Report

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Experimental mapping of short-wavelength phonons in proteins

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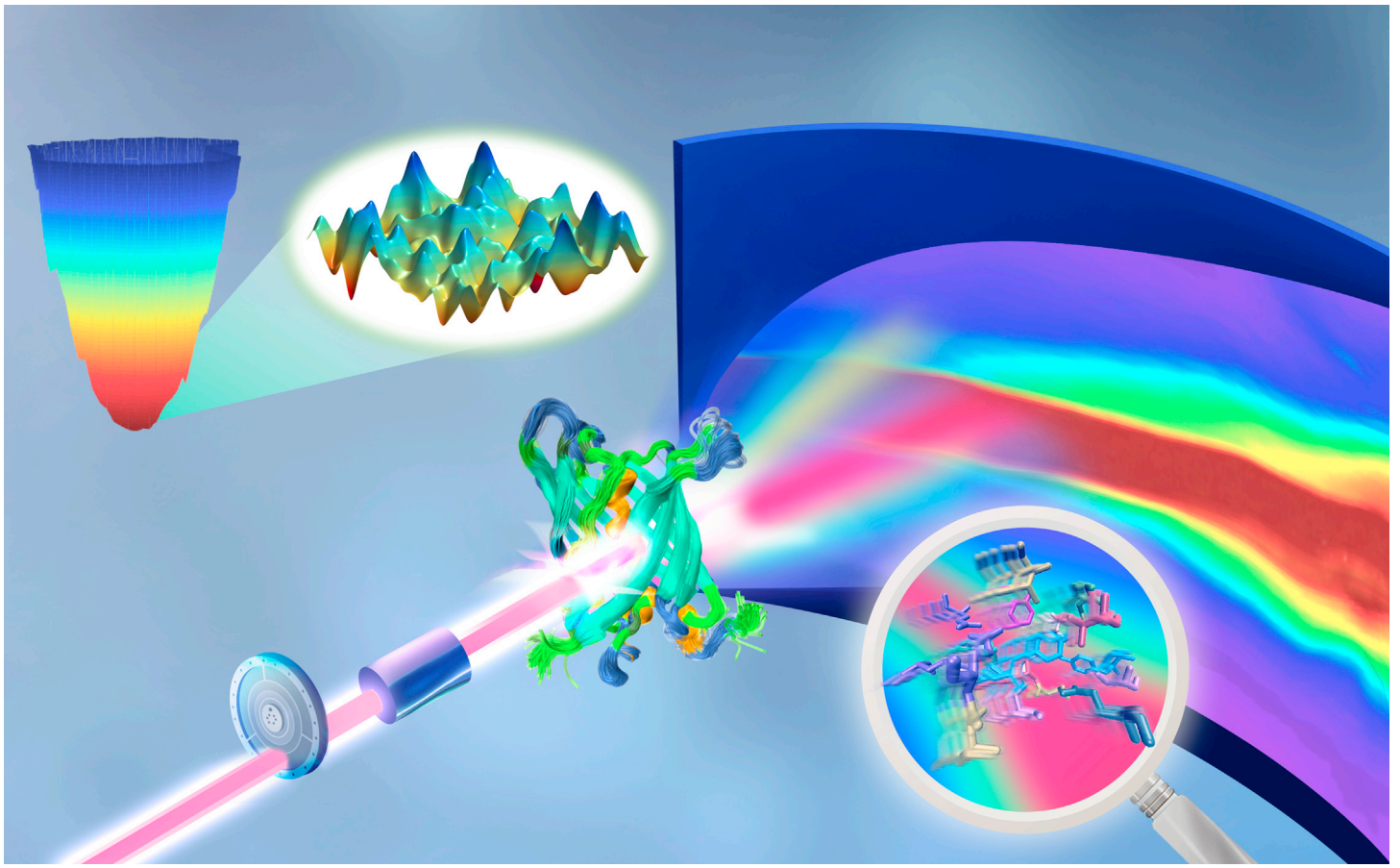
Graphical abstract



Public summary

- Quantum phenomena in biology have long fascinated people around the world
- This work presents a direct experimental observation of phonons, the quantum vibrations in a protein
- The collective excitations or phonons in proteins were detected by utilizing inelastic neutron scattering technique at Oak Ridge National Laboratory
- Our results illustrate the flexibility-activity relationship in proteins by mapping the temperature and hydration dependence of these collective excitations

Report

Experimental mapping of short-wavelength phonons in proteins

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Phonons are quasi-particles, observed as lattice vibrations in periodic materials, that often dampen in the presence of structural perturbations. Nevertheless, phonon-like collective excitations exist in highly complex systems, such as proteins, although the origin of such collective motions has remained elusive. Here we present a picture of temperature and hydration dependence of collective excitations in green fluorescent protein (GFP) obtained by inelastic neutron scattering. Our results provide evidence that such excitations can be used as a measure of flexibility/softness and are possibly associated with the protein's activity. Moreover, we show that the hydration water in GFP interferes with the phonon propagation pathway, enhancing the structural rigidity and stability of GFP.

