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Collective Excitations in Protein as a Measure of Balance Between its Softness and Rigidity

Utsab R. Shrestha,[†] Debsindhu Bhowmik,[‡] Kurt W. Van Delinder,[†] Eugene Mamontov,[§] Hugh O'Neill,[⊥] Qiu Zhang,[⊥] Ahmet Alatas,[∥] and Xiang-Qiang Chu*[,†]

[†]Department of Physics and Astronomy, Wayne State University, Detroit, MI 48201, United States

[‡]Computational Science and Engineering Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, United States

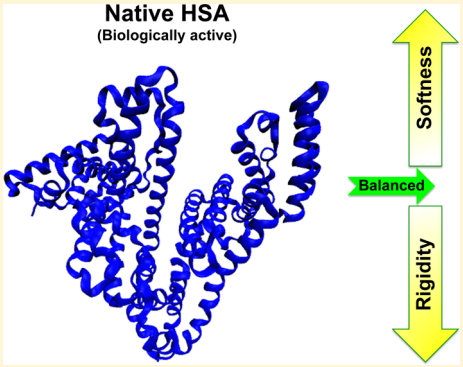
[§]Chemical and Engineering Materials Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, United States

[⊥]Biology and Soft Matter Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, United States

[∥]Advanced Photon Source, Argonne National laboratory, Argonne, IL 60439, United States

S *Supporting Information*

ABSTRACT: In this article, we elucidate the protein activity from the perspective of protein softness and flexibility by studying the collective phonon-like excitations in a globular protein, human serum albumin (HSA), and taking advantage of the state-of-the-art inelastic X-ray scattering (IXS) technique. Such excitations demonstrate that the protein becomes softer upon thermal denaturation due to disruption of weak noncovalent bonds. On the other hand, no significant change in the local excitations is detected in ligand- (drugs) bound HSA compared to the ligand-free HSA. Our results clearly suggest that the protein conformational flexibility and rigidity are balanced by the native protein structure for biological activity.



INTRODUCTION

It is well accepted that the protein dynamics play a significant role in enzyme catalysis together with its native structure.[1,2] However, understanding of the relationship among a protein's structure, dynamics, and function is still a major challenge in biophysical research.[3−6] Protein internal motions on the time scale from femtoseconds to milliseconds[7−9] are similar to those motions in supercooled liquids, glasses, and polymers.[10,11] Enzymatic reactions occur on the time scale of few microseconds to milliseconds due to slow binding and conformational changes in proteins.[12] Nevertheless, recent studies have revealed that the catalytic reactions are also coupled to the fast motions (femtoseconds to picoseconds) in protein due to the formation and disruption of covalent bonds, hydrogen bonds, and transfer of electrons, protons, or hydride ions among different functional groups.[13−15] The long-range structural order in protein is minimal compared to the solid crystals. However, the secondary structures, α-helices, and β-sheets, exhibit a significant fraction of higher-order structure, which is stabilized by the weak bonds like hydrogen bond, Van der Waals interaction, and salt bridges.[16] Such structures exhibit the internal dynamics, which gives rise to the phonon-like excitations in protein.[16] According to the multidimensional protein energy landscape,[9,17] the rapid fluctuations in protein structures correspond to the jumps between the conformational substates in local conformational minima. Such motions largely influence the stability and the flexibility of protein and hence are of considerable interest.[3,18−20]

Inelastic X-ray scattering (IXS) technique is a unique tool to study the collective density fluctuations in amorphous materials, glasses, or liquids.[21−23] High energy resolution of the order of milli-electronvolt (meV) achieved by IXS spectrometers allows a precise investigation of highly damped collective modes and the phonon dispersion in topologically disordered systems.[24−26] Previously, there are evidence from inelastic neutron scattering (INS) experiments of collective motions in proteins, which are similar to the boson peak in the glass-forming liquids due to the collective excitations or density fluctuations.[27−31] Furthermore, IXS, INS, Brillouin neutron spectroscopy (BNS), and molecular dynamic (MD) simulations have revealed the propagation of acoustic phonons in proteins and their hydration water in the longer wavelength limit corresponding to the length scale larger than ∼ 1 nm.[32−42] However, very few studies reported investigation of local excitations within the protein secondary structure on a scale shorter than 1.5 nm.[38,40,43,44] A recent IXS experiment by Wang et al. has studied the collective excitations in a globular protein lysozyme and concluded that the phonon energy softening and phonon

