Collective Dynamics of Lysozyme in Water: Terahertz Absorption Spectroscopy and Comparison with Theory

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To directly measure the low-frequency vibrational modes of proteins in biologically relevant water environment rather than previously explored dry or slightly hydrated phase, we have developed a broadband terahertz spectrometer suitable for strongly attenuating protein solutions. Radiation is provided by harmonic multipliers (up to 0.21 THz), a Gunn oscillator (at 0.139 THz), and the UCSB free-electron lasers (up to 4.8 THz). Our spectrometer combines these intense sources with a sensitive cryogenic detector and a variable path length sample cell to detect radiation after it is attenuated by more than 7 orders of magnitudes by the aqueous sample. Using this spectrometer, we have measured the molar extinction of solvated lysozyme between 0.075 and 3.72 THz (2.5−124 cm^{-1}), and we made direct comparison to several published theoretical models based on molecular dynamics simulations and normal-mode analysis. We confirm the existence of dense, overlapping normal modes in the terahertz frequency range. Our observed spectrum, while in rough qualitative agreement with these models, differs in detail. Further, we observe a low-frequency cutoff in terahertz dynamics between 0.2 and 0.3 THz, and we see no evidence of a predicted normal mode at ∼0.09 THz for the protein.

Introduction

Molecular dynamics simulation and normal-mode analysis of biopolymers predict a large number of functionally relevant, collective modes at terahertz frequencies (corresponding to periods of ∼1 ps).[1−9] Recent advances in terahertz sources and detection methods have stimulated experimental researchers to employ absorption spectroscopy to directly probe and confirm these postulated collective vibrational modes.[10−20] Because of the overwhelming attenuation of the terahertz radiation by water, however, these pioneering studies have been limited to dry or, at best, moist samples. Successful isolation of the low-frequency vibrational activities of solvated biomolecules in their natural water environment has remained elusive.

We have recently reported the first successful isolation of terahertz molar extinction (or molar absorptivity) of a protein, bovine serum albumin (BSA), in aqueous solution.[21] Unfortunately, however, BSA has not previously been the focus of theoretical investigations, and thus our earlier work did not provide an opportunity for direct comparison between theory and experiment. In contrast, both molecular dynamics simulations[9,16] and normal mode calculations[7] have been reported regarding the low-frequency normal modes of the monomeric protein hen egg white lysozyme, inviting the detailed terahertz absorption measurement of this protein. Thus motivated, we report here the absorption spectrum of lysozyme in aqueous solution between 0.075 and 3.72 THz.

Experimental Methods and Materials

Materials and Solution Preparation. For this study, we employed chicken hen egg white lysozyme (Sigma, St. Louis, MO, without further purification), a small monomeric protein with molecular weight of 14.3 kDa. We prepared water−lysozyme solutions buffered at pH 3 (50 mM phosphate in distilled deionized water) with 0.05% sodium azide to prevent bacterial growth. We also employed a lysozyme-free buffered blank as the reference.

Densimetrically Determined Molarities of Lysozyme and Water. To precisely determine the molarities of both lysozyme and buffered water in solution, we first measured the masses of both lysozyme and water prior to solution mixing. This solute-to-solvent mass ratio remains the same for successful solvation where the solution is uniform and clear without any precipitation. We then carefully determined the final solution density by measuring the mass of 1 mL of solution, and we repeated this density measurement nine times for each solution to estimate confidence limits. We calculated the molar concentration of lyzoyme in solution as follows:

$$[\text{lysozyme}] = \frac{\rho_{\text{solution}}}{MW_{\text{lysozyme}}} \cdot \frac{m_{\text{lysozyme}}}{m_{\text{lysozyme}} + m_{\text{water}}}$$

where [lysozyme] is the molarity of lyzosyme, ρ_{solution} is the final solution density, MW_{lysozyme} is the molecular weight of lysozyme, and m_{lysozyme} and m_{water} are the respective masses of the solute and solvent. The molarity of water in solution was similarly determined:

$$[\text{water}] = \frac{\rho_{\text{solution}}}{MW_{\text{water}}} \cdot \frac{m_{\text{water}}}{m_{\text{lysozyme}} + m_{\text{water}}}$$

where [water] is the molarity of water in solution, and MW_{water} is the molecular weight of water. Thus determined molar concentrations of both lysozyme and water enable us to precisely extract and remove the dominating water background absorption.