INTRODUCTION

The motion of life, specifically protein dynamics, plays a vital role in biological activities.[1] The timescale of internal motions in protein ranges from femtoseconds to milliseconds,[2] analogous to supercooled liquids and glasses.[1] Such internal motions are essential for enzyme catalysis, conformational transitions, binding affinities, transfer of ions, transport of metabolic energy (ATP hydrolysis), and the folding-unfolding mechanism.[3–5] The conventional ideas of biological processes come from the observation of slow binding and conformational changes in proteins occurring on a timescale of a few microseconds to milliseconds.[6] However, recent studies have stressed that harmonic vibrations (on the order of femtoseconds to subpicoseconds) are precursors of the biological activities in proteins and enzymes.[3–5,7]

Despite a long history of study of protein dynamics, it remains elusive how the softness of a protein is associated with its activity.[8] Moreover, many studies have reported that protein becomes flexible or soft upon hydration required for activity.[9–11] Hydration water is believed to affect fast and slow motions in proteins by forming hydrogen bond networks.[12–17] However, the collective excitations (fast motion) in proteins are highly damped and short-lived (on the order of tens of femtoseconds to picoseconds) because of glass-like behavior,[18] making them experimentally challenging to measure.

Phonon-like collective excitations in proteins have been investigated using inelastic X-ray scattering (IXS).[19–23] However, IXS has a limited energy resolution, especially for studying low-frequency collective excitations.[24] Compared with IXS, coherent inelastic neutron scattering (INS) technique is exceptionally suitable for investigating low-frequency collective excitations[24,25] and has been applied successfully to study collective density fluctuations in amorphous materials, glasses, or liquids.[25–27] Moreover, INS has a significant advantage when studying biological samples because it has much better resolution at the biologically relevant energy and length scales but causes almost no radiation damage compared with IXS. However, a large amount of fully deuterated protein samples is required for high-resolution INS experiments because of the huge incoherent scattering cross-section of hydrogen atoms to neutrons, hindering mapping and analysis of such collective motions in past decades.

In this work, a powerful state-of-the-art INS spectrometer allowed us to map the temperature and hydration dependence of collective excitations in proteins with high precision and relate them to a protein's biological activity. Here we report INS study of a model protein, perdeuterated green fluorescent protein (dGFP), dry and hydrated, to investigate low-frequency intra-protein collective motions on the order of femtoseconds to subpicoseconds.[19–22,28] GFP has a remarkable regular structure that is stable and resistant to unfolding so that even the water molecules on the outside of the barrel form "stripes."[29] Therefore, GFP is a suitable model system for INS measurements. In our experiment, we observed propagating acoustic[21,23,28,30,31] and non-propagating localized[19–22] modes from both samples. Further analysis of such excitations helped us to understand the relations between protein flexibility/softness and its activity from a physical perspective.

RESULTS

The INS experiments were performed on a fine-resolution Fermi chopper spectrometer, SEQUOIA,[32] at the Spallation Neutron Source (SNS), Oak Ridge National Laboratory (ORNL). Details regarding the measurements, sample preparation, and rationale behind using the perdeuterated samples for INS measurements are described in the supplemental information. The hydrated sample of dGFP had a hydration level of $h \sim 0.37$ (i.e., 0.37 g of D_2O per 1 g of dry dGFP). Such a hydration level forms at least a monolayer of hydration water around the protein and is linked to its bioactivity.[21,33,34] The UV-visible (UV-vis) emission spectra of protonated GFP (hGFP) and dGFP samples are substantially the same (Figure S1), confirming that the deuteration has no adverse effect on the protein's activity in the *in vitro* experiment. Here we considered dry (yellow) and hydrated[9–11] (bright green) samples of dGFP inactive and active states, respectively, in terms of their optical properties (Figure 1).[33,34] The measured INS spectra or dynamic structure factors $S(Q, E)$ are shown in Figure S2 for dry and D_2O-hydrated dGFP samples at different temperatures as a function of neutron momentum and energy transfers (Q and E).

The static structure factors $S(Q)$ of dry and hydrated samples are compared at a series of temperatures, T = 150–270 K (Figures 1A and 1B). $S(Q)$ was calculated using the relation $S(Q) = \int S(Q, E)\, dE$, where the integration of energy depends on each Q value (Figure S2). While integrating the energy transfer ($\pm E$) at each specific Q value, the same energy ranges were considered for dry and hydrated samples. For the dry sample, a small peak at $Q \sim 0.6$ Å^{-1} represents the distance between the secondary structures (i.e., central α-helix and β-barrel strand, approximately equal to 10.5 Å). The prominent peak observed at $Q_p^{dry} \sim 1.4$ Å^{-1} corresponds to the spacing between the β-barrel strands being nearly equal to 4.5 Å. The additional broader peak at $Q \sim 2.9$ Å^{-1} represents the local structure, a fluorophore, and amino acid residues.[29,35] On the other hand, for the hydrated sample, the peak at $Q \sim 0.6$ Å^{-1} shifts to a slightly lower Q, possibly because of a slight expansion of the β-barrel to accommodate the water molecules.[18] The prominent peak at $Q_p^{dry} \sim 1.4$ Å^{-1} becomes broader and then shifts toward a slightly higher Q value, $Q_p^{hyd} \sim 1.6$ Å^{-1}. The additional broader peak at $Q \sim 2.9$ Å^{-1} flattens out because of the change in the local structure (Figures 1A, 1B, and S3). There is also a sign of ice crystals in the hydrated sample (see supplemental information for more details).