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population enhancement in hydrated protein (both native and denatured) is induced by the hydration shell.[39] On the other hand, MD simulation results clearly suggested that the strong coupling between the dynamics of a globular protein, such as maltose binding protein and its hydration shell, exists rather than protein dynamics being slaved to the hydration water.[34] Specifically, the acoustic phonon propagations in protein and its hydration water are almost identical.[34−36] This result suggests that collective excitations of the same frequency exist in both subsystems, but are not necessarily slaved to the hydration water in case of protein,[34] because dry proteins also show similar collective excitations, as reported in the previous studies.[31−33] Such phonon modes from the protein and its hydration shell are difficult to decouple through the analysis of experimental data, but they have been successfully decoupled and studied by MD simulations.[34]

For proteins, as a dynamic entity similar to the glasses, it has been a major concern whether the phonon modes exist in native and denatured proteins and how such modes are correlated with the activity of enzymes.[39] In general, thermal denaturation of globular protein is irreversible in nature and largely influences the secondary structures.[45,46] It dismantles the secondary structures in such a way that the protein conformation becomes unfolded or possibly aggregated, which makes protein biologically inactive.[47−50] In this study, we investigated the collective excitations in hydrated samples of native and denatured human serum albumin (HSA) as a model protein using IXS technique. HSA is the most prominent protein present in the plasma since ∼ 60% of the total protein content in the blood serum is HSA.[51] It consists of 585 amino acids having the molecular weight of 66.5 kDa.[51] The molecular structure of HSA consists of three domains (I, II, and III) with two subdomains (A and B) each. It has important properties of binding and transporting insoluble molecules, such as fatty acids, porphyrins, and a large variety of drugs.[51] Thus, HSA influences the drug delivery and efficacy that ultimately affect the pharmacokinetic/pharmacodynamic properties of drugs.[52,53] According to Sudlow's nomenclature, it possesses two major binding sites: hydrophobic subdomains IIA (site I) preferred by heterocyclic anions and IIIA (site II) favored by the aromatic carboxylates.[54,55] An anticoagulant drug like warfarin and a nonsteroidal anti-inflammatory drug like ibuprofen bind to Sudlow's site I and site II, respectively.[55] Such strong binding affinity to HSA helps drugs transport and release on the specific targets. Therefore, the interaction of these drugs with HSA has significant implications for the pharmaceutical drug design industries.[53] However, little is known regarding how such drugs affect the local structure, dynamics, and local flexibility of HSA. Therefore, we have also studied here the collective excitations from the samples of warfarin and ibuprofen (pharmaceutical drugs) bound to HSA using IXS. Furthermore, we introduce the idea of protein softness and rigidity as revealed by the collective phonon-like excitations within the protein secondary structure to address the concerns outlined above.

Using the IXS spectrometer, we measured the coherent dynamic structure factor as a function of energy and momentum transfers, $S(Q, E)$ within the protein secondary structure of few angstroms (Å) at two temperatures, T = 200 and 300 K (i. e., below and above the dynamic transition temperature, T_D ∼ 220 K;[56,57] specifically, 300 K is the physiological temperature), where both the propagating collective modes and the nonpropagating localized modes are observed. Remarkably, we detected the phonons in both native and denatured HSA, consistent with the recently reported results by Wang et al.[39] Such phonons are due to the fluctuations of the protein folded structure in the native sample, or the unfolded domains in the denatured sample, and not merely induced by the surrounding hydration shell. Surprisingly, we found that the collective excitations in the thermally denatured sample of HSA show more softening compared to those in the native sample due to disruption of its secondary and tertiary structures, that further causes loss in protein activity. Thus, it gives the notion that the degree of flexibility and stability (a well-accepted measure of protein function) are balanced by the native protein structure for mediating the biological activity. Furthermore, according to the results from the experiments on HSA bound with the drugs, warfarin, and ibuprofen, the protein softness or flexibility remains unaltered upon binding to the drugs at the physiological conditions. Therefore, binding of such drugs does not really affect the local structure and flexibility of HSA. Based on these results, we address and generalize the following major concepts: (i) the phonon-like excitations exist in proteins both in native and denatured states; however, the degree of softness or flexibility determines whether the proteins are functional or not; (ii) the collective excitations are highly structure-dependent, and (iii) the idea of protein softness (flexibility) and rigidity can be defined in terms of collective excitations that can be further used as a novel approach to understand the enzyme activity.

