presents the estimated temperature dependence of the relaxation time of the methyl group rotation. It is clear that the slow relaxation process varies stronger with temperature than methyl group rotation. Interestingly, at high temperatures above 295 K, the slow relaxation becomes faster than the methyl group rotation. This result can be explained well with the heterogeneous molecular basis of both protein structures and dynamics between the protein core and surface. Most of the large amplitude motions referred to as the slow relaxation process are attributed to secondary structures’ conformational relaxation of the “protein surface” driven by water translational motions through the hydrogen-bond fluctuations. As temperature increases, the hydrogen-bond network fluctuates faster.
In contrast, the activation of methyl group rotation is sterically hindered in the confined hydrophobic structure of the native “protein core” and thus not affected by the dynamics of water molecule. This might be the reason why at a high temperature like 320 K, methyl group rotation is slower than the slow relaxation process.

6.4.5 Comparison between the temperature dependence of the slow relaxation process and enzymatic activity

We discussed the relationship between the well-known dynamic transition of protein and the onset of its enzymatic activity in Chapter IV. In Chapter V, we proposed
that the slow relaxation process is the mode that activates dynamic transition and is closely related to the enzymatic activity at room temperature. Therefore, a comparison between the temperature dependence of the slow relaxation process and enzymatic activity can provide more details about their relationship.

In general, the enzymatic activity of protein exhibits Arrhenius behavior until it is not measurable at low temperatures. The activation energy for the biological reaction of lysozyme was reported in \(^{23}\). Figure 2.10 represents the several lysozyme-substrate binding processes until the cleavage reaction. It includes 3 intermediate processes where productive and unproductive complexes are created.

It is worth noting that the energy of protein-substrate binding ~ 71 kJ/mol is similar to the energy of the cleaving reaction ~ 79 kJ/mol. Therefore, the binding process is also very critical for determining enzymatic activity. The activation energy of the slow relaxation process ~ 43 kJ/mol is only about half of the energy of the biological processes. The difference means there should be some pre-factor(s) bridging the gap, such as entropic rearrangements in protein-substrate binding processes.

6.5 Conclusions

The temperature dependence of the slow relaxation process was studied in this chapter. At temperatures lower than \(T_D < 225 \text{ K}\), the methyl group rotation dominates the dynamics in both dry and wet proteins in a frequency range lower than 100 GHz. The slow relaxation process appears at \(T \sim 225 \text{ K}\) in the accessible frequency window. This
result supports the conclusion that the slow relaxation process is responsible for the dynamic transition of protein.

The characteristic relaxation time of the slow relaxation process was estimated after the neutron scattering spectra were corrected for the methyl group rotation or/and the possible fast process. It has stronger temperature dependence than the methyl group rotation and can be approximated by an Arrhenius-like dependence with the activation energy $\sim 43$ kJ/mol. The presented results lead us to speculate that: i) the slow relaxation process may be more localized motions like secondary relaxation in glass-forming liquids $^{55,115}$, and ii) the dynamic transition may be just the result of the slow relaxation process moving into the accessible frequency window at $T > T_D$.

The estimated activation energy of the slow relaxation process represents only about half of the energy required for the biological processes. The reason for this difference remains unknown.
CHAPTER VII

THE ROLES OF SOLVENTS IN PROTEIN DYNAMICS

7.1 Introduction

A thorough understanding of protein dynamics at the molecular level is essential not only for elucidating the mechanism of protein function, but also for finding the pathway to control protein stability. It is well known that protein dynamics are strongly influenced by solvents. One of the best examples evidencing the role of a solvent in protein dynamics is dynamic transition.

A number of studies carried out using neutron scattering, molecular dynamics simulation, and Mössbauer spectroscopy have supported the idea that protein dynamics are “slaved” or “coupled” to dynamical properties of solvents, since the translational motions of solvents are critical for triggering the dynamic transition.

Water- and glycerol-embedded proteins show dynamic transition at \( T \approx 200 \) K and \( 270 \) K respectively, close to the dynamic crossover temperature, \( T_C \) of the solvents. Trehalose-embedded proteins do not show the dynamic transition at \( T \) range up to \( 300 \) K because \( T_C \) of trehalose is higher than \( 390 \) K. At high \( T > 270 \) K, trehalose suppresses the dynamics of protein more strongly than water and glycerol.
It is worth noting that a recent light scattering study reported that glycerol is more effective in suppressing protein dynamics than trehalose at $T \sim 150 \text{ K}^{80,93}$. This result is consistent with the influence of glycerol and trehalose on the kinetics of CO-myoglobin geminate rebinding. It was shown that at $T < 270 \text{ K} (T_c \text{ glycerol})$ the escape rate of CO is faster in proteins placed in trehalose than in glycerol$^{131-133}$. These observations contradict the idea that protein motions and activity remain more strongly suppressed in a solvent of higher $T_g$ than in a solvent of lower $T_g$. The solution to this contradiction is still a subject of active discussion.