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Absorption Measurements. We have developed a terahertz absorption spectrometer appropriate for strongly absorbing media such as water.[21,22] Radiation is provided by harmonic multiplication of the output of a microwave synthesizer (0.075−0.244 THz, 20−30 mW, continuous wave), a Gunn oscillator (0.139 THz, 28 mW, continuous wave), and the UCSB free-electron lasers (0.3−4.8 THz, ~1 kW, pulsed). The spectrometer combines these intense sources with a cryogenic composite silicon detector (QMC Instruments, NEP $\sim 10^{-12}$ W/$\sqrt{\text{Hz}}$) and is capable of detecting radiation after it is attenuated by more than 7 orders of magnitudes by the sample medium.

A variable path length sample cell is employed to precisely measure the change in absorbance with path length. A polyethylene bag filled with liquid sample is squeezed between two parallel transparent polyethylene[23] windows (~2.5 cm diameter). Fine adjustments, reproducible to ~0.2 μm, of the distance between the polyethylene windows result in precise change in sample path length. The terahertz extinction of the sample is determined by

$$\Delta A = \Delta(-\ln[I_t]) = \alpha \cdot \Delta l$$

where ΔA is the change in absorbance, I_t is the transmitted intensity, α is the extinction coefficients of the sample, and Δl is the change in path length.

At each wavelength, we examined an average of nine distinct path lengths, with increments ranging between 2 and 20 μm depending on the absorption strength of the sample, and repeated the absorbance measurements approximately eight times to estimate confidence limits.[21,22] Using our spectrometer, we have precisely measured the extinctions of both protein solutions and their associated buffer blanks between 0.075 and 3.72 THz (Figures 1 and 2). Spectra are recorded, point-by-point, by repeating these measurements at each frequency.

Molar Absorptivity of Solvated Protein. We assume that the terahertz extinction of solution is a weighted sum of solvent and solute absorptions by their respective molar concentrations,

$$\alpha_{\text{solution}} = \sigma_{\text{lysozyme}} \cdot [\text{lysozyme}] + \sigma_{\text{bulk water}} \cdot [\text{bulk water}]$$

where α_{solution} is the measured solution extinction, [lysozyme] and [bulk water] are the molarities, and σ_{lysozyme} and σ_{water} are the molar extinctions of the lysozyme and the buffered water, respectively. Because the buffer blank is free of lysozyme by definition, molar extinction of bulk water, $\sigma_{\text{bulk water}}$, is obtained using the measured extinctions of the buffer blank. The solvent baseline, $\sigma_{\text{bulk water}} \cdot$[bulk water], is the water extinction spectrum scaled by the concentration of bulk water in solution, [bulk water]. Subtracting this from the total solution extinction allows us to measure $\sigma_{\text{lysozyme}} \cdot$[lysozyme] and to determine the absorption contribution from the lysozyme solute, and its molar extinction, σ_{lysozyme}.

Results and Discussion

Solutions of lysozyme absorb terahertz radiation less effectively than samples of the equivalent protein-free buffer (Figures 1 and 2). This striking feature indicates that the protein solute does not absorb as strongly as the water it displaces. We scaled the buffer spectrum by the densimetrically determined molar concentration of water in solution (see Experimental Materials and Methods) to obtain a measure of solvent background extinction. As compared to this baseline ("Solvent Baseline" in Figure 2), the lysozyme solution now exhibits an excess extinction, over most, but not all, of the frequency range, which can be ascribed to the protein solute. This is consistent with the theoretically postulated broad distribution of normal modes of biopolymers in the terahertz frequency range. However, at the lowest frequencies (0.075−0.105 THz), the same solvent baseline ("Baseline 1" in Figure 1) still appears to be stronger than the solution extinction. This apparent contradiction may be resolved if we extend our analysis to include water in the protein's hydration shell and assume that this dynamically bound water contributes to solution absorption as part of the solvated protein dynamics and at higher frequencies.