Here we hypothesized that the softness or stiffness (opposite of softness) of a protein upon hydration depends predominantly on the tertiary structure in 3 dimensions. The temperature dependence of $S(Q)$ can be observed above $Q \sim 1.6$ Å^{-1} for dry and hydrated dGFP, where $S(Q)$ values decrease with an increase in temperature. The overlap of $S(Q)$ below $Q \sim 1.4$ Å^{-1} confirms the scattering signal dominated by protein in both samples, nearly independent of temperature (Figures 1A, 1B, and S3). However, above $Q \sim 1.4$ Å^{-1}, the higher intensity in the hydrated sample indicates that the protein hydration shell makes it more rigid than the dry sample.

The measured INS signal arises from the detection of collective excitations in dGFP (Figure 2). The dynamic coherent structure factor $S(Q, E)$ was fitted using a dampened harmonic oscillator (DHO) model with the following expression:

$$S(Q,E) = \left[I_0(Q)\delta(E) + g(E) I_1(Q) \frac{\Gamma(Q)}{\pi} \left\{ \frac{1}{(E-\Omega(Q))^2 + (\Gamma(Q))^2} - \frac{1}{(E+\Omega(Q))^2 + (\Gamma(Q))^2} \right\} + (A \cdot E + B) \right] \otimes R(Q,E) \qquad \text{(Equation 1)}$$

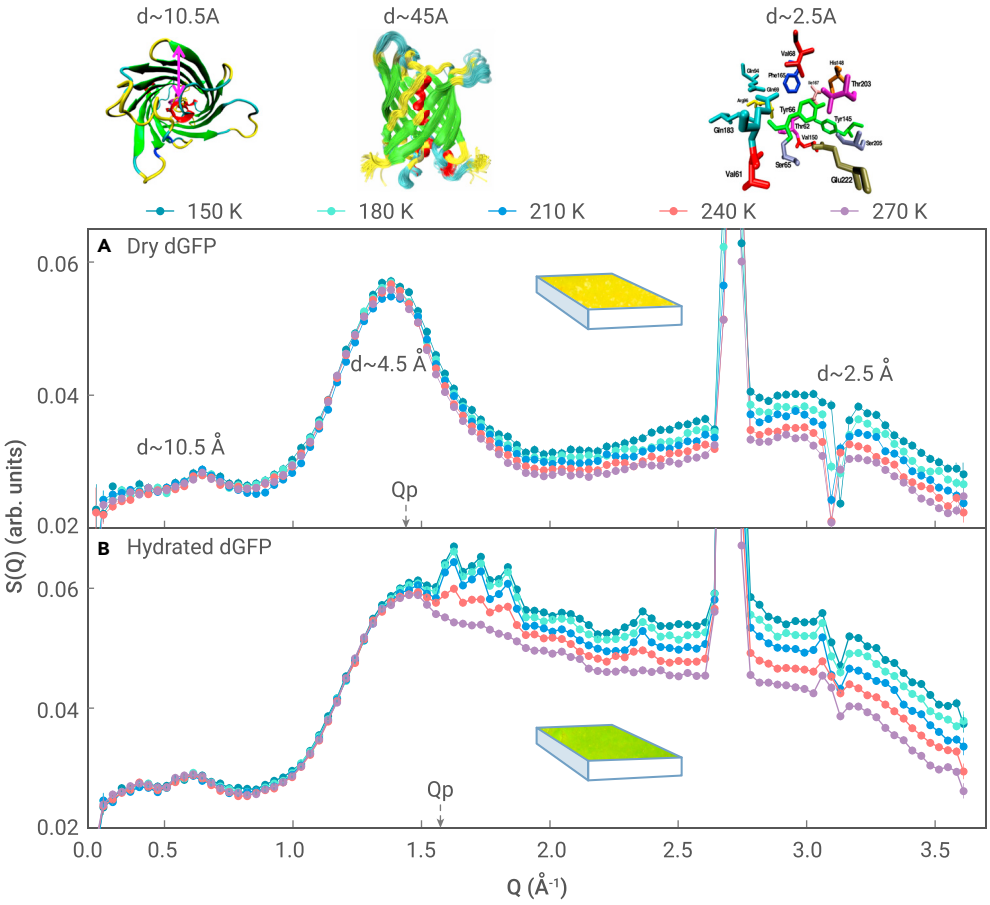

Figure 1. Static structure factors of dry and D_2O hydrated dGFP samples, measured by INS at temperatures of 150–270 K (A and B) Static structure factors of (A) dry and (B) D_2O-hydrated dGFP samples, measured by INS as functions of wave-vector transfer Q at temperatures of 150–270 K. The error bars are within the size of the symbols.