Our results in Chapters IV, V and VI demonstrated that there are three main relaxation processes in protein molecules: the methyl group rotation, the fast fluctuation and the slow relaxation process. It was shown that the slow relaxation process is responsible for the dynamic transition and is probably connected to the activation of protein function. It dominates at $T > T_D$, is strongly stretched, and overlaps with methyl group rotation and the fast process. At $T < T_D$, the fast process dominates high-frequency dynamics and methyl group rotation dominates the low-frequency dynamics. However, the effect of solvents on the dynamic processes of protein remains unclear.

The main goal of this chapter is to study the role of the solvents glycerol and trehalose in protein dynamics, as compared to the dynamics of dry and wet lysozyme. Neutron scattering measurements were performed in a broad frequency range from 100 MHz to 1 THz and in a broad $T$ range from 10 to 300 K. The fast and slow relaxation processes were characterized after the spectra were corrected for methyl group rotation. It turned out that the fast process at $T \sim 150 \text{ K}$ is suppressed more strongly in glycerol-solvated lysozyme than in dry, wet, and trehalose-solvated lysozyme, whereas at $T \sim 320$
K, the fast process is suppressed more strongly in protein placed in trehalose than in protein placed in glycerol. The slow relaxation process appears only in lysozyme placed in glycerol or water at $T > T_D$. This result supports the idea that the dynamic transition is related to the slow process entering the accessible experimental frequency window.

7.2 Samples and techniques

Sample preparation was described in section 3.4.1. The weight ratio of lysozyme to D$_2$O, glycerol, and trehalose is 1:0.42, 0.46, or 0.50, 1:1 and 1:1, respectively. Energy-resolving neutron scattering measurements were performed at 150, 200, 250, 295, and 320 K as explained in sections 4.2 and 5.2.1. Neutron scattering data were treated in the same way as described in sections 3.2.2 and 4.2.

7.3 Results

Figure 7.1 presents the $<\chi^2(T)>$ of all samples, estimated using a Gaussian approximation (equations (3.21) and (4.1)). As discussed in Chapter IV, lysozyme shows the onset of methyl group rotation at $T \sim 100$ K.

Only wet lysozyme (WL) and lysozyme placed in glycerol (LG) show the traditional onset of anharmonicity at $T_D \sim 200$ K and 270 K, respectively, as well as the onset of the methyl group rotation. At $T < T_D$, the $<\chi^2(T)>$ of wet lysozyme and lysozyme placed in glycerol is slightly lower than that of dry lysozyme (DL). The $<\chi^2(T)>$ of the lysozyme/trehalose sample (LT) is lowest over the entire temperature range up to 300 K.
Figure 7.1 $<x^2(T)>$ vs $T$. (See text in section 7.4.1 for LT corrected for trehalose.)

Figure 7.2 presents low-frequency $S(Q,\nu)$ summed over all measured $Q$ for wet lysozyme ($0.46\, h$) and lysozyme placed in glycerol at 250 K and the resolution function (vanadium). Wet lysozyme has a much stronger QES intensity than lysozyme placed in glycerol at $T \approx 250$ K, i.e. 50 K above $T_D$ of wet lysozyme, but 20 K below $T_D$ of lysozyme placed in glycerol. $S(Q,\nu)$ of lysozyme placed in glycerol is very similar to that of dry lysozyme. Therefore, the same relaxation mode that dominated the spectra of dry lysozyme dominates the low-frequency spectra of lysozyme placed in glycerol at $T < T_D$. The relevant motions are mainly methyl group rotation.
Figure 7.2 The low-frequency $S(Q, \nu)$ of dry lysozyme, wet lysozyme (0.46 h), and lysozyme placed in glycerol at $T \sim 250$ K. The spectrum of vanadium at $T \sim 295$ K presents the resolution function.

