1. INTRODUCTION

The emphasis in the studies of dynamics of hydrated proteins has been on two processes, namely, the structural $\alpha$-relaxation and the secondary $\beta$-relaxation of hydration water, analogous to those of glass-formers. Their relaxation times $\tau_{\alpha}(T)$ and $\tau_{\beta}(T)$ were determined by dielectric relaxation, nuclear magnetic resonance, and neutron scattering. The phenomenon that attracted most interest is the protein dynamical transition (PDT), first observed in M"ossbauer spectroscopy and in neutron scattering by a change in temperature dependence of the mean-squared displacements (MSD) at temperature $T_{\beta}$ which depends on the experimental timescale. The PDT is believed to be essential for biological activity of the protein. If not masked by the methyl group contribution, another change in $T$-dependence of the MSD occurs at $T_g$ below $T_{\beta}$ independent of the experimental timescale, analogous to that found by neutron scattering in glass-formers of different kinds.

The crossover of the MSD at $T_g$ was explained in terms of the caged molecular dynamics and the associated nearly constant loss changing intensity at $T_g$ as proffered by the coupling model. The PDT at $T_d$ was explained by the $\beta$-relaxation of hydration water of the Johari-Goldstein (JG) kind entering the experimental time window. It is not due to the $\alpha$-relaxation as proposed in ref 15, simply because its relaxation time $\tau_{\alpha}(T)$ at $T = T_d$ is orders of magnitude too long for it to be involved. Advances were made in characterizing the $\alpha$-relaxation and the hydration water $\beta$-relaxation and understanding their roles in the changes in MSD observed at $T_g$ and at $T_d$ of the PDT. Notwithstanding, the question that remains is whether there are still some features of the dynamics not yet been explored, and if found, the new features may deepen the current level of knowledge of the dynamic processes in hydrated proteins. Our exploration is motivated by the new findings in recent experimental studies and theoretical interpretations.

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of the dynamics of highly asymmetric mixtures (HAM) of two glass-formers A and B with glass transition temperature $T_g$ of A much higher than $T_g$ of B and particularly at low concentration of the fast component B. The dynamics of HAM are composed of three processes, $\alpha_1$, $\alpha_2$, and JG $\beta$. The $\alpha_1$ and $\alpha_2$ are cooperative relaxations that give rise to glass transition at temperatures $T_g^{\alpha_1}$ and $T_g^{\alpha_2}$, respectively, and can be observed by calorimetry. The slower $\alpha_1$ is dominated by the slower component A with participation of the component B, and its relaxation times $\tau_{\alpha_1}(T)$ has Vogel–Fulcher–Tammann (VFT) temperature dependence, leading to the higher $T_g^{\alpha_1}$. The $\alpha_2$ is contributed by the component B in the presence of the slower A component with relaxation times $\tau_{\alpha_2}(T)$ shorter than $\tau_{\alpha_1}(T)$. At temperatures above $T_g^{\alpha_1}$, $\tau_{\alpha_2}(T)$ also has a VFT dependence. However, at temperatures below $T_g^{\alpha_1}$, $\tau_{\alpha_2}(T)$ changes to assume an Arrhenius dependence because the B molecules are confined by the immobile A molecules. The confinement by the frozen matrix of component A causes localization of the $\alpha_2$-relaxation at temperatures below $T_g^{\alpha_2}$ to make it in some way like a secondary relaxation. Despite $\tau_{\alpha_2}(T)$ being Arrhenius below $T_g^{\alpha_1}$, the $\alpha_2$-relaxation will subsequently become vitrified at $T_g^{\alpha_2}$. Both $T_g^{\alpha_2}$ and $T_g^{\alpha_1}$ increase monotonically with a decrease in the concentration of the faster component $c_B$, although a different interpretation was also reported.34–37 At lower $c_B$, the difference ($T_g^{\alpha_1} - T_g^{\alpha_2}$) is larger and $\tau_{\alpha_2}(T)$ becomes more separated from $\tau_{\alpha_1}(T)$, making the former easier to determine.36,37 The $\beta$-relaxation involves the more local motions of the B molecules coupled to the A molecules at temperatures below $T_g^{\beta_1}$ and $T_g^{\beta_2}$. This $\beta$-relaxation has properties indicating that it is strongly connected to each of the two $\alpha$-relaxations and thus it is of the JG kind.37,38 For example, its relaxation time $\tau_{\beta}(T)$ changes temperature dependence from Arrhenius below $T_g^{\beta_1}$ to a stronger Arrhenius dependence above $T_g^{\beta_1}$ and exhibits another change on crossing $T_g^{\beta_2}$ to assume a super-Arrhenius $T$-dependence above $T_g^{\beta_2}$. This double change in temperature dependence is easier to observe in HAM at lower concentration of the fast component $c_B$.34–37 An example of HAM showing all the properties described above is 50% methyl-tetrahydrofuran (MTHF) in polystyrene with a high molecular weight of 60,000 g/mol.30 The relaxation times $\tau_{\alpha_1}(T)$, $\tau_{\alpha_2}(T)$, and $\tau_{\beta}(T)$ and their properties of this example are summarized in Figure S1 in the Supporting Information.

