

Neutron inelastic scattering as a high-resolution vibrational spectroscopy: New tool for the study of protein dynamics

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Abstract. We have applied inelastic neutron scattering (INS) to the understanding of protein dynamics. INS spectrum of staphylococcal nuclease (SNase) at 25 K in the energy range between 100 and 4000 cm^{-1} is compared with the result of normal mode calculation. The theoretical spectrum is in general agreement with experiment and is used to assign the peaks. INS spectra show some significant differences for the folded and the unfolded SNase. The intensity distribution of INS spectrum is different from protein to protein reflecting the differences in amino acid composition. INS is unique and effective for the study of protein dynamics, especially for comparison with theory.

1. Introduction

It is widely accepted that protein is flexible and mobile. The flexibility and mobility, that is, protein dynamics are essential for protein function. In order to investigate protein dynamics quantitatively, we need to measure the dynamics in detail. Inelastic neutron scattering (INS) is one of the effective methods to measure protein dynamics quantitatively [1]. INS gives an essentially vibrational spectrum, which is complementary to the IR or Raman spectroscopy. One advantage of INS over these spectroscopic methods is that the observed spectrum is quantitatively compared with the theoretically calculated spectrum. The estimation of transition dipole moments or changes in electronic polarizability is necessary to calculate the IR or Raman spectrum, respectively, which is not easy calculation. Moreover, all vibrational modes are not necessarily observed by optical vibrational spectroscopy. INS is free from such a selection rule.

The disadvantages of INS come from the fact that we need a reactor or an accelerator for the neutron source. The places where we can carry out INS experiment are quite limited. Even though we can access to the neutron facilities, the allocated machine time is quite limited. Since the incident neutron flux is not necessarily high, we need a large amount of sample proteins and/or a considerably long measurement time to obtain good quality data. Therefore, INS is not a universal method, but a quite unique method for

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quantitative analysis of protein dynamics. Recent progresses of neutron sources and neutron spectrometers as well as the progress in the method to purify a large amount of protein in molecular biology allow us to obtain high-resolution spectra over the whole frequency range of interest (0–4000 cm^{-1}).

Using Staphylococcal nuclease (SNase), we have been investigating the protein dynamics with INS over a wide range of frequency [2,3]. We succeeded to record INS spectrum of SNase at 25 K in the region between 200 and 3500 cm^{-1} with good quality [2]. The spectrum was compared with the normal mode analysis of SNase [2]. The calculated spectrum, which was obtained using a force field that was not refined to fit the present experimental data, is in general agreement with experiment and are used to assign the peaks. However, the agreement between experiment and theory is not perfect. The study obviously made an essential contribution to the improvement of theoretical studies.

Protein dynamics are closely related to the function and the unique tertiary structure is required to express function. Thus, two simple questions raised are: whether there are specific dynamical properties to the folded native protein; and whether dynamical property is different from protein to protein. We want to reveal the changes in protein dynamics upon folding. In this regard, SNase is a unique and useful model protein. The fragment of SNase which lacks the C-terminal 13 residues is in partially unfolded state under a physiological condition [4,5]. We can measure INS spectrum for the folded and the unfolded states under the same condition. We also intend to reveal the protein individuality in terms of dynamics. For the purpose, we selected photoactive yellow protein (PYP). Both PYP and SNase are categorized into the α/β protein. While SNase is an enzyme, PYP is thought to be a photoreceptor protein and expected to show large conformational change upon light absorption [6,7]. In this paper, we will compare the INS vibrational spectra of the unfolded SNase and PYP with the unfolded SNase.

2. Experimental

2.1. Sample preparation

SNase, its deletion mutant (fragment) which lacks C-terminal 13 residues, and PYP were overexpressed with *Escherichia coli*, and isolated by urea extraction [8–10]. SNase and its mutant were purified with ion-exchange chromatography. PYP was also purified with ion-exchange column after reconstituted with p-coumaric acid. Purified proteins were dissolved into D_2O and lyophilized. This procedure was repeated two to three times to substitute labile protons to deuterons completely. Lyophilized powder of each protein was equilibrated with 90% relative humidity of D_2O to ensure hydration. The estimated hydration level was 0.39 g, 0.29 g, and 0.38 g D_2O per g protein for SNase wild type, SNase fragment and PYP, respectively.

2.2. Neutron scattering experiment

Inelastic neutron scattering experiment was performed with the TOSCA spectrometer, an indirect geometry time-of-flight spectrometer at the ISIS pulsed spallation neutron source at the Rutherford Appleton Laboratory [11]. The final neutron energy was 3.95 meV, and spectra were acquired at a single scattering angle of 135° . The energy resolution, $\Delta E/E$ is about 2% between 400–1600 cm^{-1} and increases to more than 3% in 2400–4000 cm^{-1} range.