In Figure 7.3, the strongest QES intensity in the frequencies lower than 8 GHz is observed in wet lysozyme at $T \sim 295$ K. Lysozyme placed in glycerol clearly shows a stronger QES intensity than lysozyme placed in trehalose at $T \sim 320$ K. However, lysozyme placed in trehalose also has a strong QES intensity at $T \sim 320$ K that is very similar to the QES spectrum of dry lysozyme at $T \sim 295$ K.
Figure 7.3 The low-frequency $S(Q, \nu)$ of dry and wet lysozyme, and vanadium at $T \sim 295$ K, and the spectra of lysozyme placed in glycerol and lysozyme placed in trehalose at $T \sim 320$ K.

Figure 7.4 shows the high-frequency $S(Q, \nu)$ of dry lysozyme, wet lysozyme (0.50 $h$), lysozyme placed in glycerol, and lysozyme placed in trehalose at $T \sim 150$ K. As we discussed in Chapter VI, at $T \sim 150$ K, the methyl group rotation provides only a negligible contribution to the high-frequency dynamics of protein in a frequency range from 10 GHz to 3 THz (Figure 6.2(a)). Therefore, the high-frequency QES spectra are dominated by the fast process. There is no doubt that lysozyme placed either in glycerol or trehalose, as well as dry and wet lysozyme have a significant QES intensity at the low $T \sim 150$ K. It should be pointed out that the QES of lysozyme placed in glycerol exhibits the lowest intensity, and lysozyme placed in trehalose and dry lysozyme have the highest
QES at $T \sim 150$ K. The QES of wet lysozyme seems to be slightly more suppressed than those of lysozyme placed in trehalose and dry lysozyme.

Figure 7.4 The high-frequency $S(Q, \nu)$ of dry lysozyme, wet lysozyme, lysozyme placed in glycerol, and lysozyme placed in trehalose at $T \sim 150$ K.

At $T \sim 295$ K, wet lysozyme shows the highest QES intensity (Figure 7.5). The QES of lysozyme placed in glycerol at $T \sim 320$ K exhibits a stronger intensity than that of dry lysozyme at $T \sim 295$ K and of lysozyme placed in trehalose at $T \sim 320$ K. It seems that, as well as the fast process, the methyl group rotation that dominates the dynamics of dry lysozyme is still the dominant contributor to the dynamics of lysozyme placed in trehalose. However, lysozyme placed in glycerol at $T > T_D$ exhibits an additional process – the high frequency tail of the slow relaxation process.
Figure 7.5 The high-frequency $S(Q, \nu)$ of dry and wet lysozyme at $T \sim 295$ K, and of lysozyme placed in glycerol and lysozyme placed in trehalose at $T \sim 320$ K.

Figure 7.6 presents the high-frequency $S(Q, \nu)$ of lysozyme placed in glycerol at $T \sim 150, 250, \text{ and } 320$ K. It is obvious that the QES of lysozyme placed in glycerol increases with $T$. A strong increase of QES intensity for lysozyme placed in glycerol appears between $T \sim 250$ K (20 K lower than $T_D$) and 320 K (50 K higher than $T_D$.}
Figure 7.6 The high-frequency $S(Q, \nu)$ of lysozyme placed in glycerol at $T \sim 150$, 250, and 320 K.

7.4 Discussion

7.4.1 Solvent effects on the $\langle \chi^2(T) \rangle$ of proteins

Analysis of $\langle \chi^2(T) \rangle$ (Figure 7.1) shows that the methyl group rotation is also activated in the proteins placed in glycerol and in trehalose. Consistent with previous literature, wet lysozyme and lysozyme placed in glycerol exhibit a dynamic transition at $T \sim 200$ K and 270 K, respectively, but dry lysozyme and lysozyme placed in trehalose do not show any dynamic transition up to $T \sim 320$ K.
It is interesting that the $<x^2(T)>$ of the lysozyme/trehalose sample appears much lower than the $<x^2(T)>$ of lysozyme placed in glycerol at $T < T_D$ and the $<x^2(T)>$ of dry lysozyme. This result contradicts the light scattering results and some literature data indicating that at low temperatures, the fast dynamics of protein are more suppressed in glycerol than in trehalose and that the dynamics of lysozyme in trehalose is similar to that of dry lysozyme throughout the entire $T$ range.

