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# How can we derive hydration water dynamics with incoherent neutron scattering and molecular dynamics simulation?

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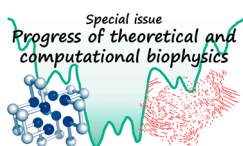
Incoherent neutron scattering (INS) is one of the useful experimental methods for studying protein dynamics at the pico-nanosecond timescale. At this timescale, protein dynamics is highly coupled with hydration, which is observed as protein dynamical transition (PDT). INS is very sensitive to hydrogen atomic dynamics because of the large incoherent scattering cross section of hydrogen atom, and thus, the INS of a hydrated protein provides overall dynamic information about the protein, including hydration water. Separation of hydration water dynamics is essential for understanding hydration-related protein dynamics. $H_2O/D_2O$ exchange is an effective method in the context of INS experiments for observing the dynamics of protein and hydration water separately. Neutron scattering is directly related to the van Hove space-time correlation function, which can be calculated quantitatively by performing molecular dynamics (MD) simulations. Diffusion and hydrogen bond dynamics of hydration water can be analyzed by performing MD simulation. MD simulation is useful for analyzing the dynamic coupling mechanism in hydration-related protein dynamics from the viewpoint of interpreting INS data because PDT is induced by hydration. In the present work, we demonstrate the methodological advantages of the $H_2O/D_2O$ exchange technique in INS and the compatibility of INS and MD simulation as tools for studying protein dynamics and hydration water.

Key words:    Quasi-elastic neutron scattering, $H_2O/D_2O$ exchange, Protein dynamics, Protein hydration

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Proteins, biologically functional molecules, work in an aqueous environment at ambient temperature. Protein structure fluctuates thermally, and its dynamics is strongly influenced by hydration [1–3]. In fact, a theoretical study demonstrated that the low-frequency collective modes in proteins are strongly coupled to hydration. The hydration-coupled modes contribute dominantly to the motional amplitude of proteins [4]. Except for stochastically rare conformational changes from the micro to the milli second timescales, protein structural dynamics under thermal equil-

◀ *Significance* ▶

Dynamical coupling between protein and hydration water is one of the essential subjects of protein biophysics. In this paper, we report incoherent neutron scattering with $H_2O/D_2O$ exchange enables to derive hydration water dynamics. All atom MD simulations reveal hydrogen bond dynamics specific to hydration water. These results are comparable with each other and give us deep insight for hydration water dynamics.

ibration is characterized at the pico-to-nanosecond timescale and on order of Angstrom units in the spatial scale [5]. Incoherent neutron scattering (INS) facilitates analysis of protein dynamics in these time and space regions [6,7]. Vibrations, relaxation, and diffusion dynamics are characterized using INS [8]. Molecular vibrations appear as inelastic scattering over a wide energy range, and the inelastic contribution can be approximated using the Debye-Waller factor. The relaxation and diffusion dynamics appear as quasi-elastic neutron scattering (QENS), which accounts for a broad part of the spectrum around elastic scattering. One of the hydration-related protein dynamics observed by INS is protein dynamical transition (PDT) [1,5,9]. This is observed as an abrupt increase in mean-square displacements (MSDs) at approximately 240 K with increasing temperature. PDT is enhanced significantly above the threshold hydration level by percolation transition of hydration water and is inhibited at lower hydration levels [3,10].