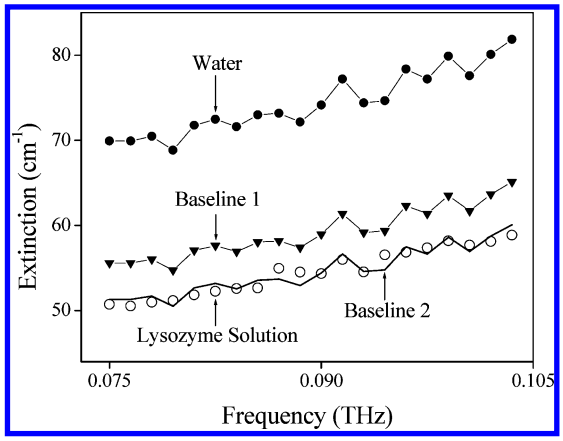

Figure 1. Between 0.075 and 0.105 THz, the lysozyme solution does not exhibit any significant spectral feature that distinguishes it from the buffered water blank. Solvent "Baseline 1" neglects any increased hydration of the protein solute and is simply the water spectrum scaled by the total water concentration. We rescale the buffer blank extinction ("Baseline 2") to bring it into agreement with the lysozyme solution. The difference between "Baseline 1" and "Baseline 2" gives rise to a measure of bound water concentration. The resulting bound water/protein ratio agrees well with other published estimates[26,27] (see text). The key to this interpretation is our assumption that the bound water contributes to solution extinction at higher frequencies. Such difference in dielectric response between bound and bulk water is supported by recent literature.[27,28] Measurement uncertainties (not shown) are within 0.5% of extinction. Systematic noise dominates and is reflected in the measurement scatter.

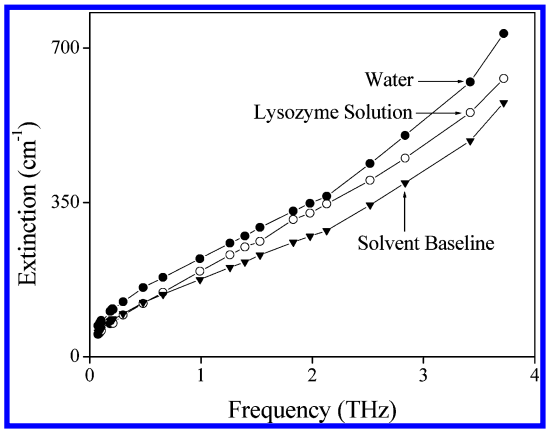

Figure 2. The terahertz extinction of buffered water (●) is greater than that of lysozyme solutions (○), indicating that the protein solute absorbs terahertz radiation less strongly than does the water it displaces. We obtained the absorption solvent baseline (▼) by scaling the buffer blank extinctions with the densimetrically determined water concentration. This baseline is subsequently adjusted to correct for the additional hydration of lysozyme in water (hydration corrected baseline is not shown) to accurately assess solvent background absorption.

Hydration Shell Estimation. Apart from systematic noise, there are no identifiable spectral features in the spectrum of lysozyme solutions between 0.075 and 0.105 THz that are distinct from those of the water spectrum (Figure 1). This provides a strong indication that only the bulk water fraction

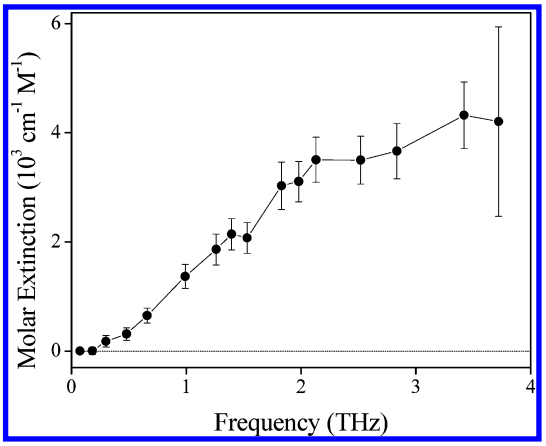

Figure 3. The terahertz molar extinctions of solvated lysozyme (along with its hydration shell) provide a measure of its low-frequency vibrational dynamics. Above ~0.2 THz, we observe a broad spectrum with an initial fast rise in absorption, and a high-frequency plateau/saturation above ~2 THz.