The $<x^2(T)>$ of the lysozyme/trehalose sample obtained directly from elastic neutron scattering measurements included the contribution of protein and the trehalose motions. Given the 63 % deuteration of trehalose determined by $^1$H NMR measurement and the 1:1 weight ratio of trehalose to lysozyme, the remaining H-atoms of trehalose contributed 33 % of the total scattering from the lysozyme/trehalose sample. The scattering ratio of H-atoms in trehalose to total H-atoms in the sample with a 1:1 weight ratio of trehalose to lysozyme:

$$\frac{S_{\text{Trehalose}}}{S_{\text{Total}}} = \frac{\frac{N_{\text{H-Trehalose}}}{M_{\text{Trehalose}}}}{\frac{N_{\text{H-Trehalose}}}{M_{\text{Trehalose}}} + \frac{N_{\text{H-Lysozyme}}}{M_{\text{Lysozyme}}}} = \frac{22 \times 0.37}{342} + \frac{22 \times 0.37}{342} + \frac{695}{14360} = 0.33 \quad (7.1)$$

where, $N_{\text{H-Trehalose}}$: the number of non-exchanged H-atoms in one trehalose molecule

$N_{\text{H-Lysozyme}}$: the number of non-exchanged H-atoms in one lysozyme molecule

$M_{\text{Trehalose}}$: the molecular weight of one trehalose molecule

$M_{\text{Lysozyme}}$: the molecular weight of one lysozyme molecule.

Therefore, the $<x^2(T)>$ of the lysozyme/trehalose sample should be corrected for the contribution of trehalose in order to estimate the $<x^2(T)>$ of lysozyme. The correction was made by
\[ \langle x^2(T) \rangle_{LT\text{-corrected}} = \frac{\langle x^2(T) \rangle_{LT\text{-measured}} - 0.33 \langle x^2(T) \rangle_{\text{Trehalose}}}{0.67} + C. \]

\( \langle x^2(T) \rangle_{\text{Trehalose}} \): Mean-squared atomic displacement of pure trehalose

\( C \): Normalization factor

The final \( \langle x^2(T) \rangle_{LT\text{-corrected}} \) was obtained from a normalization by the \( \langle x^2(T) \rangle \) of dry lysozyme at the lowest \( T \), using \( C \). The \( \langle x^2(T) \rangle_{LT\text{-corrected}} \) approaches the \( \langle x^2(T) \rangle \) of dry lysozyme, but is slightly lower than the \( \langle x^2(T) \rangle \) of dry lysozyme at a high temperature (Figure 7.1). This result suggests that trehalose is more effective in suppressing the dynamics of protein at high temperatures than in a dry state.

7.4.2 Solvent effects on protein dynamics at temperatures below \( T_D \)

Figure 7.7 shows the HFBS- and DCS-combined \( \chi''(\nu) \) of dry and wet lysozyme, lysozyme placed in glycerol, and lysozyme placed in trehalose at \( T \sim 150 \) K at a broad frequency range. At lower frequencies, all QES spectra are expected to be dominated by methyl group rotation at \( T \sim 150 \) K (Figure 6.3). At higher frequencies, the contribution of methyl group dynamics is negligible and the difference in the QES spectra originates from the fast process.

In general, fast dynamics exist both in dry and solvated proteins even at temperatures lower than the \( T_g \) of the solvents. Glassy solvents like water and glycerol cause the fast dynamics of proteins to become more suppressed at low temperatures than in a dry state. This is probably due to the volume-reducing confinement created by the glassy solvent molecules. We suspect that the size of the cage formed by neighboring
groups of atoms decreases by the water and glycerol molecules at low temperatures. This confinement restricts the motions of groups of atoms in protein molecules.

The solvent molecules contain functional groups that can form hydrogen bonds with the surface polar groups of protein molecules. These hydrogen bonds can fluctuate at $T \sim 150$ K. However, the hydrogen bond fluctuation at $T$ lower than $T_g$ of the solvent is too restricted to overcome the confinement, leading to localized motions smaller than the fast motions in dry lysozyme. Therefore, the coupling between protein dynamics and the dynamics of the surrounding solvent molecules is considered important at low temperatures.