The hydrated powder of each protein was packed into an aluminum bag of 5 cm height and 2 cm width with a path length 0.2 mm. Total amount of powder was 1–1.2 g. The estimated transmission

was 0.88–0.90, indicating that the multi-phonon effect was negligible. The data collection time was 36–48 hours. The measurement was carried out at both 25 and 300 K. Biochemical and biophysical properties of these proteins were not changed after the neutron exposure.

3. Results and discussion

Figure 1 shows INS spectra of SNase at room temperature and at 25 K. Although the curve for low temperature is shifted for the sake of clarity, the baselines are almost identical for both spectra as shown previously [2]. It is clear that the intensity of low energy region for room temperature is higher than that for low temperature, indicating the increase of thermal motions at room temperature. The low temperature spectrum is more structured than the room temperature spectrum, which is mainly due to Debye–Waller factor [2]. The activated anharmonic motions as well as multi-phonon scattering would also affect the room temperature spectrum.

The low temperature spectrum can be explained qualitatively with the calculated normal modes [2]. Using the results of normal modes calculation with CHARMM, we can assign the peaks of INS spectrum [2]. For example, the peaks at around 2900 cm^{-1} is assigned as the CH stretch band, and the peak around 1500 cm^{-1} is a CN stretch band. The vibrations between 700 and 1000 cm^{-1} are mostly CH_3 rotation, CH_2 rotation and CC stretch. The sharp peak at 235 cm^{-1} is due to methyl torsions. In the range between 350 and 500 cm^{-1} the vibrations are delocalized over the protein and involve essentially coupled skeletal angle and dihedral displacements. The typical assignment for the spectrum is given in Table 1. Quantitative coincidence between the experiment and the theory is not necessarily obtained. The observed peak positions are shifted from the expected positions, and intensity distribution is different between the experiment and the theory. This fact strongly indicates that the improvement of theoretical model including the estimation of force constants is required for the entire understanding of experimental spectrum of a protein. Recently, it is shown that the room temperature spectrum is qualitatively simulated by molecular dynamics simulation, while normal mode calculation cannot explain the experimental room temperature spectrum [12]. This fact suggests a significant contribution of anharmonic motions to the spectrum at room temperature. Consequently, INS is considered to be a unique and effective for the improvement of the theoretical dynamical model of proteins.

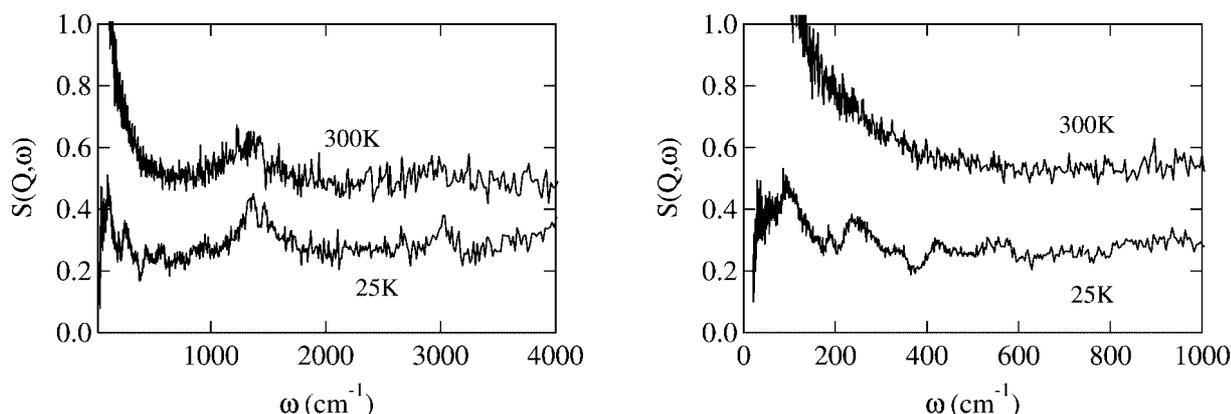


Fig. 1. Experimental INS spectra of staphylococcal nuclease at 300 K and at 25 K. (Left) entire energy region, (right) lower energy region. For the sake of clarity, the 300 K spectrum is shifted 0.2 unit along $S(Q, \omega)$ axis.