MATERIALS AND METHODS

Sample Preparation. Human serum albumin (Sigma Aldrich A3782) was used as purchased for the experiments. Thermally denatured HSA was prepared by incubating 25 mg/ml HSA at 85 °C for 10 h, and 100 °C for 20 min, before freezing at −80 °C followed by lyophilization. Ibuprofen (I4883) and warfarin (A2250) were also purchased from Sigma-Aldrich. For protein−ligand interaction studies, ibuprofen and warfarin were dissolved in ethanol and added to a HSA solution (0.242 mM or 16 mg/ml) to a final concentration of 2.42 mM (0.5 mg/ml and 0.75 mg/ml ibuprofen and warfarin, respectively). All the samples were incubated at room temperature for 1 h before lyophilization.

The samples were prepared for two separate IXS experiments. In experiment 1, the native HSA and the thermally denatured HSA samples were measured. In experiment 2, we prepared and measured another set of native HSA, HSA bound to warfarin (HSA/warfarin) and HSA bound to ibuprofen (HSA/ibuprofen). All the samples were hydrated with H_2O with hydration level h ∼ 0.4 (0.4 g of H_2O per gram of HSA), which forms at least a monolayer of hydration shell, but is not sufficient to form the secondary layer and more.[38] Thus, in the first approximation, the three-dimensional water−water hydrogen bond cannot be formed and therefore, only the scattering signal from the vibrations of protein and water coupled to the protein surfaces are measured.

Inelastic X-ray Scattering Measurements and Data Analysis. The high-resolution IXS spectra were measured at the beamline 3-ID-C, Advanced Photon Source (APS), Argonne National Laboratory using X-ray beam of energy 21.6 keV.[58−60] The data were collected in the Q-range of 2.0 − 32.0 nm^{-1} using four spherically bent silicon analyzers with the energy resolution of 2.1 meV, and the energy transfer (E) or the dynamic range was ± 25 meV, appropriate for the detection of collective excitations in proteins. The data fitting was done

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using the peak analysis software PAN in the package data analysis and visualization environment (DAVE) developed at NIST Center for Neutron Research (NCNR).[61]

The measured IXS spectrum can be expressed as,

$$S(Q, E) = [S_m(Q, E) + A{\cdot}E + B] \otimes R(E) \quad (1)$$

where $S(Q, E)$ is the dynamic coherent structure factor, $S_m(Q, E)$ is the model function, $(A{\cdot}E{+}B)$ is the linear background, $R(E)$ is the resolution function of the instrument, and $\otimes$ is the convolution operator. The model function, $S_m(Q, E)$ can be further expressed as the sum of the delta or Lorentzian function for the central peak (depending upon the dynamic nature of the sample in the specific Q-range) and the damped harmonic oscillator (DHO) function for the Brillouin side peaks due to the collective excitations. The energy resolution function, $R(E)$ is measured from the plexiglass at its structure maxima ($Q \sim 10$ nm^{-1}). All the data were fitted within the dynamic range of ± 20 meV with eq 1 using the following model function,

$$S_m(Q, E) = \left[I_0(Q)\delta(E) + g(E)I_1(Q)\frac{\Gamma(Q)}{\pi}\left\{ \frac{1}{(E - \Omega(Q))^2 + (\Gamma(Q))^2} - \frac{1}{(E + \Omega(Q))^2 + (\Gamma(Q))^2} \right\} \right] \quad (2)$$

or

$$S_m(Q, E) = \left[I_0(Q)L(Q, E) + g(E)I_1(Q)\frac{\Gamma(Q)}{\pi} \left\{ \frac{1}{(E - \Omega(Q))^2 + (\Gamma(Q))^2} - \frac{1}{(E + \Omega(Q))^2 + (\Gamma(Q))^2} \right\} \right] \quad (3)$$