Since the $T_g$ of dry proteins is much higher than the $T_g$ of water, it is reasonable to consider hydrated proteins as HAM and to expect similar dynamics. This hypothesis is supported by a close correlation between solvent and protein dynamics based on experimental and simulation evidence,38–41 like the coupling between components A and B found in the $\alpha_1$-, $\alpha_2$-, and JG $\beta$-relaxations in HAM,39–47 and also in peptide solutions.42,43

In this paper, we verify by experiments the anticipated analogy of the dynamics in hydrated proteins with those in HAM. The $\alpha_1$ process is predetermined by the protein but facilitated by the coupling with the hydration water. Its $\tau_{\alpha_1}(T)$ has Vogel–Fulcher–Tammann (VFT) dependence above $T_g^{\alpha_1}$. The $\alpha_2$ process is contributed mainly by the hydrated water coupled to protein. The $T$-dependence of its $\tau_{\alpha_2}(T)$ changes from VFT dependence at temperatures above $T_g^{\alpha_2}$ to Arrhenius below $T_g^{\alpha_1}$, which continues with temperature falling until the $\alpha_2$-relaxation is vitrified at $T_g^{\alpha_2}$. The JG $\beta$ process originates from the hydration water, and for this reason, it is also called the $\nu$-relaxation.2,4,5 Below $T_g^{\alpha_2}$, its $\tau_{\beta}(T)$ or equivalent $\tau_{\beta}(T)$ has an Arrhenius dependence in response to confinement by the frozen $\alpha_1$ and $\alpha_2$ processes combined. By increasing temperature to cross $T_g^{\alpha_2}$, $\tau_{\beta}(T)$ assumes a stronger Arrhenius dependence due to devitrification of the $\alpha_2$ process. A further increase in temperature and after crossing $T_g^{\alpha_1}$, the $\alpha_1$ process is also devitrified. Consequently, $\tau_{\beta}(T)$ has its temperature dependence changed to super-Arrhenius or VFT-like, in response to the equilibrium liquid state of the hydrated protein above $T_g^{\alpha_1}$. The VFT-like dependence of $\tau_{\beta}(T)$ continues with increasing temperature until $\tau_{\beta}(T)$ matches the timescale of either the Mößbauer or the neutron scattering spectrometer, giving rise to the PDT. Thus, if verified, the dynamics of hydrated proteins are richer than presently known and the additional properties of the $\alpha_1$, $\alpha_2$, and $\nu$ processes that we found enhance the current knowledge and impact theoretical interpretation.