, where $R(Q, E)$ is the instrumental resolution function, and $(A \cdot E + B)$ is the background term. The additional "slope" term $(A \cdot E)$ in the background of Equation 1 was included to improve the overall fit quality[36] even though the positions of the fitted inelastic features were not affected by the fit with or without this term. Such a sloped background term likely originates from the additional experimental background (e.g., from epithermal neutrons) that is roughly constant as a function of time of flight and yields a sloped background upon data conversion to energy transfer. $I_0(Q)$ and $\delta(E)$ are the elastic intensity and the delta function, respectively, in the elastic component, and $I_1(Q)$, $\Omega(Q)$ and $\Gamma(Q)$ are the inelastic intensity, excitation energy, and damping factor, respectively, of the DHO function describing the collective phonon excitations. Also, the Bose thermal factor $g(E)$ or the temperature-dependent correction in the DHO function[36] is given by

$$g(E) = \frac{1}{1 - exp(-E/k_B T)} \qquad \text{(Equation 2)}$$

, where k_B is the Boltzmann constant and T is the absolute temperature. The DHO model has been implemented successfully to study Brillouin-like inelastic side peaks in liquids[24,36–38] and amorphous materials,[39] including biomolecules.[19,23,30,31,40,41]

Figures 2A and 2B illustrate the fitting of measured INS spectra $S(Q, E)$ using Equation 1 at T = 150 K and Q = 0.64 Å^{-1}. The Brillouin-like side peaks of DHO are observed at the energy positions (Ω) that indicate the excitation energy of the collective motions. The dispersion curves of collective excitations can be determined from these DHO side peaks at all measured temperatures for both samples (Figures 2C, 2D, 3, and 4), where the black dashed lines represent the acoustic branches of phonon energy dispersion fitted with Equation 3 in the region $Q \lesssim$ 1.1 Å^{-1}. The dispersion curves overlaid on top of 2-dimensional (2D) raw INS spectra for dGFP samples at T = 150 K and Q = 0.64 Å^{-1} confirm that the calculated excitation energies are larger than the instrumental resolution (Figures 2C and 2D). Furthermore, the bending of the energy dispersion curve occurs at the boundary of the acoustic branch (i.e., $Q_m \sim 1.1$ Å^{-1}), suggesting the existence of a pseudo-Brillouin zone[26] with a finite group velocity up to the value of Q_m, after which a plateau develops or $\Omega(Q)$ starts to deviate from the usual Q dependence. The value of $Q_m \sim 1.1$ Å^{-1} corresponds to the topological disorder length scale in the sample or half of the distance to the nearest pseudo-reciprocal lattice point.[42] The plateau, or the lack of significant dispersion at higher Q-values above Q_m, indicates the end of such wave propagation, exemplifying the localized collective modes.[39,43]

Furthermore, SEQUOIA[32] has an energy resolution of less than 2 meV and a dynamic range of ~30 meV (Figure S2), capable of resolving acoustic phonons in proteins.[32] As a result, we observed low-frequency coherent motions in dry and hydrated samples of dGFP, as confirmed by the energy dispersion curves (Figures 3 and 4). Such motions arise from periodic vibrations of predominant secondary structures, such as β-barrels, in dGFP. Here the phonon-like propagation takes place on a length scale larger than 5 Å, where Q_m = 1.1 Å^{-1} can be considered a boundary above which the excitations are localized. This size resembles the protein secondary and tertiary structures participating in biological activity. On the other hand, the localized excitations at $Q > Q_m$ are mainly attributed to vibrations of fluorophore plus amino acid residues for the dry sample and fluorophore plus amino acid residues plus D_2O for the hydrated sample, which are on a length scale of 2.0–5.0 Å.[35]

The energy dispersion curves (Ω versus Q) show the propagation of acoustic modes in the longer wavelength-limit $Q \lesssim Q_m$ at all measured temperatures of T = 150–270 K for dGFP samples (Figures 3A, 3B, and 4). We designate these modes as acoustic because extrapolation of the excitation energy in the dispersion curve is approaching the value zero in the limit of low Q. Because of amorphous protein samples that lack a preferred orientation, it is not easy to explicitly differentiate between longitudinal and transverse acoustic modes.[26,39,44] However, the calculated values of low-frequency phonon-like excitations can be attributed to the transverse acoustic mode, which is in good agreement with recently published molecular dynamics simulations[45] and experiments.[30,46,47] Furthermore, for $Q \gtrsim Q_m$, an apparent softening of the localized intra-protein vibrations with an increase in temperature is observed, as indicated by the decrease in the excitation energy in both samples, whereas the acoustic mode is nearly temperature independent. It suggests that dry and hydrated proteins become softer as the temperature increases.