where $I_0(Q)$ and $\delta(E)$ are the elastic intensity and delta function, respectively, in the elastic component, and $I_1(Q)$, $\Omega(Q) = \hbar\omega$, and $\Gamma(Q)$ are the inelastic intensity, the excitation energy, and the damping factor, respectively, of DHO function for collective excitations. Also, the Bose thermal factor $g(E)$ for the temperature-dependent correction in DHO function[22] is given by,

$$g(E) = \frac{1}{1 - \exp\left(\frac{-E}{k_B T}\right)} \quad (4)$$

where k_B is the Boltzmann constant and T is the absolute temperature. The DHO function has been successfully implemented to study the Brillouin-like inelastic side-peaks in liquids,[22,26,41,42] amorphous materials,[37] and biomolecules.[33,35,36,39,40] $L(Q,E)$ is the Lorentzian function given by,

$$L(Q, E) = \frac{1}{\pi}\frac{\Gamma'(Q)}{\Gamma'^2(Q) + E^2} \quad (5)$$

where $\Gamma'(Q)$ is the half width at half-maximum (HWHM) of the Lorentzian. eqs 2 and 3 were implemented to fit the data in the Q-range of 2.1−9.3 nm^{-1} and 12.1−31.2 nm^{-1}, respectively. Here, the possible rapid diffusion process in the samples may necessitate the use of eq 3 incorporating the Lorentzian function for the central peak at higher Q-values.

RESULTS AND DISCUSSION

Structural Features of Hydrated Proteins. At first, the structural properties and the quality of the hydrated protein samples were investigated by measuring the static structure factor, $S(Q)$, over the wide momentum transfer range, Q = 2.0−32.0 nm^{-1} as shown in Figure 1. The static structure factor

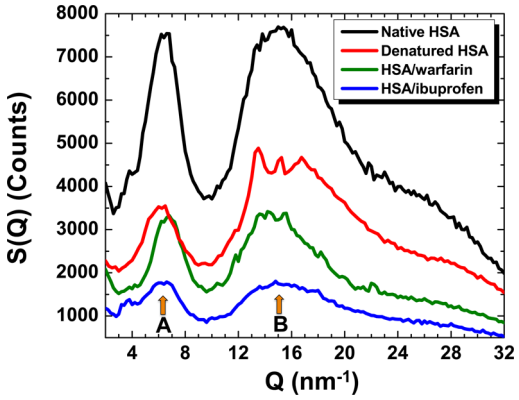

Figure 1. Static structure factor $S(Q)$ as functions of Q measured at T = 300 K for the native HSA, thermally denatured HSA, warfarin bound to HSA (HSA/warfarin), and ibuprofen bound to HSA (HSA/ibuprofen), represented by black, red, green, and blue curves, respectively.

is calculated from the integration of $S(Q, E)$ over an energy range measured at each Q-value given by the relation, $S(Q) = \int S(Q, E)\, dE$. Clearly, the two major peaks are observed from all the samples at $Q \sim 6$ nm^{-1} (**A**) and ~ 15 nm^{-1} (**B**), which have also been observed in different hydrated proteins, such as lysozyme,[39] green fluorescent protein,[29] beta-lactoglobulin,[40] alpha-chymotrypsinogen A,[43] myoglobin, hemoglobin, and C-phycocyanin.[62] Thus, such a structural property can be considered a universal feature of all the proteins. Peak **A** corresponds to the protein−protein interaction at a relatively larger length scale (in the order of 1 nm), whereas peak **B** represents the spatial order of the secondary structure, β-sheets of length ∼ 4.5 Å and α-helix repeats of ∼ 5 Å.[39,40] The absence of a structural peak of bulk water at ~ 19 nm^{-1} assures that the scattering data collected are exclusively from the hydrated protein samples.[35,40,63] Furthermore, the distorted peak **B** in $S(Q)$ of the thermally denatured HSA confirms the significant loss in the secondary structure, particularly α-helices, and tertiary structure due to disruption of noncovalent bonds, such as hydrogen bonds, and Van der Waals interactions.[39] Moreover, we did not see any significant change in the $S(Q)$ of HSA due to binding with the drugs (warfarin and ibuprofen). This is because HSA consists of large hydrophobic cavities in subdomains IIA (site I) and IIIA (site II) that hold drugs without affecting its local and the overall native conformation.[53]