2. RESULTS AND DISCUSSION

2.1. Neutron Scattering Measurements of Hydrated Myoglobin. As mentioned before, in HAM, the observation of the three processes, $\alpha_1$, $\alpha_2$, and JG $\beta$, their properties, and inter-relationship is best brought out at lower concentrations of the faster component because $\tau_{\alpha_1}(T)$, $\tau_{\alpha_2}(T)$, and $\tau_{\beta}(T)$ become more widely separated. Many neutron scattering and dielectric relaxation studies of hydrated proteins in the past were performed at higher hydration levels $h$, and this explains why together the three processes and their properties were not made known. Therefore, we made neutron scattering measurements of hydrated myoglobin at $h = 0.30$ and dielectric relaxation at slightly lower $h = 0.28$ in order to better resolve
first by the weaker relaxation strength of the $\alpha_2$-relaxation and second by myoglobin being probed in H-MYO/D$_2$O instead of water, while the $\alpha_2$-relaxation is predominately contributed by water. The secondary change in T-dependence of $(\chi''(T))$ is the PDT at $T_\text{D} \approx 250$–255 K and $T_\text{D} \approx 275$ K, respectively, for 1 and 13 $\mu$eV energy resolutions.

Differential scanning calorimetry (DSC) is a conventional method to detect glass transition and glass transition temperature. The technique was applied to hydrated biomolecules, and the results of the studies before 1994 were reviewed by Sartor et al.45 According to the review,44 the DSC measurements of myoglobin crystals and hydrated powders in 1986 by Doster et al.45 had an increase in heat capacity observed at $\sim$220 K and was attributed to glass–liquid transition of water. This temperature of $\sim$220 K is much higher than the onset temperature of the glass transition of 162–170 K for vitrified and freezable water in hydrated methemoglobin (MethHb) in the 1992 study by Sartor et al.46 The sharp glass transitions in myoglobin crystals and hydrated powders in calorimetry by Miyazaki et al.37 with $T_\text{g}$ values at 188 and 216 K are in conflict with those by Doster et al.45 Although the DSC $T_\text{g}$ of hydrated myoglobin powder (0.4 g/g) was reported in Figure 4 of the 2010 paper of Doster et al.44 to be $\sim$170 K. The 1994 paper by Sartor et al.44 not only addressed the discrepancy between the DSC $T_\text{g}$ values but also resolved the discrepancy by their own DSC studies of hydrated lysozyme, hemoglobin, and myoglobin powders to show that their heat capacity slowly increases with increasing temperature, without showing an abrupt increase characteristic of glass–liquid transition. Their study further showed that annealing from $\sim$150 K up to the denaturation temperature has a substantial calorimetric effect, which may be confused with glass transition. The results led them to suggest that the DSC glass transition in hydrated hemoglobin, myoglobin, and lysozyme occurs over a broad temperature range that extends from $\sim$150 K up to the denaturation temperature, and no single glass transition temperature from DSC can be assigned to the three hydrated proteins.44 Bearing in mind the problem of DSC data revealed by Sartor et al., we nevertheless made our own DSC measurements on the same hydrated sample H-MYO in D$_2$O at $h = 0.30$ studied by neutron scattering. The DSC measurement was carried out by cooling the sample directly to $\sim$100 K and then heating back to 300 K at a rate of 5 K/min. The results during heating were recorded and presented in Figure S3 of the Supporting Information without any annealing. A small endothermic hump observed at $\sim$200 K in our sample seems to indicate $T_\text{g} \approx 204$ K, but it could result from the unfreezing of the fastest portion of the broad distribution of relaxation time of the structural dynamics in the hydrated proteins, as suggested by Sartor et al.45 Despite the uncertainty of DSC data, we still have a reliable determination of $T_\text{g} \approx 198$ K from neutron scattering. As we shall see, this $T_\text{g}$ is supported by dielectric relaxation data to be presented next.