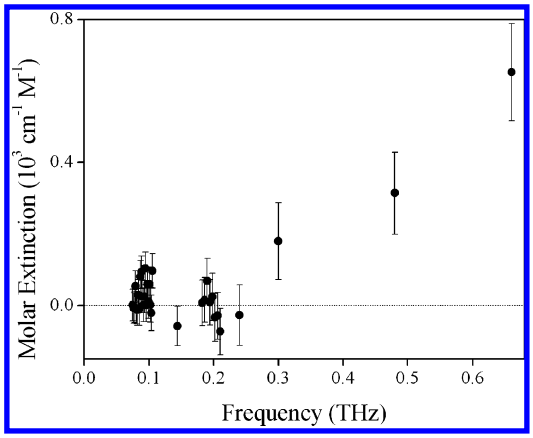

Figure 4. We see no evidence of vibrational dynamics below ~0.2 THz in solvated lysozyme. Instead, we observe a critical on-set of extinction between 0.2 and 0.3 THz. This low-frequency cutoff may simply arise from the finite size of the protein.

of lysozyme solution is contributing to terahertz absorption in this frequency range. The observed solution extinction strengths, however, are less than we would expect for the absorbance of the amount of water, densimetrically determined, in solution ("Baseline 1" in Figure 1). At this juncture, were we to subtract the densimetrically rescaled water from the solution we would determine that the protein exhibits negative absorption. This is clearly not possible. This, in turn, suggests that at least some of the water is no longer "visible" at these wavelengths, presumably because it is bound to the protein and acting as "part" of it rather than remaining as "bulk water".[24−26] In other words

$$[\text{bulk water}] = [\text{water}] - [\text{bound water}]$$

where [bound water] is the molarity of bound water.

The magnitude of rescaling (or the difference between "Baseline 1" and "Baseline 2" in Figure 1) gives rise to a measure of bound water concentration. The ratio of the bound water concentration and the densimetrically determined lysozyme concentration indicates that there are approximately (173 ± 40) water molecules associated with each lysozyme, or (0.22 ± 0.05) grams of water per gram of protein. This experimental estimation of hydration is in excellent agreement with the previously published values of <0.27 g of water per gram of hen egg white lysozyme,[27] and ~140 bound water per protein molecule for the related human and tortoise proteins,[26] as well as a very compelling confirmation of the dielectric distinction of hydration versus bulk water observed in recent high-resolution quasi-elastic neutron scattering measurements.[28] Thus motivated, we have used this hydration estimation to adjust the solvent baseline (Figures 1 and 2) over the entire observed spectrum, allowing us to extract the molar extinction of the solvated protein and its hydration shell (Figures 3 and 4).

Terahertz Dynamics of Solvated Lysozyme. The molar extinctions of solvated lysozyme exhibit a rapid rise with frequency followed by a nearly constant extinction from approximately 2 THz to the highest frequency investigated (3.72 THz). At our frequency resolution ($\Delta f = 1.5$ GHz below 0.105 THz, $\Delta f = 4$ GHz between 0.18 and 0.21 THz, and $\Delta f \approx 0.25$ THz above 0.3 THz), distinct narrow spectral features are not apparent.

Low-Frequency Cutoff. Theoretical models, such as molecular dynamics and normal mode calculations, have long predicted low-frequency vibrational modes in proteins in the vicinity of 0.1 THz.[1,2,5,7,8] Specifically, normal-mode analysis has predicted the first vibrational mode of chicken egg white lysozyme to be at 0.09 THz.[7] However, we see no evidence of vibrational dynamics in solvated lysozyme below ~0.2 THz (Figures 1 and 4). Instead, we observe a critical on-set of protein dynamics between 0.2 and 0.3 THz (Figure 4).