The neutron scattering function is expressed by the dynamic structural factor, $S(Q,\omega)$, which is a Fourier transformation of van Hove's correlation function in real space and time, $G(r,t)$ [11]. The neutron scattering function is separated into coherent and incoherent scatterings. INS probes the self-correlation of a single particle, which is useful for characterizing molecule dynamics, while the molecular structure is determined by the analysis of coherent neutron scattering. In Table 1, the neutron cross sections of the main constituent atoms in proteins are listed. The cross sections of incoherent and coherent scattering depend on the element species. Because X-rays are scattered by electrons, scattering cross section is almost proportional to atomic number. This is not the case for neutrons. Particularly noteworthy in Table 1 are large incoherent cross section of hydrogen and its significant isotope effect. The cross section of hydrogen is larger than that of deuterium by one order of magnitude. Typically, half of the constituent atoms of proteins is hydrogen atoms. In INS experiments, protein dynamics are observed through scattering from the hydrogen atoms in protein. Therefore, deuteration isotope labeling can be used for observing site-specific dynamics [12,13]. For studying protein dynamics in the hydrated state by INS, $D_2O$ is used for hydration during sample preparation to minimize the scattering due to hydration water. Moreover, $H_2O/D_2O$ exchange is rather effective for analyzing the protein and hydration water separately [10,14,15].
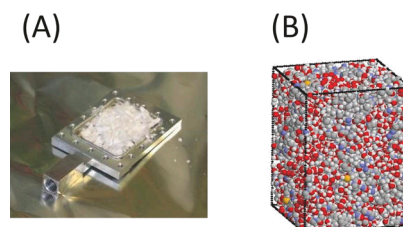
Molecular dynamics (MD) simulation is a powerful method for studying protein dynamics [16]. From MD results, van Hove's correlation function, which is directly related to the neutron scattering function, can be calculated [8]. Neutron scattering spectra calculated from MD simulation of crystalline protein is comparable quantitatively to the experimental data obtained using hydrated powder protein samples [17,18]. Moreover, MD simulation is an effective tool for analyzing the dynamics of hydration water, such as diffusion and hydrogen-bonding dynamics [19]. The analysis provides a reasonable interpretation of the neutron spectrum of hydrated proteins [20]. Combined analysis of INS with MD is effective for elucidating the dynamic coupling between protein and hydration water.

In the present work, we analyze the dynamics of hydration water in hydration-induced PDT of staphylococcal nuclease (SNase) by performing an INS experiment with $H_2O/D_2O$ exchange of hydration water and MD simulation.

## Materials and Methods

### Sample preparation

SNase was expressed in *Escherichia coli* and purified by urea extraction and with SP-Sepharose Fast Flow column chromatography. The purified protein was dialyzed against $D_2O$ and lyophilized. The resultant powder was dissolved in $D_2O$ and lyophilized. The procedure was repeated more than three times to ensure complete substitution with $D_2O$. The obtained lyophilized protein was used as the dehydrated sample (Fig. 1(A)), and its hydration level was estimated as to be 0.11 g $D_2O$/g protein by thermogravimetry [3]. Hydrated SNase was prepared by equilibration of the dehydrated sample with a saturated KCl, NaCl, or NaBr solution of $D_2O$. The corresponding hydration levels were 0.44, 0.36, and 0.30 g $D_2O$/g protein, respectively. The water content of the dehydrated SNase was almost identical to the number of water molecules in its crystal structure (1EY0.pdb). The hydration level of 0.44 g $D_2O$/g protein corresponds roughly to a single hydration layer [3].

**Table 1** Coherent, incoherent, and total neutron cross sections of a few elements.

| Elements | $\sigma_{coh}$ ($10^{-24}$ cm$^2$) | $\sigma_{inc}$ ($10^{-24}$ cm$^2$) | $\sigma_{tot}$ ($10^{-24}$ cm$^2$) |
|---|---|---|---|
| H | 1.76 | 80.27 | 82.03 |
| D | 5.59 | 2.05 | 7.64 |
| C | 5.55 | 0.001 | 5.55 |
| N | 11.01 | 0.50 | 11.51 |
| O | 4.23 | 0.00 | 4.23 |



(A)                    (B)

**Figure 1** (A) Lyophilized protein power sample in aluminum neutron cell and (B) snapshot from MD simulations of hydrated crystalline model system containing four SNase molecules at h=0.40 g $H_2O$/g protein.
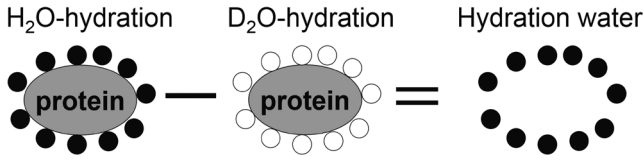
**Figure 2**  Concept of H$_2$O/D$_2$O exchange for estimating scattering intensity of hydration water based on difference in scattering intensity between H$_2$O- and D$_2$O-hydrated proteins.

### Neutron scattering experiment

Neutron scattering experiments were performed with the triple axis spectrometer LTAS in the JRR-3M reactor, which has an energy resolution of ~106 μeV, in Tokai, Japan. The q-range covered was 0.4–2.0 Å$^{-1}$. Neutron scattering measurements were performed at intervals of 10 K between 80 K and 300 K. First, the temperature was reduced to 80 K. Then, the measurements were performed at the target temperature. The obtained data were corrected by empty cell background subtraction. Data analysis was performed without multiple scattering correction because the sample transmission value was relatively large, approximately 90%.