### 2.2. Dielectric Spectra of Hydrated Myoglobin

Comparison of the dielectric loss $\varepsilon''(\nu)$ spectra of hydrated myoglobin H-MYO at $h = 0.28$ over 11 decades of frequencies from 103 to 263 K are shown in Figure 2 together with the dielectric $\varepsilon'(\nu)$/log $f$ of the real part $\varepsilon'(\nu)$ with respect to log $f$. The latter enables all three processes, $\alpha_1$, $\alpha_2$, and $\nu$- or JG- $\beta$-relaxation, to be resolved in the high temperature range from 203 to 263 K. The source of the H-MYO sample in the dielectric study was the same as that in the neutron scattering study. Dielectric measurements were made on both the heating and cooling paths of the sample, and the same results were obtained. The possibility of the $\alpha_1$ process might be a polarization process of Maxwell–Wagner type due to enhanced conductivity of the sample. An effective criterion is the comparison of the values of $\varepsilon'''$ and $\varepsilon''$ at the frequency of the loss peak. In the case where $\varepsilon''' = \varepsilon''$, there is strong indication that the loss peak is of Maxwell–Wagner type. It was found otherwise, and therefore, the $\alpha_1$ process is correlated with the segmental-like mobility of the hydrated protein. Further support comes from the fact that its timescale and temperature dependence to be shown in Figure 3 are consistent with the calorimetric glass transition at 204 K of the H-MYO hydrated at 0.30 (see Figure S3 in the Supporting Information). Regarding the rather high strength of the $\alpha_1$ process, as has been already reported for many hydrated...
From Figure 3, the arrow indicates the temperature at which $\tau_\alpha(T) = 5$ ns (dotted horizontal line), which is five times the neutron experimental timescale $\tau_{\text{exp}} = 1$ ns. The latter is commonly used to compare with the temperature $T_\alpha$ at the onset of the rise of the MSD defining the PDT in Figure 1a.\textsuperscript{9,15,56} It can be seen from Figure 3 that $T_\alpha$ is about 250 K, in good agreement with a value of 250–255 K determined by the MSD in Figure 1, assuming that the change in $T_\alpha$ with the increase in $h$ from 0.28 to 0.30 is not large and within the accuracy of determining $h$. The $\alpha$2 was observed dielectrically before in hydrated lysozyme and myoglobin by others,\textsuperscript{1,3,6} but the various interpretations given are different from this paper.

In contrast to the other works, our dielectric data of hydrated myoglobin data at $h = 0.28$ are more complete in showing not only all three processes at the same time but also their individual and inter-related properties as well as the two glass transition temperatures $T^\alpha_{\text{gs}}$ and $T^\beta_{\text{gs}}$, and verifying the dielectric $\tau(T)$ determines the PDT temperature $T_\alpha$ determined by neutron scattering (see Figure 1a) by the criterion $\tau_\alpha(T)$ equal to five times the experimental timescale (see Figure 3).

Our conclusion that $\alpha$2 is the structural relaxation of water component is furthermore supported by the $^2$H NMR study of H-MYO/D$_2$O ($h = 0.35$),\textsuperscript{57} which reported that $^2$H spin–lattice relaxation is exponential above $\approx 195$ K but nonexponential below (see Figure S4 in the Supporting Information). This observation means that the D$_2$O subsystem becomes non-ergodic on the experimental timescale $T_{\text{g}}(195$ K) = 0.02 s upon cooling through this temperature. In Figure 3, we find $\tau_\alpha(195$ K) $\approx T_{\text{g}}(195 K)$, while $\alpha$2 is faster and slower than $\alpha$1 at higher and lower temperatures, respectively. These findings show that $\alpha$2 is the ergodicity-restoring process of the D$_2$O subsystem and, hence, it can be identified with its structural relaxation.
K, as shown in Figure 5 for hydrated elastin at $h = 0.23$. Found by a dielectric study of hydrated elastin at $h = 0.23^{54}$ and to ps and still $T_f^{d}$ has not been reached. By contrast, in hydrated globular proteins, the $\nu$-relaxation is in the liquid state when $T_\nu(T_d)$ matches $5\tau_{\text{exp}}$ of either Mössbauer or neutron scattering spectroscopy, i.e., $T_d$ is above $T_f^{\text{ul}}$ and $T_\nu(T)$ has VFT dependence (see deuteron NMR data in Figure 3). On the other hand, in hydrated elastin, the $T_d$ satisfying the rule $T_\nu(T_d) = 5\tau_{\text{exp}}$ is far below $T_f^{\text{ul}}$ (see the broken line at 5 ns in the inset of Figure 5), and thus, the $\nu$-relaxation is confined in the glassy matrix and $T_\nu(T)$ has Arrhenius $T$-dependence. This difference in the property of the $\nu$-relaxation at temperature where $T_\nu(T) = 5\tau_{\text{exp}}$ between hydrated globular proteins and hydrated elastin is important. It is the reason why the protein dynamic transition (PDT) was observed in hydrated globular protein but not in hydrated elastin.\(^{10}\) Since the dynamics of hydrated collagen are similar to those of hydrated elastin as shown by dielectric data in ref 10, we predict that the PDT cannot be observed by neutron scattering as well.