The observed cutoff is consistent with the acoustic behavior of small, solid objects. An isolated particle cannot support vibrational modes at arbitrarily low frequencies;[29,30] instead, it will exhibit a cutoff frequency related to the velocity of sound in the particle and an acoustic wavelength roughly twice the particle's diameter. While this analysis will depend on the particle's shape, the roughly spherical nature of lysozyme suggests this may be a reasonable approximation. Using an estimated lysozyme sound velocity of 1958 m/s (ref 31) and diameter of ~5 nm (ref 32), we obtain a low-frequency limit of ~0.2 THz. While this agreement is perhaps better than this simple model deserves, we nevertheless propose that the critical behavior observed between 0.2 and 0.3 THz may simply arise from the finite size of the solvated protein.

The above analysis suggests that the lowest frequency vibrations of lyzozyme are perhaps surprisingly weakly coupled to the bulk solvent: if the low-frequency modes were well coupled to the solvent, the notion of a lowest "acoustic wavelength" and a cutoff in vibrational modes of the particle would be meaningless. A reasonably well-defined feature, the cutoff in the absorption spectrum, implies that the low-lying modes are not over-damped from being in water.

This interpretation of the low-frequency cutoff of the absorption spectrum as a measure of the lowest possible vibrational modes, however appealing, excludes the theoretically predicted first mode at ~0.09 THz. We cannot rule out that the absorption strength of these modes at the low-frequency edge of the spectrum is not sufficient to be detected at the current level of sensitivity, that the apparent cutoff in Figure 4 does not define the very lowest modes in the protein. If there are lower modes, at present levels of sensitivity, we have not been able to observe them.

Comparison with BSA. The terahertz absorption spectra of two distinct solvated proteins, lysozyme (this study) and BSA,[21] differ in detail (Figure 5). The overall molar extinction of BSA is several times stronger than that of lysozyme (Figure 5, top). Normalization by their respective number of amino acid residues per molecule (lysozyme contains 129 residues, and BSA contains 585) brings the overall absorption strength into reasonable agreement (Figure 5, bottom), indicating that the

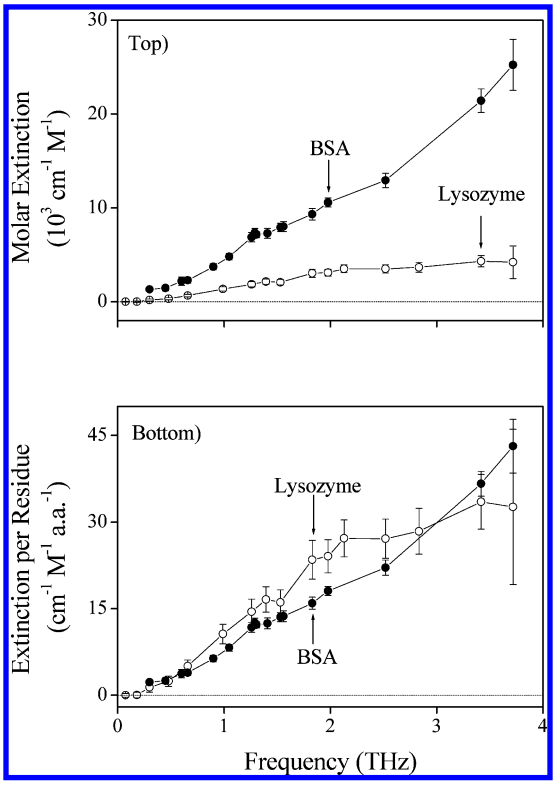

Figure 5. (Top) The absolute teraherz spectra of solvated lysozyme (○) and BSA[21] (●) differ significantly. For example, the monotonic increase in extinction observed for BSA tapers off and saturates above ~2 THz for lysozyme. (Bottom) The apparent greater overall absorption of the BSA is largely mitigated by normalizing the spectra by the number of amino acid residue.