Table 1

Peak assignment for 25 K INS spectrum of staphylococcal nuclease with the aid of normal mode analysis [2]

Experiment (cm^{-1})	Theory (cm^{-1})	Assignment ^a
235	269	CH ₃ -t
400–450	395–435	CCC-def, CCN-def, skeletal
477	471	CCC-def, CCN-def, skeletal
470–590	–	Water O-H · · O-b
720–775	720–795	CH ₂ -r, CH-b
837	835	CH ₂ -r
936	958	CH ₂ -r, CH ₃ -r
1136	1136	CH-b, CH ₂ -tw, CH ₃ -r
1284	1291	CH ₂ -tw, CH ₂ -w, CH-b, CH ₃ -sb
1326		
	1352	CH ₂ -w, CH ₂ -tw, CH-b, CH ₃ -sb
1386		
1455	1431	CH ₂ -b, CH ₃ -ab, CH-ip
1555	1530	CN-s
	1686	CN-s
	2430	ND-s
2952	2920	CH-s

^aThe abbreviation used are as follows: t = torsion; def = deformation; b = bend; r = rock, tw = twist; w = wag; sb = symmetric bend; ab = antisymmetric bend; ip = in-plane bend; s = stretch.

We are interested in the dynamical properties specific to the folded protein. In other words, can we expect that the INS spectrum in high energy region reflects the protein conformational state? In order to approach this problem, we tried to measure the INS spectra for the folded SNase and the unfolded SNase. The large fragment of SNase that lacks 13 residues from its C-terminus takes a compact denatured structure [4,5]. Therefore, we can measure INS spectra for the folded state and the unfolded state under the same experimental condition. This is great advantage for the INS experiment. Generally, protein unfolding is brought by thermal denaturation, acid denaturation or denaturant denaturation. Because INS measures thermal motions and INS mainly comes from incoherent scattering of hydrogen, these procedures affect the INS spectrum even without unfolding. It would be quite difficult to distinguish the changes in vibrational modes due to unfolding.

Figure 2 compares the INS spectrum of the SNase mutant with that of wild type SNase at 25 K. The folded SNase and the unfolded fragment gave quite similar spectra, especially in higher energy region. If we examine them very carefully, we notice some differences. For example, the peak at about 3000 cm^{-1} is sharper for the folded state than for the unfolded state. The peak at about 150 cm^{-1} is distinct for the folded state, while the peak at about 770 cm^{-1} is distinct for the unfolded state. This would reflect the formation of the secondary structures. Since the local environment of each amino acid residue formed by non-covalent bonds should be different between the folded and unfolded states, peak shifts and/or peak broadening can be expected. In order to reveal such differences, we need more precise measurement with higher energy resolution.

According to the theoretical studies by Nishikawa and Go, the contribution from vibrational modes with higher energy than 30 cm^{-1} to the atomic displacements of each amino acid residue is independent

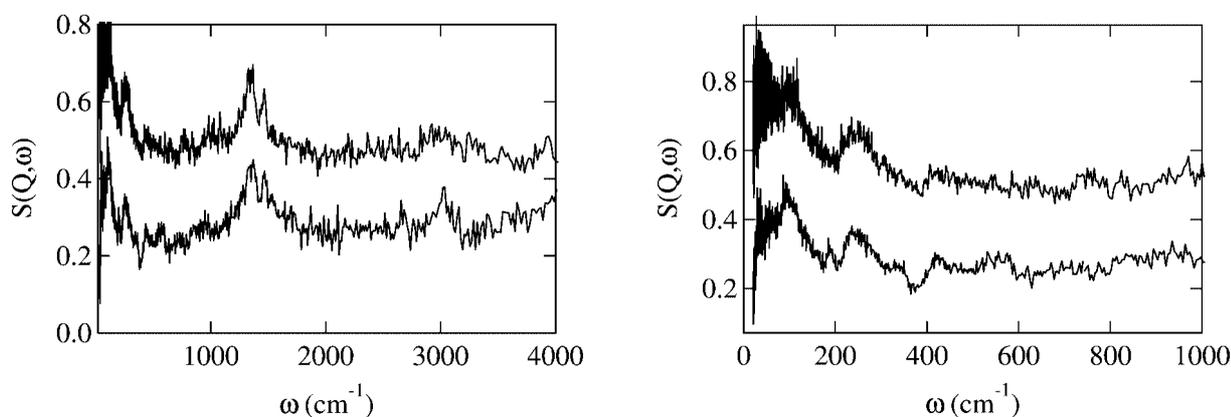


Fig. 2. Comparison of INS spectra between wild type SNase (folded form) and the truncated mutant (unfolded form). (Left) entire energy region, (right) lower energy region. In both panels, the upper and the lower curves represent the mutant spectrum and the wild type spectrum, respectively. For the sake of clarity, the spectrum for the mutant is shifted 0.2 unit along $S(Q, \omega)$ axis.