2.5. Dielectric and Neutron Scattering Data of Ribonuclease A. It is worthwhile to briefly mention the dielectric relaxation\(^{10}\) and neutron scattering data\(^{15,55}\) of the globular protein ribonuclease A (RNase A) at hydration level $h = 0.4$. Like hydrated lysozyme and myoglobin, the dynamics of RNase A were shown\(^{15}\) to have all the $\alpha 1$, $\alpha 2$, and $\nu$ processes, the nearly constant loss of caged molecules, and most of their properties. The protein dynamic transition was observed by neutron scattering, and it conforms to the rule $T_\nu(T_d) = 5\tau_{\text{exp}}$ verified in the other hydrated globular proteins. The dielectric spectra are shown in Figures S5 and S6 of the Supporting Information. Hence, the data of hydrated RNase throw another support of the generality of the dynamics of hydrated proteins proffered in this paper.

3. SUMMARY AND CONCLUSIONS

In summary, we have provided neutron scattering, dielectric relaxation, and deuteron NMR data in three hydrated globular proteins, myoglobin, BSA, and RNase, and the fibrous elastin to show the presence of three relaxations, $\alpha 1$, $\alpha 2$, and $\nu$, with properties that are inter-related, analogous to the $\alpha 1$, $\alpha 2$, and JG $\beta$-relaxations in highly asymmetric mixtures of two molecular glass-formers. There are two glass transition temperatures $T_f^{\text{ul}}$ and $T_f^{\text{al}}$ corresponding, respectively, to vitrification of the $\alpha 1$ and $\alpha 2$ processes. The $\alpha 2$-relaxation responds to vitrification of $\alpha 1$-relaxation by changing the $T$-dependence of its relaxation time $\tau_{\alpha 2}(T)$ on crossing $T_f^{\text{al}}$. The $\nu$-relaxation responds to the two vitrifications of $\alpha 1$- and $\nu$-relaxations by changing the $T$-dependence of its relaxation time $\tau_{\nu}(T)$ on crossing $T_f^{\text{al}}$ and $T_f^{\text{ul}}$. The $\nu$-relaxation generates the protein dynamic transition (PDT) at $T_d$ where $T_\nu(T_d)$ matches approximately five times the experimental instrument timescale $\tau_{\text{exp}}$ provided that $T_d > T_f^{\text{al}}$. The $\nu$-relaxation is in the liquid state and $T_\nu(T)$ has VFT-like temperature dependence. The $\nu$-relaxation of the hydrated globular proteins considered in this paper satisfies the condition $T_d > T_f^{\text{al}}$ and the PDT is generated and detected. On the other hand, if $T_d < T_f^{\text{al}}$, the $\nu$-relaxation is confined within the glassy state and $T_\nu(T)$ has Arrhenius temperature dependence. This contrasting condition $T_d < T_f^{\text{al}}$ of the $\nu$-relaxation prevails in hydrated elastin, which renders the $\nu$-relaxation ineffective in generating the PDT, and explains why PDT was not found by neutron scattering before in the case of hydrated elastin. Thus, the dynamics of hydrated proteins are exactly the same as those of highly asymmetric mixtures of glass-formers and are richer and diversified than...
presently known. The advances made by this study should have impact on future research efforts in the dynamics of hydrated proteins and applications.