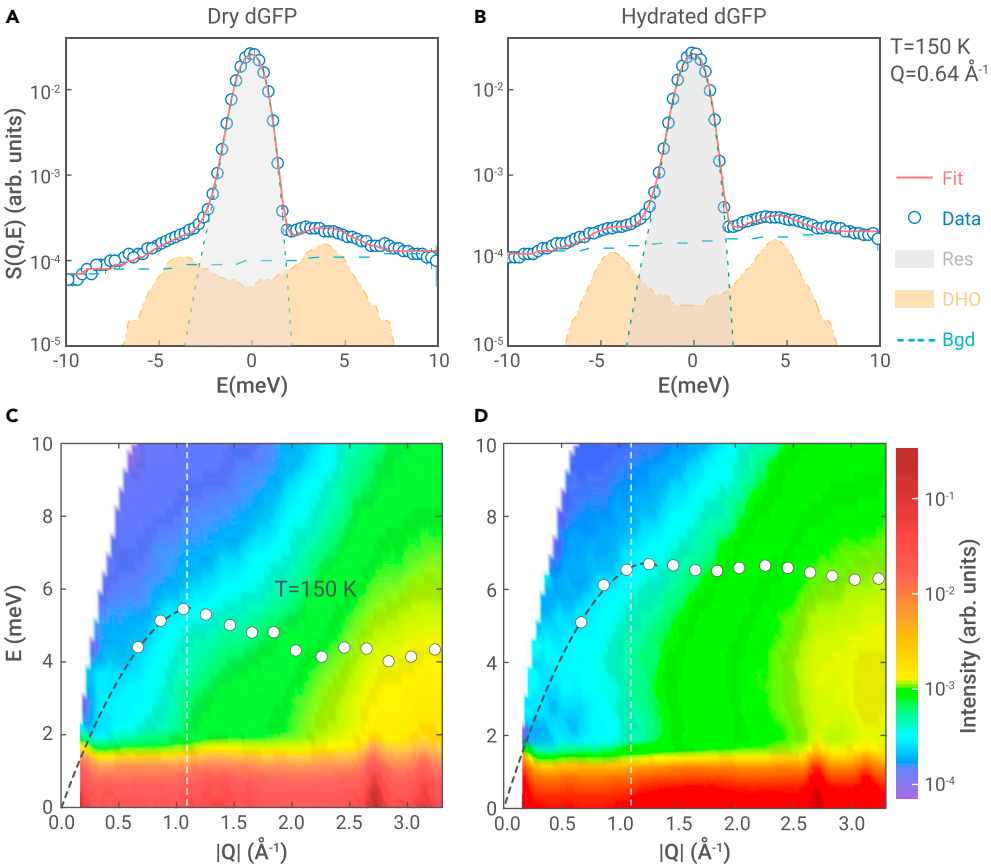

Figure 2. Dynamic coherent structure factors of dry and hydrated dGFP samples fitted with the DHO model. (A and B) The measured coherent inelastic neutron scattering (INS) spectra fitted with the DHO model as expressed in Equation 1 at wave-vector transfer Q = 0.64 Å^{-1} and T = 150 K. (C and D) 2D contour plots of coherent INS spectra at T = 150 K for both samples. The phonon-like dispersion curves are represented by open circles, and the black dashed lines indicate the acoustic branch as separated by the white dashed lines at Q = 1.1 Å^{-1}. Note that the error bars are smaller than the size of the symbols and cannot be shown in the figures.

The apparent increase in the half-width at half-maximum (HWHM) of the inelastic side peaks or the phonon damping parameter (Γ) with temperature (Figures 3C and 3D) suggests an increase in damping of intra-protein collective vibrations because of emerging relaxation and diffusion processes or anharmonic motion. It corresponds to the lifetime ($\tau = 2/\hbar\Gamma$) of the collective excitations[39] and is fitted with the relation $\Gamma(Q) = AQ^2$ at low Q values (dashed