Figure 7.7 $\chi''(\nu)$ of dry lysozyme, wet lysozyme, lysozyme placed in glycerol and lysozyme placed in trehalose at $T \sim 150$ K in a broad frequency range.
Recent light scattering and simulation\(^{80,93,115,116}\) studies have contributed to an understanding of the solvent effects on protein dynamics at low temperatures. Their results showed that QES intensities at \(T \sim 150\) K are stronger in lysozyme placed in trehalose and in pure trehalose than in lysozyme placed in glycerol and in pure glycerol. Based on the stronger QES of trehalose than of glycerol, it was suggested that trehalose itself shows strong fast conformational fluctuations even in a solid state, and thereby leads to a weaker suppression of the fast motions in proteins. We suspect that the flexible ether bond in trehalose (Figure 3.22) allows the protein placed in trehalose to fluctuate even at low temperatures. At the same time, the more suppressed dynamics of glycerol result in a stronger restriction of fluctuation in protein placed in glycerol. Simulation studies\(^{115}\) showed that the dynamic behavior of protein follows the dynamics of pure glycerol up to 1 ns. Therefore, a strong coupling between the fast dynamics of proteins and the dynamics of the surrounding solvents occurs even at low temperatures.

However, a definite understanding of the microscopic mechanism that explains the strongest effect of glycerol to suppress the fast process of protein at low temperatures has not yet been determined. It is probably because the fluctuation of glycerol at low temperatures is strongly suppressed. The suppressed fluctuation of glycerol transfers to the lowest fluctuation of protein due to coupling between solvents and protein molecules.

7.4.3 Solvent effects on protein dynamics at temperatures above \(T_D\)

Figure 7.8 presents combined high- and low-frequency \(\chi''(\nu)\) spectra of dry and wet lysozyme, lysozyme placed in glycerol, and lysozyme placed in trehalose at high \(T\).
The spectra of dry lysozyme at $T \sim 295$ K and lysozyme placed in trehalose at $T \sim 320$ K at frequencies lower than 10 GHz are dominated by methyl group rotation. However, the spectra of wet lysozyme at $T \sim 295$ K and lysozyme placed in glycerol at $T \sim 320$ K show a strong additional contribution, the slow relaxation process. The slow process appears differently in wet lysozyme and lysozyme placed in glycerol.

Figure 7.8 $\chi''(\nu)$ of dry lysozyme, wet lysozyme, lysozyme placed in glycerol, lysozyme placed in trehalose at $T \sim 295$ or 320 K in a broad frequency range.

A clear relaxation peak appears in the wet sample, while only the high-frequency tail of the slow relaxation process appears in the spectrum of lysozyme placed in glycerol. Assuming that the shape and the amplitude of the peak remain the same for wet protein.
and protein placed in glycerol, we estimated the maximum of the peak in the latter case using the observed tail of the slow process (Figure 7.8). It appears to be ~ 0.06 GHz.

Wet lysozyme at \( T \sim 320 \text{ K} \) exhibits the relaxation frequency of the slow relaxation process around 30 GHz (Figure 6.6). This result indicated that the relaxation time is 500 times faster in wet lysozyme than in lysozyme placed in glycerol at the same temperature. This difference is close to the difference in viscosity: at \( T \sim 320 \text{ K} \), bulk water has 260 times lower viscosity than bulk glycerol.

At \( T \sim 320 \text{ K} \), the dynamics of lysozyme at frequencies above 50 GHz is most suppressed in trehalose. The difference between lysozyme placed in trehalose and dry lysozyme would be even larger if they had been measured at the same temperature. This result is consistent with the literature and indicates that at high \( T \), trehalose suppresses the dynamics more strongly than any other solvents. That may be the reason for better protein stabilization in trehalose than in the dry state at high temperatures.

It is obvious that the fast process exists even at \( T \) lower than \( T_D \). For example, a clear QES is visible at \( T \sim 150 \text{ K} \) at higher frequencies where the methyl group rotation does not interfere with the fast process (Figure 7.4). The fast process, in general, increases with \( T \), but its relaxation frequency varies weakly. This is similar to the behavior of the fast process in glass-forming systems. Figure 7.6 shows the increase in the high-frequency dynamics of lysozyme placed in glycerol with \( T \). Unfortunately, determining the temperature dependence of a pure fast process is not possible unless the temperature dependence of the methyl group rotation and the slow relaxation process has been well described quantitatively. A clear result is that the fast process becomes less
important than the slow relaxation process as $T$ increases above $T_D$, whereas the fast process is important at $T$ lower than $T_D$.

7.5 Conclusions

In this chapter, we have analyzed the influence of glassy solvents on protein dynamics by using neutron scattering measurements. Fast relaxation is observed in all samples even at low $T \sim 150$ K, whereas slow dynamics were found only in wet lysozyme and lysozyme in glycerol at $T > 200$ and 270 K, respectively.