The dynamics of hydration water can be examined based on the difference in INS between H$_2$O- and D$_2$O-hydrated proteins, as follows [10,14,15],

$$S(Q,\omega)_{\text{water}} = S(Q,\omega)_{\text{H}_2\text{O-hydrated protein}} - S(Q,\omega)_{\text{D}_2\text{O-hydrated protein}}$$

(1)

The concept is depicted in Figure 2.

### MD simulation of protein crystalline

MD simulations of crystalline SNase were performed at a constant temperature (300 K) and pressure (1 atm), which were maintained following the Berendsen method (relaxation time of 1 ps for both) by using the program AMBER [18]. The crystal structure of SNase (PDB code: 1EY0.pdb) was used as the initial simulation structure. The simulated system was constructed to reproduce the crystal unit cell,

and it had a space group symmetry of $P4_1$ (Fig. 1(B)). Missing residues 1–5 and 142–149 in 1EY0.pdb were modeled on the basis of the NMR structures of SNase (PDB code: 1JOR.pdb). The simulations in the protein crystal explain quantitatively the neutron scattering functions with lyophilized SNase [17,18]. The simulation system consisted of four protein molecules with either 1,110 or 1,494 water molecules, which correspond to h=0.30 and 0.40 g H$_2$O/g protein, respectively. Water molecules were placed randomly in the empty space in each system. For bulk water, 440 water molecules were set in a rectangular box. The periodic boundary condition was imposed, and the particle mesh Ewald method was used with a cutoff of 10.0 Å. The AMBER *ff14SB* force field and the TIP3P water model were employed [21]. After energy minimization (2000 steps), 2 ns MDs were performed to equilibrate the systems at 300 K and 1 atm. Successive 4 ns trajectories were obtained for the analyses. For hydrogen bond analysis, pairs of water molecules were selected as being hydrogen-bonded only if their inter-oxygen distance was smaller than 3.5 Å and simultaneously the O–H...O angle was greater than 120°.

## Results and Discussion

### Selective observation of protein and hydration water dynamics by H$_2$O/D$_2$O exchange

Figure 3(A) shows the INS profiles of the D$_2$O- and H$_2$O-hydrated proteins, and the hydration water. The protein dynamics can be observed with the D$_2$O-hydrated sample, where the INS due to hydration water (D$_2$O) is minimized. As shown in Figure 2, the INS due to the hydration water is obtained by the difference in the scattering intensities of an H$_2$O-hydrated sample and a D$_2$O-hydrated sample according to equation (1).

Figures 3(B) and (C) show the INS due to hydration water at h=0.44 and that due to bulk liquid water (H$_2$O) at 300 K, respectively, where the strong broad QENS spectrum is observed. The scattering function can be expressed approximately as follows [22]:
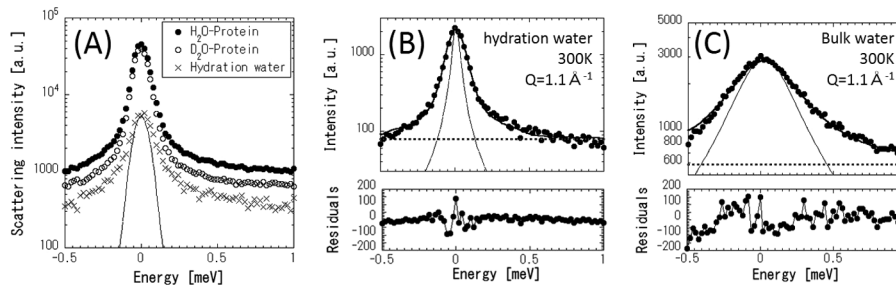