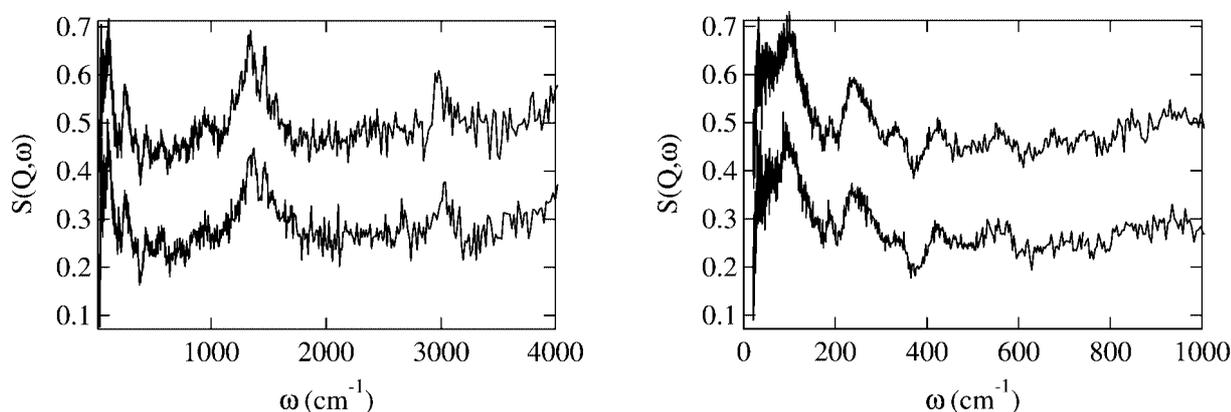


Fig. 3. Comparison of INS spectra between SNase and PYP. (Left) entire energy region, (right) lower energy region. In both panels, the upper and the lower curves represent the PYP spectrum and the SNase spectrum, respectively. For the sake of clarity, the PYP spectrum is shifted 0.2 unit along $S(Q, \omega)$ axis.

of the residue position, while the contribution from the modes with the energy lower than 30 cm^{-1} shows the residue number dependence [13]. This example suggests the importance of the measurement of INS spectrum of lower energy than 100 cm^{-1} . We cannot access this energy range with TOSCA. Using the LAM-40 spectrometer installed at the spallation neutron source at Tsukuba (KENS), Japan, we demonstrated that the differences in dynamical properties between the folded and the unfolded state are observed with INS spectrum below 150 cm^{-1} at room temperature [3]. Further intensive measurements are required to identify the vibrational modes and anharmonic motions specific to the folded state.

Finally, we examined whether INS can reveal the protein individuality. Figure 3 shows the comparison of INS spectra between SNase and photoactive yellow protein (PYP). Both proteins are categorized into α/β proteins [14,15]. PYP contains *p*-coumaric acid as a chromophore [15]. The two spectra are quite similar in their appearance, especially in high energy region. This is reasonable, because covalent bonds responsible to the vibrational spectrum are common for both proteins. However, the intensity distribution is different each other. This is also reasonable, because the composition of amino acids, and

thus the composition of covalent bonds, is different. The differences in the range 100–600 cm^{-1} would be a reflection of the differences in the composition of secondary structures and the tertiary structure [1]. Theoretical analysis of the INS spectrum of PYP is now under way in our group.

PYP is a putative photoreceptor for the negative phototaxis of halophilic purple sulfur bacteria [16]. PYP shows a large conformational change upon light absorption [6,7]. The conformational change is responsible for the signal transduction. SNase is an enzyme for hydrolysis of nucleic acid. Structural change during enzymatic function would be localized and not so large. Therefore, dynamical properties supporting these conformational changes are expected to be quite different each other. These dynamical modes would be delocalized and extended to all over the protein or to all over the domain. Kinetic energies of such modes are very low as compared with the localized vibrational modes. Therefore, the differences in dynamical properties between SNase and PYP and other proteins would be observed by INS of lower energy region than 200 cm^{-1} as is theoretically suggested [13]. In general, the INS spectrum in the high energy region is useful to show common dynamical properties to proteins, but with current instrumentation cannot easily be used to describe the protein individuality. In order to discuss the dynamical properties specific to the folded state and necessary for the function, it is required to measure INS spectra of lower energy region than 200 cm^{-1} more precisely and intensively. Such a trial is also under way in our group.

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