Collective Excitations in Hydrated Proteins. The dynamic coherent structure factor, $S(Q,E)$ measured by IXS from different protein samples and the corresponding fitting by the DHO model (eq 1) are shown in Figure 2 at Q = 2.7 nm^{-1} and T = 300 K. The same data fittings at a medium Q = 19.6 nm^{-1} and a high Q = 31.2 nm^{-1} at T = 300 K are shown in the Figures S1 and S2 in the Supporting Information (SI). Figure 2A, B are from experiment 1 (native/denatured HSA), whereas the panels (C) and (D) are from experiment 2 (HSA bound with drugs). The green open circles represent the data points, whereas the black, red, and blue solid curves represent the

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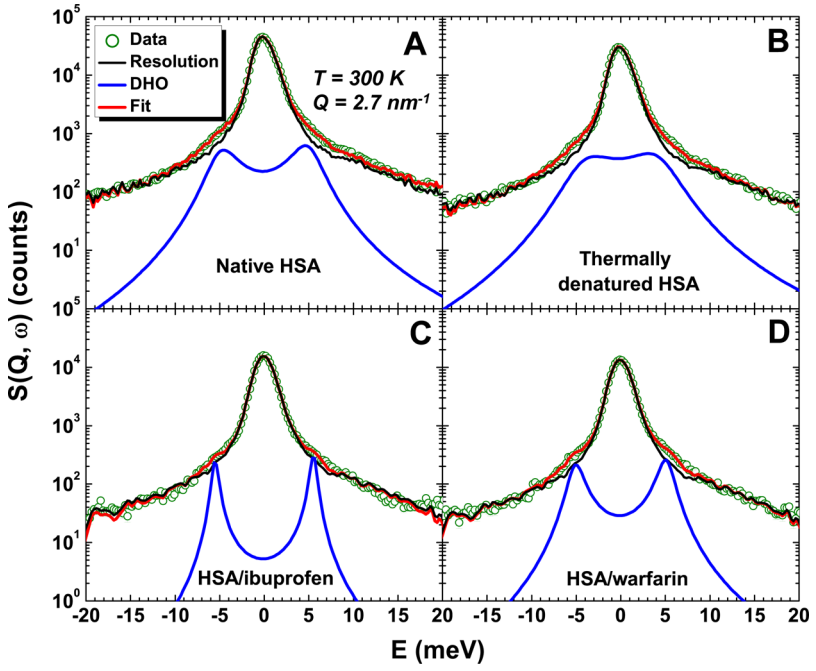

Figure 2. Dynamic coherent structure factor measured from (A) native HSA, (B) thermally denatured HSA, (C) HSA/ibuprofen, and (D) HSA/warfarin and corresponding fitting with eq 1 at $Q = 2.7$ nm^{-1} and $T = 300$ K. The green circles, black, blue, and red curves indicate the experimental data, resolution function, DHO function with two Brillouin-side peaks, and the fitted curve, respectively.