**Figure 3**  (A) Neutron scattering profiles at Q=1.1 Å$^{-1}$ of H$_2$O- and D$_2$O-hydrated proteins at h=0.44 g D$_2$O/g protein and 300 K, and that of hydration water. INS data of hydration water are obtained by subtracting D$_2$O-hydrated protein from H$_2$O-hydrated protein, according to equation (1). The thin line indicates the instrumental resolution function. Quasi-elastic neutron scattering (QENS) of hydration water (B) and bulk water (C) at 300 K. Closed circle and the solid lines denote the observed QENS data and the curve fitted using equation (2), respectively. The thin and dotted lines indicate Lorentzian and constant background, respectively. The under-panel shows the difference between the observed and the fitted QENS intensities.
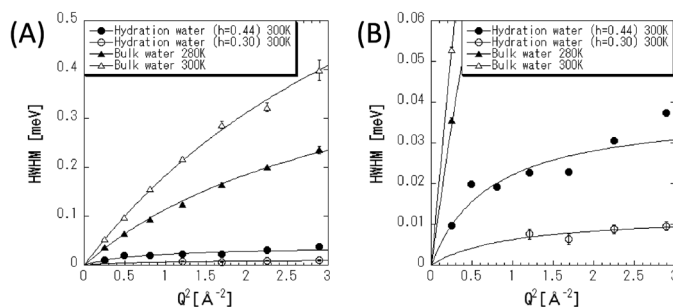
**Figure 4**  (A) Half-width at half-maximum of Lorentzian function in fitting equation (2) for bulk water and hydration water against $Q^2$. The solid curves correspond to the data fit with the jump-diffusion models. Closed circle, open circle, closed triangle, and open triangle denote hydration water with h=0.44 at 300 K, hydration water with h=0.30 at 300 K, bulk water at 280 K, and bulk water at 300 K, respectively. (B) Enlarged view of (A).

$$S(Q,\omega) = A(Q)[EISF \cdot \delta(\omega) + (1 - EISF) \cdot L(Q,\omega)] + B(\omega) \quad (2),$$

where $A(Q)$ is the Debye–Waller factor; EISF is an elastic incoherent structural factor; $L(Q,\omega)$ is a Lorentzian function with half width at half maximum (HWHM), $\Gamma(Q)$; and $B(\omega)$ is the constant background due to inelastic scattering. The scattering profile can be fitted reasonably by equation (2) at all Q values. EISF decreased to be zero substantially in the present case. This is reasonable for water molecules because EISF is related to the probability of finding an atom at the same position at t=0 and ∞. Therefore, the elastic term was not included in the subsequent data fitting. Figure 4 shows the variation of $\Gamma(Q)$ with $Q^2$ for the hydration water and the bulk liquid water. The $\Gamma(Q)$ of bulk water increased with Q from the origin. This result indicates that translational diffusion was dominant over localized motions such as rotation. We analyzed the translational diffusion dynamics due to jump-diffusion model by using the following equation [22]:

$$\Gamma(Q) = \frac{D_{trans}Q^2}{1 + D_{trans}Q^2\tau} \quad (3)$$

where $D_{trans}$ and $\tau$ are the diffusion constant and residence time, respectively, and the obtained values are listed in Table 2. $D_{trans}$ and $\tau$ of bulk liquid water are consistent with the values obtained in a previous work [23]. In case of the hydra-

**Table 2**  Dynamic parameters of liquid bulk water and protein hydration water at two hydration levels, h=0.30 and 0.44 g D$_2$O/g protein, obtained by quasi-elastic neutron scattering.

| Sample | $D*10^{-5}$ [cm$^2$/s] | $\tau$ [ps] |
|---|---|---|
| h=0.44, 300 K | 0.799±0.073 | 39.75±0.22 |
| h=0.30, 300 K | 0.190±0.131 | 121.62±40.57 |
| Bulk water, 280 K | 2.225±0.032 | 3.01±0.08 |
| Bulk water, 300 K | 3.372±0.042 | 1.44±0.10 |

tion water, $\Gamma(Q)$ values are significantly smaller than that of the bulk liquid water. This suggests that the diffusional dynamics of hydration water in protein is suppressed owing to the interaction with protein surface.

Figures 5(A) and (B) shows the MSDs of protein and its hydration water, respectively, at different hydration levels as a function of temperature, as obtained in the INS experiments. As for the protein dynamics in Figure 5(A), the MSDs increase monotonously with temperature, and the MSDs of the samples with h=0.36 and 0.44 deviate from those of the samples with h=0.30 above 240 K, which indicates PDT. Figure 5(B) shows that the MSDs of hydration water, which are almost comparable to the MSDs of the proteins below the PDT temperature at all hydration levels. At h=0.30, the MSDs of the protein and the hydration water are similar across the entire temperature range. At h=0.36 and 0.44, the
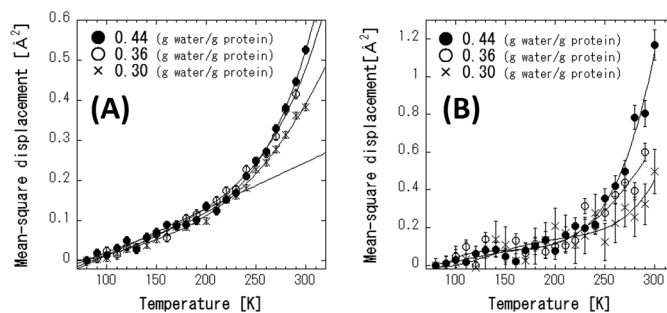