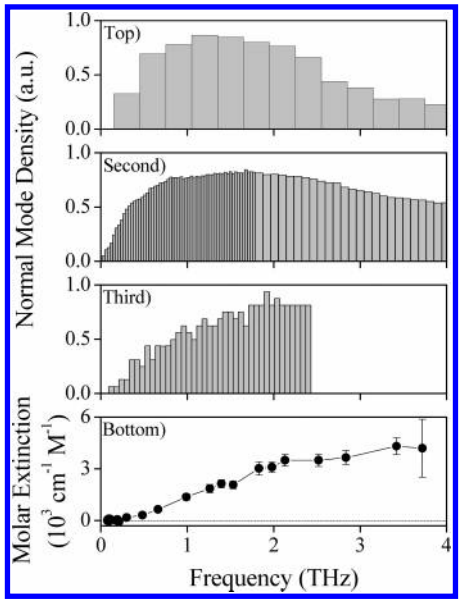

Figure 6. We observe a reasonable agreement between theory (top three panels, normal mode densities obtained from original publications without further modification[7,9,16]) and experiment (bottom panel). The initial fast rise in lysozyme extinction is approximately captured in vibrational mode densities calculated using both normal-mode analysis (top[7] panel) and molecular dynamics simulations (second[9] and third[16] panels). (Several models are presented in ref 9; here (second panel), we have employed the averaged density of states model based on normal-mode calculations of 10 different structures, as this method is shown by Karplus and co-workers to improve agreement with available inelastic neutron scattering experiments when applied to bovine pancreatic trypsin inhibitor.[9]) Above ~2 THz, however, the ability of current theory to predict the experimentally observed absorption spectrum is less universal.

density of amino acid residues in solution dominates the overall absorption strengths. Nevertheless, the spectral shapes of the two proteins exhibit subtle but experimentally significant differences (Figure 5). After the initial fast rise in absorption common to both proteins, the BSA spectrum continues to rise monotonically up to 3.72 THz, whereas the extinction of the solvated lysozyme appears to plateau at approximately 2 THz. Such a departure may arise from the specific charge distribution in each protein and the protein-specific macromolecular modes.

Comparison with Theoretical Calculations. The existence of published molecular dynamics[9,16]- and normal mode[7]-based models of the dynamics of lysozyme (Figure 6, theoretical values are taken from original publications[7,9,16] without further modification) provide an opportunity to compare our experimental results with the predictions of these computational efforts. Vlijmen et al.[9] and Markelz et al.[16] carried out molecular dynamics simulations using the CHARMM force field in the presence and absence of explicit water bath, respectively. Both results suggest broad mode density distributions in our investigated frequency range (Figure 6, second and third panels). Normal mode calculations by Levitt et al.[7] suggest a prominent spectral peak at ~1 THz that rapidly tapers off both in the lower and in the higher frequency range (Figure 6, top).

Precise comparison between experiment and these computational studies (using their original literature values[7,9,16]) is difficult because the experimental observable, terahertz extinction, depends on both normal mode densities and the transition dipole moments of these modes. Yet, to zeroth order, the nonperiodic distribution of charge, in the organized but aperiodic protein structure, should allow coupling of the radiation field to all modes in this range. Perhaps consistent with this, we observe a reasonable qualitative agreement between these theoretical predictions and our experimental results (Figure 6, bottom): Above the cutoff, the initial monotonic rise in lysozyme extinction is approximately captured by both molecular dynamics simulations[9,16] and normal-mode analysis.[7] The ability of theory to predict the experimentally observed plateau above ~2 THz, however, is less universal. While the observed saturation is in good qualitative agreement with the molecular dynamics simulation by Markelz et al.[16] (available up to 2.4 THz), it does not appear to support the large and fast depletion in spectral density predicted via normal-mode analysis[7] between 2 and 4 THz, and the relatively weaker and slower reduction predicted above 2 THz via the molecular dynamics simulations of Karplus and co-workers.[9]

Conclusions

We have successfully measured terahertz absorbance of solvated lysozyme and employed the experimental absorption spectrum as a test for previously published theoretical models of the protein's collective dynamics. Our experiments generally confirm theoretical predictions of densely overlapping normal modes in the terahertz frequency range[7,9,16] with the measured extinction dropping to zero between 0.2 and 0.3 THz. There remain, however, important differences between current theory and our experimental results. We also observe subtle yet statistically significant differences between the absorption spectra of BSA and lysozyme, presumably due to the differences in their molecular details. Together these observations suggest that terahertz absorption measurements may be a valuable technique to provide detailed information regarding protein-specific dynamics and to test theoretical models of protein dynamics.

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