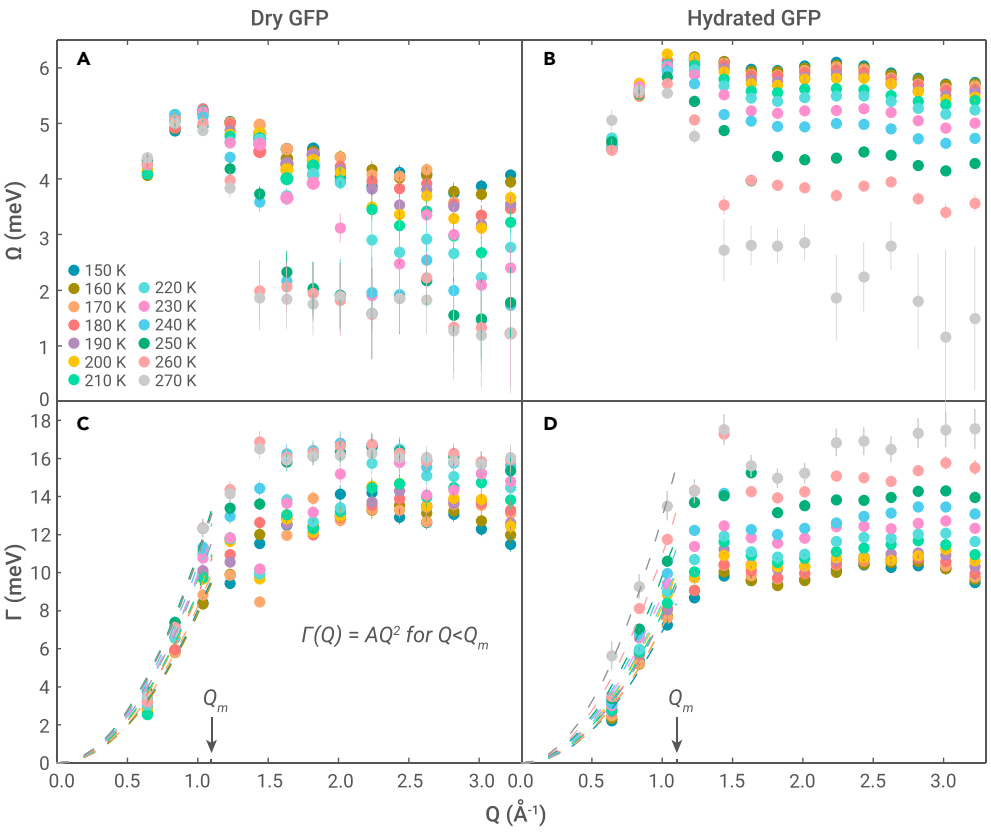

Figure 3. Collective excitations in dry and hydrated dGFP samples (A and B) Energy dispersion curves from (A) dry and (B) hydrated dGFP samples at temperatures of 150–270 K. (C and D) The half-width at half-maximum or damping parameter (Γ) of DHO functions as a function of wave-vector transfer Q at temperatures of 150–270 K with a temperature step of 10 K. The dashed lines represent the fitting of Γ with relation $\Gamma(Q) = AQ^2$ at low Q values as in ordinary glasses.[43] Q_m refers to the values of Q, above which $\Omega(Q)$ and $\Gamma(Q)$ start to deviate from their usual Q dependence.