**Figure 5**  MSD of protein (A) and hydration water (B) at three hydration levels obtained by INS experiments. Closed circle, open circle, and cross denote h=0.44, 0.36, and 0.30, respectively. The thin line in (A) indicate the regression line below 240 K for h=0.44.
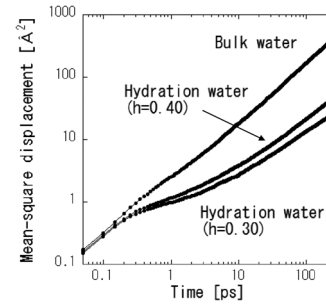
MSDs of the hydration water are greater than those of the proteins above the PDT temperature, and the increment of MSD is greater for h=0.44 than it is for h=0.33. These results indicate that the dynamics of the protein and its hydration water are tightly coupled to each other, and PDT can be controlled by hydration water dynamics. There is a threshold hydration level, h=0.36, for the onset of PDT [10,24]. Dynamics of the hydration water is activated by the percolation transition, and consequently PDT is facilitated by the activated hydration water dynamics via hydrogen bond between them. The percolation of hydration water is the origin of the onset of PDT [10]. The present results are consistent with the previous findings [10,24].

## Diffusion and Hydrogen bond dynamics of hydration water by MD simulation

In MD simulation with a crystalline protein system, larger scale motions of protein are suppressed due to the direct contact among neighboring proteins (crowded environment). Due to this effect, the results from MD simulation in this system can reproduce neutron scattering data quantitatively, because samples in INS is powder state, where the direct interaction exists. Furthermore, in principle, incoherent neutron scattering is able to observe the single atomic dynamics. The amplitude of the fluctuation is an order of a few Å. This is much smaller than the periodic box length. Thus, the MD with the periodic box condition is relevant to compare with the observed protein dynamics. The hydration-dependent protein dynamics in MD simulation are compatible with neutron scattering experiments [17,18]. To examine the hydration-level-dependent diffusion and the hydrogen bond dynamics of the hydration water, MD simulations of an all-atom model were performed with hydrated crystalline SNase. From the MD simulation of two hydration levels, h=0.30 and 0.40, at 300 K, the MSDs of hydration water and bulk water were estimated. Hydrogen bond dynamics were analyzed as well. The selected two hydration levels were below and above the hydration threshold for PDT onset [10].

The obtained time-dependent MSDs of hydration water and bulk water are shown in Figure 6. Life time of hydrogen bond between hydration water and protein is pico-second time scale [19]. The hydration water molecules sometimes move to the different hydration sites or the outer hydration layers, and then do not always stay in the same hydration site in this time scale. They are not always oscillating in the harmonic potential. Thus, the dynamics of hydration water can be described as Brownian motion rather than harmonic motion. The Brownian processes in the short-time and long-time are considered as ballistic motions and diffusion motions, respectively [25]. The MSDs of both hydration water and bulk water increase rapidly until 0.2 ps, which can be ascribed to ballistic motions. The ballistic motions of hydration water are indistinguishable from those of liquid water, suggesting that these motions are not affected by the interaction with protein. Over longer timescales, the MSDs



**Figure 6** Time-dependent MSDs of hydration water at two hydration levels and bulk water at 300 K obtained by MD simulations, averaged over all water molecules in the system at time zero.

of the hydration water are significantly smaller than those of the bulk water. This reduction in the amplitudes of water dynamics can possibly be ascribed to the interaction of water with protein, such as a hydrogen-bond or van der Waals contact and so on. The diffusion constants estimated at 10 ps of bulk water and hydration water at h=0.40 and h=0.30 are $2.7 \times 10^{-5}$ cm²/s, $1.32 \times 10^{-5}$ cm²/s, and $0.15 \times 10^{-5}$ cm²/s, respectively. These values are reasonably comparable to the diffusion constants obtained in INS experiments (see Table 2). Under thermal equilibrium, the hydrogen bonds of water molecules fluctuate temporally. To examine the dynamics of the hydrogen bonds associated with the water molecules, a hydrogen bond population operator, h, was introduced [26]. Its value was unity when a given tagged pair of water molecules was hydrogen-bonded and was zero otherwise. The set of values h for all pairs of water molecules specifies the bond organization at a given instant. The average number of hydrogen bonds in an equilibrium of N water molecules is