Significantly, at the low $T \sim 150$ K, the fast dynamics of lysozyme placed in glycerol is more suppressed than dry and wet lysozyme, and lysozyme placed in trehalose. This observation suggests glycerol gives more confined environments to the protein than other solvents.

Water is the solvent that provides the highest mobility associated with the relaxation rate in protein molecules at $T > T_D$. Glycerol gives less flexibility (lower relaxation rate of the slow process) to protein than water at the same temperature. The effect of trehalose on the fast dynamics of protein is very similar to the dry state. However, at a high $T \sim 320$ K, the fast fluctuations in protein are suppressed more strongly in trehalose than in the dry state.

If the fast dynamics is important for the long-term stability of protein at low temperatures, glycerol is a better cryogenic solvent for bio-preservation than trehalose and water. However, trehalose is a better glassy solvent than glycerol for protein preservation at high temperatures, because the slow relaxation process is totally
suppressed in lysozyme placed in trehalose, while it dominates the dynamics of protein placed in glycerol.
CHAPTER VIII

SUMMARY

We investigated the influence of hydration and temperature on protein dynamics on a pico- to nano-second time scale by using neutron and light scattering spectroscopy. The main goal of this study was to understand i) the basics of protein dynamics, ii) the microscopic nature of processes contributing to relaxation on a pico- to nano-second time scale iii) the relationship between protein dynamics and functions, and iv) the microscopic nature of functionally important motions of protein. Detailed analysis of the spectroscopic results showed that protein has three main dynamic processes: methyl group rotation, fast fluctuation, and a slow relaxation process. We discussed their hydration- and temperature-dependencies and roles in biochemical activity.

We found a low-temperature onset of anharmonicity in $<x^2(T)>$ at $T \sim 100$ K in addition to the well-known dynamical transition at $T \sim 200 – 220$ K. It is worth emphasizing that the low-temperature anahrmonicity was observed in all samples regardless of a hydration level, whereas the well-known dynamical transition was observed only in wet proteins at hydration levels higher than $\sim 0.2$ $h$. Analysis of the temperature, wave vector, and frequency dependence of neutron scattering parameters for dry lysozyme evidenced that methyl group dynamics is the major contributor to the low-
temperature onset of anharmonicity. The MD-simulations separating the contribution from methyl groups supported our interpretation.

The influence of hydration on protein dynamics was investigated using neutron and light scattering measurements at room temperature. In addition to methyl group dynamics, fast and slow relaxation processes were identified. The slow relaxation process was attributed to larger-amplitude motions, but it was still localized to displacements $\sim 3$ Å. The three different dynamic modes have very particular dependence on hydration: i) methyl group dynamics turned out to be rather insensitive to hydration, ii) the fast fluctuations increase very sharply at $h < 0.2$ and then slightly with further increase in the hydration level, and iii) the slow relaxation process seems to be suppressed until $h \sim 0.2$, then it increases sharply between $h \sim 0.2$ and $h \sim 0.5$ and only slightly at $h > 0.5$. Interestingly, the hydration dependence of the slow relaxation process showed good agreement with that of dynamic transition and enzymatic activity. From this result, we ascribed the slow relaxation process to the mode that is responsible for the dynamic transition and is necessary for the activation of protein function (the enzymatic activity in the case of lysozyme).

We demonstrated that the slow relaxation process appears in the given time window at $T \geq 200$ K. Temperature dependence of the characteristic relaxation time of the slow process was estimated after correcting for methyl group contribution. It behaves Arrhenius-like with the activation energy of $\sim 43$ kJ/mol. These results suggest that the dynamic transition is a result of the slow relaxation process entering the given time window. However, the microscopic mechanism of the slow relaxation process remains unclear. The activation energy of the slow relaxation process is only half of the
activation energy that controls the rate of the enzymatic activity of lysozyme. The gap between them is not clear. It may come from some environmental factors like entropic rearrangement of the substrate, which cannot be considered in protein dynamics.

An analysis of the influence of different solvents on protein dynamics demonstrates that, at low temperatures, glycerol suppresses the fast process of protein more strongly than other solvents (water and trehalose). This observation suggests that glycerol confines the protein stronger than other solvents at low temperatures. This result might provide an explanation of why glycerol is the best cryopreservant known. The fast process, however, is probably not important at high temperatures, where the slow relaxation process is dominating the protein dynamics. Trehalose suppresses protein dynamics better than other solvents at high temperatures. That might explain why trehalose provides the best protein-preservation at high temperatures.
REFERENCES


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