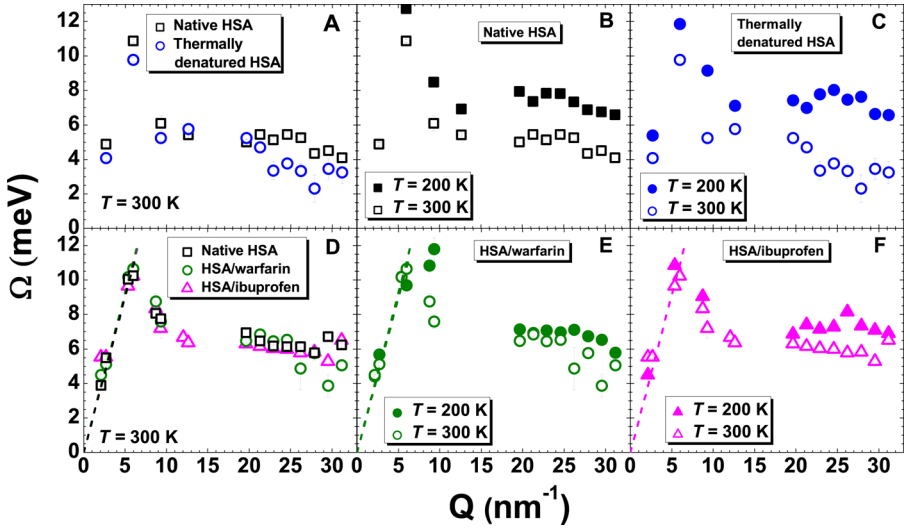

Figure 3. Phonon dispersion curves (Ω vs Q) calculated from native HSA and thermally denatured HSA samples (A–C), HSA/warfarin and HSA/ibuprofen samples (D–F) with $h \sim 0.4$ at $T = 200$ and 300 K. The dashed lines represent the fitting of dispersion curve at low Q-values (acoustic branch) with eq 6 to calculate the longitudinal sound velocity (v_L). The longitudinal sound velocity is calculated for the data that have more than two data points in the acoustic branch of the corresponding dispersion curve.

resolution function, the fitted curve and the DHO function with Brillouin-side peaks, respectively. Clearly, we can see the two small inelastic peaks on the shoulder of the central peak due to the collective excitations or density fluctuations in the samples. Such weak inelastic peaks are due to the amorphous nature of the samples similar to the glasses or glass forming liquids.[33,37,44]

Figure 3 shows the dispersion curves Ω vs Q calculated from the different protein samples. The data shown in the upper three panels are from experiment 1 and the data in the lower three panels are from experiment 2. The dispersion phenomena can be observed roughly up to the Q-value where the peak **B** of the structure factor rises (~10 nm^{-1}), which corresponds to the spatial order of the secondary structure as discussed above. Such behavior implies that the protein intermediate and short-range order can define the dispersion curve similar to the Brillouin zone in the crystals.[33] However, at the higher Q-values, the collective excitations seem to be almost Q-independent, and thus can be considered as nonpropagating, or localized. Such localized fluctuations are due to the topologically disorder length scale[64] in the sample mainly contributed by intraprotein collective vibrations due to the protein primary structure or amino acid residues, such as methyl groups, which are on the length scale of 2.0–5.0 Å. On the other hand, the linearity of the curve at the low Q-values

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indicates the acoustic longitudinal phonons that propagate with certain sound velocity in the samples. The sound velocity of such phonon is calculated from the slope of the energy dispersion curve by fitting with a relation given by

$$\Omega(Q) = \hbar v_L Q \quad (6)$$

where v_L is the longitudinal sound velocity. The calculated values of longitudinal sound velocity at room temperature (T = 300 K) listed in Table 1 are analogous to the values as reported in previous experiments.[33,35−40] The significant temperature dependence of characteristic excitation energies in native HSA, thermally denatured HSA, HSA/warfarin, and HSA/ibuprofen samples is shown in Figure 3B, C, E, and F, respectively. Clearly, the phonon softening that can be observed at higher Q-values with rise in temperature, also reported before in a similar IXS experiment,[38] must be crucial for the protein enzymatic activity at physiological temperatures.

Table 1. Longitudinal Sound Velocities Calculated from the Fitting of Dispersion Curves in the Acoustic Branch with Eq 6

samples	$v_L(m/s)$ T = 300 K
native HSA (expt. 2)	2744 ± 91
HSA/warfarin (expt. 2)	2809 ± 80
HSA/ibuprofen (expt.2)	2772 ± 194

The phonon damping constant (Γ), or the half width at half-maximum of the inelastic side-peaks, is plotted as functions of Q in Figure 4. Its Q-dependence resembles the lifetime of collective excitations in the ordinary glasses,[37,44] which follows a power law relation $\Gamma'(Q) \propto Q^2$ at low Q-values. The data in Figure 4 upper three panels are calculated from experiment 1, whereas the data in the lower three panels are from experiment 2. Clearly, the increase in the value of Γ with Q and temperature indicates the increase in the damping of collective modes due to the activation of diffusion processes in the samples.