$$\frac{1}{2}N(N-1)\langle h \rangle,$$

where $\langle h \rangle$ denotes the time average of h. The fluctuation is characterized by the correlation function, $c(t)$. The correlation function of $c(t)$ is the probability that two arbitrary water molecules that are hydrogen-bonded at time zero are bonded at time t as well, regardless of whether the bond is broken at an intermediate time [19]. Figure 7 shows the $c(t)$ of hydration water and bulk water. The initial decay at the sub-pico second scale can be ascribed to the ballistic motions observed in the time-dependent MSDs shown in Figure 6. Beyond the initial period of the ballistic motions, the decay of $c(t)$ indicates rearrangement of the hydration water cluster or network [19]. The function of the higher hydration level decays faster than that of the lower hydration level. The two hydration levels are above and below the percolation transition of hydration water [10]. The decay of c(t) originates from the extension of the hydration water cluster or network by percolation. Decay of the c(t) of the bulk water was significantly faster than that of the hydration water. This indicated the deceleration of water dynamics by hydration, and
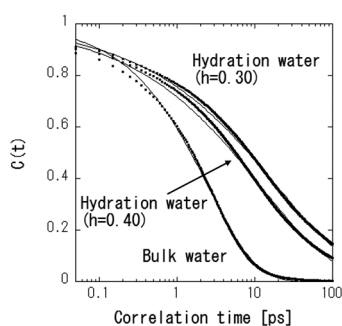
**Figure 7** Time evolution of water-water hydrogen bond correlation functions for hydration water in protein at two hydration levels and bulk water at 300 K. The thin curves indicate the data fitting by KWW function.

this result is consistent with the variation of the diffusion constant. The decay of c($t$) cannot be described by a simple exponential function, suggesting the heterogeneity of hydrogen bond dynamics. The Kohlrausch-Williams-Watts (KWW) function is often used for the heterogeneous relaxation system [27], as following,

$$c(t) = exp\left(-\left(\frac{t}{\tau_{KWW}}\right)^{\beta}\right) \qquad (4)$$

where $\tau_{KWW}$ and $\beta$ are relaxation time and the distribution of the relaxation function. For stretching exponential, $\beta$ is between 0 and 1 ($\beta$=1 is the normal exponential function.). The data fittings by KWW were reasonable. The calculated $\tau_{KWW}$ and $\beta$ are 19.6 ps and 0.43 for h=0.30, 12.1 ps and 0.44 for h=0.40, and 2.53 ps and 0.70 for bulk water, respectively. The obtained values of $\beta$ indicate that the heterogeneity of relaxation time is broader for hydration water than bulk water. The dynamics of the hydration water might be more complex than bulk water by the interaction of the protein surface. Tarek *et al.*, defined the hydrogen bond relaxation time $\tau_R$ as c($\tau_R$)=$e^{-1}$ [19]. The calculated $\tau_R$ of the bulk liquid water at 300 K and those of the hydration water at h=0.40 and h=0.30 are 2.65, 13.80, and 30.25 ps, respectively. These results are not quantitatively consistent with the residence times from a jump-diffusion model analysis of INS (Table 2). The sequence of the residence times is the same between them. Therefore, the experimentally obtained residence time can be interpreted as the hydrogen bond relaxation time of water molecules. In the future, we will determine the relationship between PDT and hydration water dynamics by MD simulation.

## Conclusion

One of the main goals of this study was to demonstrate the effectiveness of $H_2O/D_2O$ exchange and MD simulation for INS experiment. Information of hydration water dynamics can be described separately from protein dynamics by conducting INS experiments with $H_2O/D_2O$ exchange. The experimentally obtained dynamic parameters of hydration water and bulk water, such as MSDs, diffusion constants, and residence times, are compatible with the values calculated by MD simulation. The residence times of water molecules obtained using the jump-diffusion model were interpreted as the relaxation time required for the rearrangement of the hydration water cluster or network. Incoherent scattering does not intrinsically include structural information because scattering expresses the self-correlation of atomic motions, while MD simulation can provide all-atom information. INS experiments with $H_2O/D_2O$ exchange combined with MD simulation should serve as a powerful next-generation tool for studying proteins and hydration water dynamics.

## Conflict of Interest

All the authors declare that they have no conflict of interest.

## Author Contributions

H. N. conducted all experiments and MD simulations. H. N. and M. K. directed the entire project and co-wrote the manuscript.

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