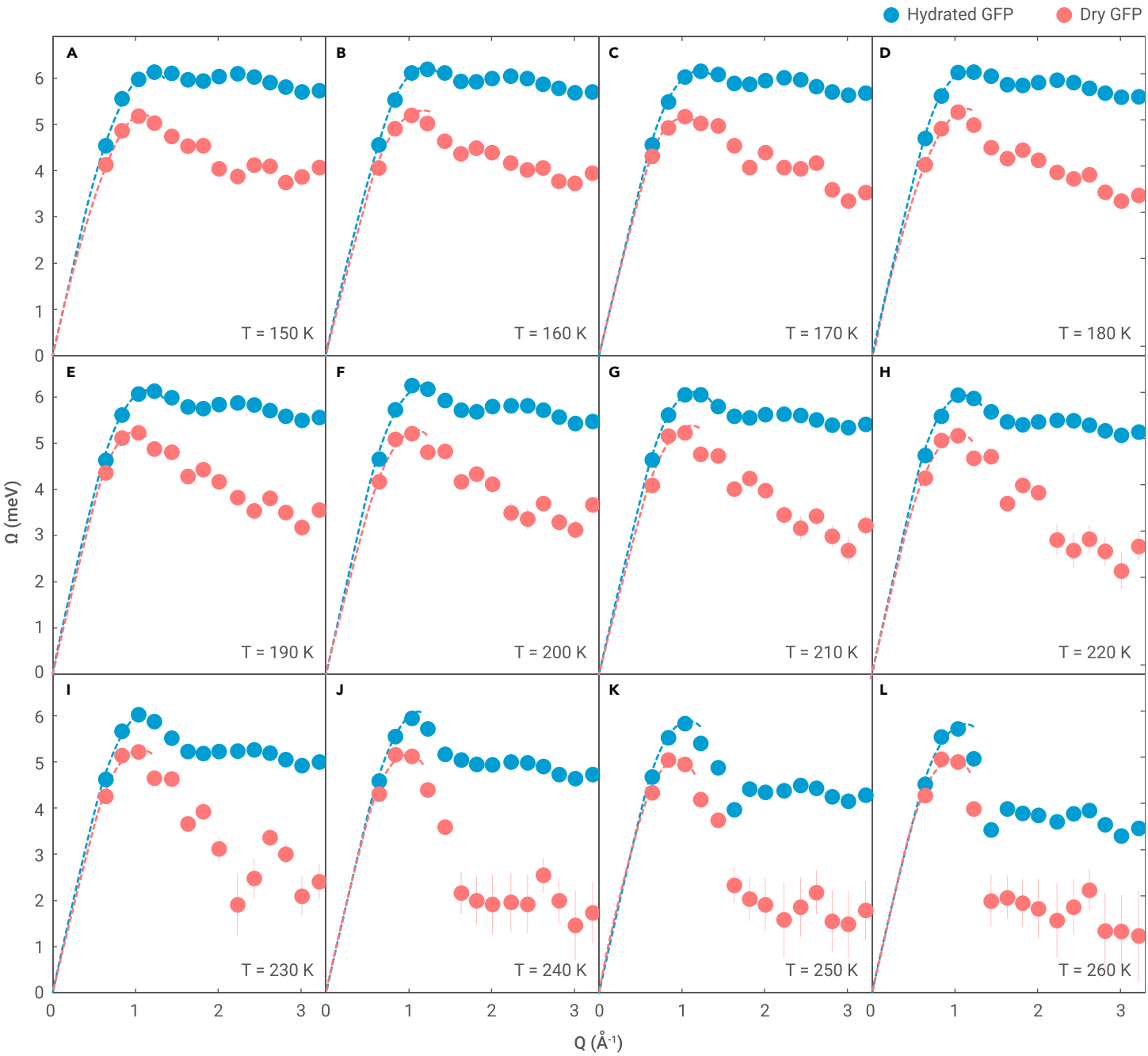

Figure 4. Comparison of phonon dispersion curves in dry and hydrated dGFP samples (A–L) The plots are for different temperatures of T = 150–260 K; the dashed lines correspond to the curves fitted with Equation 3.

lines in Figures 3C and 3D) as in ordinary glasses[43] but becomes flat or deviates from the usual Q^2-dependence for $Q > Q_m$ because of the localized excitations. Furthermore, it is interesting to note that the protein energy dispersion curve also follows the relation $\Omega(Q_m) \approx \Gamma(Q_m)$, similar to glasses and glass-forming liquids.[43] In addition, the ratio Q_m/Q_p measures the fragility of the sample and is found to be ~0.8 and ~0.7 for dry and hydrated dGFP, respectively, much larger than the values observed in glasses and glass-forming liquids such as *o*-terphenyl, glycerol, and SiO_2.[43] This signifies that GFP has considerable dynamic heterogeneity (ascribe to its secondary and tertiary structures) compared with glasses, so that a slight temperature change can reorganize structures in many orientations or a large number of conformational substates in a rugged free energy landscape (Figure 5A).[48,49] The complete set of comparison of energy dispersion curves between the dry and hydrated protein samples at temperatures of T = 150–260 K (Figure 4) suggests that the presence of water molecules in GFP increases the energy of the collective excitations and increases its rigidity.

The energy dispersion curves in the acoustic branch can be fitted with a simplified relation given by the Born-von Karman lattice dynamics theory in one dimension,[42] expressed as

$$E(meV) = 4.192 \times 10^{-3}\, v(m/s) \times Q_m(\text{Å}^{-1})\, sin\left(\frac{\pi}{2}\frac{Q(\text{Å}^{-1})}{Q_m(\text{Å}^{-1})}\right) \quad \text{(Equation 3)}$$

, where v is the velocity of the collective wave propagation. The fittings are shown in Figures 2C, 2D, and 4 as dashed lines. Although the model only considers the nearest-neighbor interactions mainly applicable to the single crystals, it successfully fits the data from the amorphous form of the protein samples.[30] The calculated velocities of the acoustic mode from dry and hydrated dGFPs are compared in Figure 5B, indicating that the propagation is slightly faster in the hydrated sample because of reduced softness. The sound velocities are barely temperature

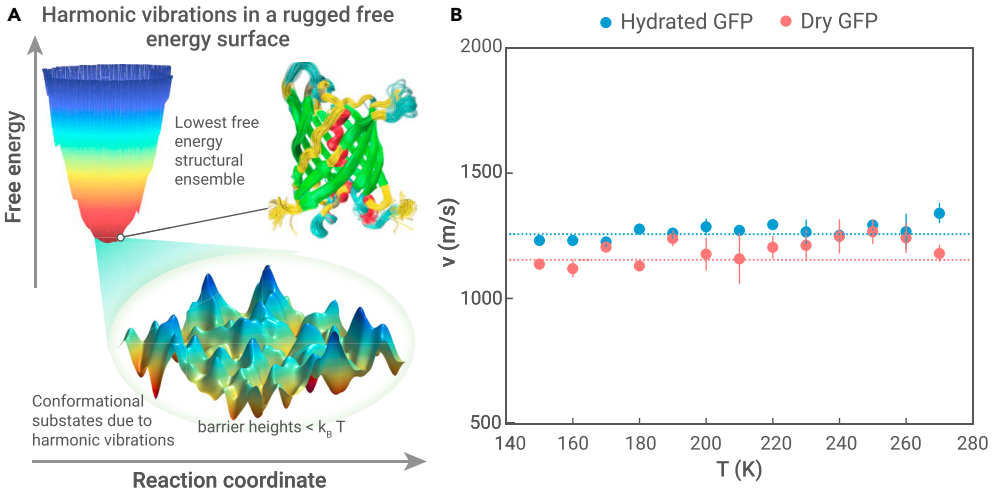

Figure 5. Schematic of the protein free energy landscape and velocities of the acoustic phonon propagation in dGFP samples (A) A schematic illustration of conformational fluctuations in a protein and its corresponding representation the in free energy landscape (FEL). A strongly funneled FEL of globular protein showing even the lowest possible free energy conformation has a rugged energy surface because of harmonic motions, where many conformational substates are separated by an energy barrier much less than k_BT. (B) The comparison of velocities of acoustic phonons in dry and hydrated dGFP samples indicates that propagation is slightly faster in the hydrated sample.