Protein Denaturation and Collective Excitations. Thermal denaturation significantly affects the protein structure by minimizing the content of α-helices and tertiary structures, whereas the β-sheets content is increased.[39,65] In addition, the thermal denaturation yields the molten globule state and aggregation of HSA.[66] Therefore, the thermally denatured protein loses its biological activity. The disruption of peak **B** in the measured static structure factor data as shown in Figure 1 clearly indicates the characteristic change in the secondary structure of thermally denatured HSA. However, our experimental results show that despite denaturation of protein, the collective excitations still exist, which is consistent with the results previously observed in denatured globular protein, lysozyme.[39] Therefore, our results indicate that the phonon-like excitations exist and propagate even through the denatured protein, which may be due to the presence of increased content of β-sheets and the unfolded domains. However, the denaturation affects the softness of the protein at the physiological condition. At room temperature (T = 300 K), as shown in Figure 3A, we observed a significant decrease in the values of excitation energy at higher Q-values (localized collective mode) in the thermally denatured HSA compared to that of native HSA. This clearly indicates the increase in the softness of protein upon denaturation. Such increased softness is due to the disruption of the weak bonds like hydrogen bonds and Van der Waals interaction (that hold the protein conformation in the native state) causing the unfolding of protein structure upon denaturation. However, at a much lower measured temperature T = 200 K, which is below the dynamic transition temperature (T_D) and at which the protein is inactive or nonfunctional,[67−69] the dispersion curves in native and thermally denatured samples are nearly identical, as shown in Figure S3 in Supporting Information. Thus, our results suggest that the protein may require certain degree of resilience for the enzymatic activity. If the protein becomes too soft at the room temperature, as we observed in the thermally denatured sample, it may lose its biological function. Therefore, the dynamical property of protein, such as softness (flexibility) and rigidity (resilience), are balanced by the biomolecules to enable their

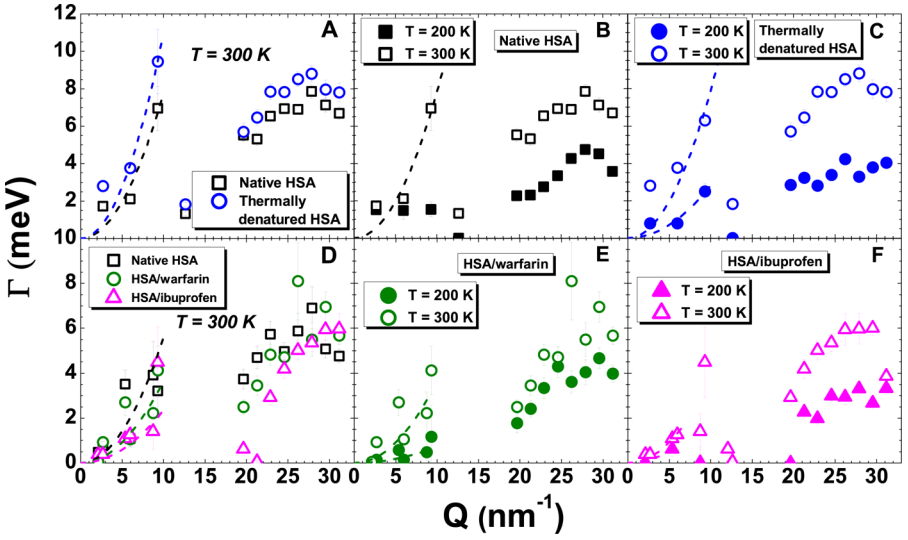

Figure 4. Damping constants of phonons calculated from the samples of native HSA, thermally denatured HSA, HSA/warfarin, and HSA/ibuprofen samples with $h \sim 0.4$ at T = 200 and 300 K. The dashed lines represent the fitting by relation $\Gamma = AQ^2$ at low Q-values (acoustic branch) similar to glasses, where A is a constant.