4. MATERIALS AND METHODS

4.1. Sample Preparation. Myoglobin (MYO) from equine skeletal muscle was purchased from Sigma-Aldrich (Shanghai, China). In order to exclude the effect of ions, the protein was dialyzed. It was then dissolved in D$_2$O to allow full deuterium exchange of all exchangeable hydrogen atoms and then lyophilized for 12 h to obtain the dry sample. The lyophilized MYO is then suspended on top of liquid D$_2$O in a desiccator to absorb D$_2$O till the desired hydration level $(h, \text{gram D}_2\text{O}/\text{gram protein}).$ The deuterium oxidized (D$_2$O, 99.9 atom % D) was purchased from Sigma-Aldrich (Shanghai, China). The hydration levels of protein samples were controlled by measuring the sample weights before and after water adsorption. The $h$ of the dry sample is 0.02, while it is 0.3 for the hydrated one, which corresponds to a case that roughly a single layer of water molecules covers the protein surface. The accuracy of $h$ is controlled within 10% error, e.g., $h = 0.3 \pm 0.03$ gram water/gram protein. All samples were sealed tightly in the aluminum cans in a nitrogen atmosphere for subsequent neutron scattering experiments.

5. EXPERIMENTAL SECTION

5.1. Elastic Incoherent Neutron Scattering. The elastic scattering intensity $S(q,\Delta t)$ is normalized to the lowest temperature ($\sim$10 K) and is approximately the value of the intermediate scattering function when decaying to the instrument resolution time ($\Delta t$). All the $S(q,\Delta t)$ data were obtained in the temperature range from $\sim$10 to 300 K during heating with a heating rate of 1.0 K/min by using the HFBS at NIST and DNA at J-PARC. The energy resolutions of HFBS and DNA are 1 meV and 13 meV, corresponding to the resolution times of $\sim$1 ns and $\sim$80 ps, respectively. The results from instruments with the two different resolutions were summed over the same q from 0.45 to 1.75 Å$^{-1}$. The mean-squared atomic displacements $(\langle x^2(t) \rangle)$ were obtained with the Gaussian approximation of $S(q,\Delta t) = \exp(-\frac{1}{2}q^2\langle x^2(t) \rangle)$ in the Gaussian approximation range from 0.45 to 0.9 Å$^{-1}$.

5.2. Broadband Dielectric Spectroscopy. Dielectric relaxation data of hydrated myoglobin H-MYO with $h = 0.28$ gram water/gram protein were obtained in the frequency range from 10 mHz to 3 GHz, by the combination of dielectric response analysis (Novocontrol Dielectric Analyzer) and coaxial reflectometry (Agilent Network Analyzer 8753ES). Myoglobin powder was hydrated, compacted, and transferred in a sealed capacitor cell by using the same procedures used for hydrated biomolecules in refs 6, 10. Temperature was varied over a wide range, spanning from the deep glassy state to ambient temperature, following isothermal steps after a suitable equilibration time. Real and imaginary parts of the spectra have been simultaneously fitted with a superposition of Havriliak–Negami and Cole–Cole relaxation functions. The derivative of the real part was used to resolve the $\alpha 1$ and $\alpha 2$ processes by suppressing the conductivity contribution, as done before in ref 6. The characteristic relaxation times reported in Figure 2 correspond to the maximum of the loss function of each process, i.e., the most probable time in the distribution of relaxation times at each temperature for each of the three relaxation process $\alpha 1$, $\alpha 2$, and $\nu$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04655.

Example of the dynamics observed in a highly asymmetric mixtures given and illustrated by the dielectric, calorimetry, light scattering, and neutron scattering data of MTHF in mixture with PS; neutron elastic intensity $S(q,\Delta t)$ of dry H-MYO and H-MYO in D$_2$O at $h = 0.30$ as well as calorimetry measurement; results from $^3$H spin–lattice relaxation studies of hydrated myoglobin; and dielectric and neutron scattering data of hydrated RNase (PDF).

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Notes

The authors declare no competing financial interest.

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