dependent, with average values of 1,194 ± 13 m/s and 1,269 ± 9 m/s for dry and hydrated dGFP samples, respectively, very close to the fast transverse sound speed of water, which is about 1,500 m/s.[44] Because these sound velocities are due to the low-frequency (transverse) acoustic mode, these values are much smaller than the velocities in proteins observed using IXS[21,23,30,31,41] due to the high-frequency (longitudinal) acoustic mode, which are close to the fast sound velocity of heavy water, about 3,300 m/s.[25,44] The calculated velocities in this study are in good agreement with the average sound velocity of about 1,200 m/s in lysozyme (in solution), obtained using ultrasound velocimetry,[46] and of 1,605 m/s in amorphous myoglobin, obtained using the Mössbauer effect.[47] Also, the sound velocity of a transverse acoustic mode in a protein active-site mimic (chloro(octaethyporphyrinato)iron(III)) has been found to be nearly 1,011 m/s, using IXS and vibrational spectroscopy techniques.[30] The slightly higher velocity in hydrated dGFP than in the dry one is due to a slight increase in stiffness caused by the presence of water molecules inside the β-barrels and water's strong coupling to the protein surface.[29,50–54] Our result is qualitatively consistent with a previously reported result from an incoherent elastic neutron scattering experiment, where dry GFP was softer than its hydrated counterpart in the temperature range of 100 K $\lesssim T \lesssim$ 240 K.[55] However, the two experiments are technically different, providing dissimilar dynamical information. Incoherent elastic neutron scattering measures the self-correlated dynamics of hydrogen atoms. In contrast, coherent INS measures the collective excitations contributed mainly by nitrogen, deuterium, carbon, and oxygen atoms in the sample.

DISCUSSION

The flexibility and stability of biomolecules are seemingly conflicting. However, both are essential for biochemical and biophysical processes such as molecular reactions, protein-ligand interactions, conformational fluctuations, and free energy of protein folding. Our work reveals the relation of fast coherent motion to a protein's intrinsic flexibility regarding temperature and hydration. The experimentally obtained energy dispersion curves show propagation of the acoustic mode in dGFP, which is nearly independent of temperature in dry and hydrated samples (Figure 5B), whereas the phonon energy of intra-protein localized collective modes decreases with temperature (Figures 3A and 3B). This indicates that dry and hydrated proteins become softer, or more flexible, as temperature increases. The larger energy values of transverse acoustic propagation and localized modes in dGFP upon hydration suggest that the protein becomes significantly more rigid, or more stable, because of accommodation of water molecules inside the β-barrels. This fact is consistent with the theory that proteins must keep their native structures thermally stable, which is necessary for their biological activity. In this way, the phonon behaviors measured in our current study bridge these two conflicting properties of proteins (i.e., stability and flexibility) and further relate them to protein bioactivities, specifically upon hydration and in their native states.

Here we observe experimentally the collective or phonon-like excitations in proteins that are due to harmonic vibrations of amino acid residues and secondary/tertiary structural segments.[19,20] Our results provide experimental evidence that the free energy landscape of a protein is rugged even in the lowest free energy basin because of harmonic motions (shown schematically in Figure 5A), where the protein structure hops around neighboring conformational substates separated by small energy barriers that are lower than k_BT. Furthermore, the collective modes can measure the softness or flexibility of the protein, which are related to the protein's activity.[19–21,56] The measured excitation energy of the order of a few meV in protein is a dynamic entity that can be used as a precursor of a protein's activity. Moreover, we observed an apparent temperature and hydration dependence of collective excitations in GFP that are connected to its optical properties (i.e., dry and hydrated samples emitted yellow and bright green fluorescent light, respectively). Interestingly, the phonon energy of acoustic and localized collective modes increases significantly upon hydration, indicating that GFP has a more rigid/stable but flexible structure in its native state. We attribute this to the mechanical packing and folding necessary for the activity of GFP upon hydration.

MATERIALS AND METHODS

The details of protein sample preparation and neutron scattering experiments are described in the supplemental information.

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AUTHOR CONTRIBUTIONS

X.C. designed the research. X.C., E.M., H.M.O., Q.Z., and A.I.K. performed the experiments. U.R.S. analyzed the data. X.C. and U.R.S. wrote the paper with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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