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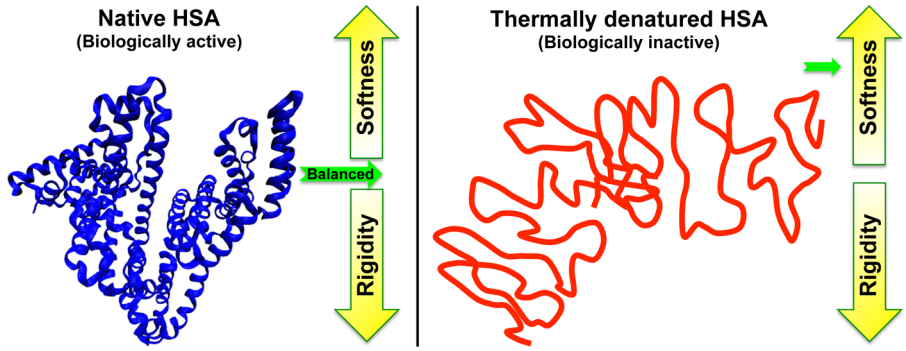

Figure 5. Schematic diagram of measure of softness and rigidity in native and denatured protein. Left: Native structure of human serum albumin (PDB: 1O9X), where the structural property, softness, and rigidity are balanced by protein for biological function. Right: Thermally denatured protein, which is softer than the native protein due to the disruption of weak bonds, such as hydrogen bonds, and Van der Waals interaction, and loses its activity.

biological activities,[70−72] as graphically presented in the schematic diagram of Figure 5.

Flexibility of the HSA Protein Remains Unaltered upon Binding to the Drugs. HSA has very strong binding sites as the site I (subdomain II A) and site II (subdomain III A). These sites are the hydrophobic cavities capable of holding significant amount of ligands or drugs.[53] Warfarin and ibuprofen are the drugs that strongly bind to the site I and site II, respectively, under physiological conditions. In most of the cases, about 99% of these drugs are bound to the protein under the normal therapeutic environments.[73] Such strong binding affinity sites in HSA is an important property for pharmacokinetic behavior that influences the efficacy and delivery rate of the drugs. A recent study suggests that these drugs also have the ability to stabilize HSA structure and protect against denaturation by chemical or thermal means.[54] Therefore, as expected, in our measured static structure factor $S(Q)$ (Figures 1, S1, and S2), no significant differences are observed in the peak position between the native unbound HSA and the drug-bound HSA samples. Similarly, at the room temperature, there is no substantial difference in the dispersion curves between the native HSA, HSA/warfarin, and HSA/ibuprofen, as shown in Figure 3D. On the other hand, in Figure 3E and F, we can clearly see the softening of the samples with the increase in temperature, as suggested by the decrease in the excitation energies at higher Q-values. These results indicate that HSA preserves its native local flexibility even when bound to the drugs with high binding affinity. Therefore, HSA acts as an efficient carrier and transporter of drugs in the plasma that is favorable for pharmaceutical drug design and delivery.

CONCLUSION

In summary, we report the collective excitations in hydrated protein samples of native HSA, denatured HSA, and HSA bound to the drugs, warfarin and ibuprofen. The collective excitations are primarily due to the protein internal dynamics. We show that both acoustic (propagating) and localized (nonpropagating) modes of phonon exist in native and denatured HSA. The acoustic mode is due to the secondary or larger structures, whereas the localized excitations are due to the fluctuations of amino acid residues, such as methyl groups. Remarkably, we found softening of localized phonons in denatured HSA compared to the native HSA, due to disruption of weak forces like hydrogen bonds and Van der Waals interaction causing the unfolding of the protein. Therefore, the protein structural property of flexibility and rigidity are balanced by the native protein for its activity. Furthermore, drugs like warfarin and ibuprofen that have high binding affinity to site I and site II of HSA, respectively, do not affect the protein structure in plasma, which is consistent with earlier crystallography study. Additionally, there is no significant change in protein dispersion curves upon binding of the drugs at room temperature. These results suggest that the dynamic properties of HSA such as collective excitations that are crucial for enzymatic activity in plasma are not affected by the drugs bound to HSA. Moreover, they also confirm that the collective excitations in protein are highly structure-dependent, similar to the glass formers. Any changes in protein local or global conformation can significantly affect its dynamic behavior, and, therefore, its function and activity. We propose the idea of protein softness and rigidity defined in terms of collective excitations that can be further used as a novel approach to understand the protein activity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.6b10245.

The data fitting for the native HSA, thermally denatured HSA, HSA/ibuprofen, and HSA/warfarin (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: chux@wayne.edu

ORCID

Xiang-Qiang Chu: 0000-0003-4320-5316

Notes

The authors declare no competing financial interest.

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