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Molecular kinetics of protein unfolding at the air/water interface probed by vibrational sum-frequency spectroscopy

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ABSTRACT

Protein behavior at aqueous interfaces is central to many soft-matter, biological, and technological systems, yet the molecular events underlying adsorption-induced structural change remain difficult to isolate. In particular, macroscopic measurements such as surface tension cannot directly reveal how proteins reorganize during interfacial adsorption. In this study, we report a time-resolved vibrational sum-frequency generation (VSFG) spectroscopy study of lysozyme adsorption and unfolding at the air/water interface, combined with simultaneous surface-tension measurements. By monitoring the interfacial CH-stretch response after subsurface protein injection, we directly correlate macroscopic changes in surface pressure with molecular restructuring of adsorbed lysozyme. During the first ~10 min after injection, the surface tension remains essentially unchanged and no lysozyme CH resonance is detected. Together with the much shorter diffusion-only estimate, this argues against assigning the full induction period to bulk transport alone. The subsequent decrease in surface tension is accompanied by a rapid increase in the interfacial CH response, indicating adsorption-induced unfolding and exposure of hydrophobic moieties toward the vapor phase. After ~30 min, the CH response saturates while the surface tension continues to evolve, revealing an additional kinetic regime associated with interfacial crowding, protein–protein interactions, and multilayer formation. Amide I VSFG spectra further show that interfacial lysozyme retains partial secondary structure, with enhanced beta-sheet contributions relative to the bulk solution. These results demonstrate how interface-selective nonlinear vibrational spectroscopy can resolve molecular kinetic steps that are hidden in surface-tension measurements alone, providing a detailed picture of protein structural dynamics at aqueous interfaces.

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I. INTRODUCTION

Protein adsorption and structural reorganization at aqueous interfaces are central to many biological, environmental, and technological processes, including foam and emulsion stabilization, biosensing, biomaterials, cosmetics, and food technology.[1] The air/water interface is also a conceptually important model hydrophobic surface, making it a useful platform for studying how proteins interact with nonpolar environments under well-defined conditions. Despite this importance, the molecular-level sequence by which proteins adsorb, unfold, and reorganize at the air/water interface remains incompletely understood, particularly because macroscopic observables such as surface tension do not directly report on protein conformation or orientation. Understanding the free energy and kinetics of protein interaction with the air/water interface is therefore important both from a fundamental perspective and for guiding applications in which interfacial protein structure controls macroscopic function. Ultimately, we wish to understand interface-induced structural transitions of a particular protein on a molecular level. To gain such general knowledge requires characterization of the interfacial properties, presumably using a variety of complementary techniques, of a suitable model protein whose solution properties are well known. Because of its wide availability, well-characterized structure, and extensive use in prior

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interfacial studies, egg-white lysozyme has been widely employed as such a model protein for investigating protein interactions with the air/water interface.[2]

Lysozyme is a relatively small ellipsoidal protein (MW = 14.3 kDa, size: ~30 × 30 × 45 Å^3) that in solution is both rigid and resistant to denaturation.[3] Both properties are generally attributed to the four disulfide bonds present in the protein's native structure.[2,3] While the energetics and mechanism of egg white lysozyme adsorption to the air/water interface have been studied by a variety of methods, two characteristics of this process—the equilibrium structure of the adsorbed protein and the mechanism of adsorption—are still incompletely understood.[3] For example, neutron reflectivity measurements at the air/water interface as a function of increasing bulk concentration suggest that the protein does not denature upon interfacial adsorption but rather assumes a preferential orientation such that its long axis points along the surface normal.[4,5] In contrast, results from FTIR[6] and x-ray reflectivity[7] have indicated that lysozyme adsorption is accompanied by unfolding. While most spectroscopic studies have examined lysozyme at the air/water interface with insufficient temporal resolution to resolve the kinetics of any unfolding process, measurements of surface properties with higher time resolution have raised still more unresolved questions. In particular, a comparison of the amount of interfacial lysozyme as a function of time after injection and surface tension suggests that the air/water interface is nearly saturated with lysozyme before the surface tension decreases.[8,9]

One challenge in generating a self-consistent picture of lysozyme adsorption from available data is that the different techniques probe notably different characteristics of the system. For instance, neutron and x-ray reflectivity are both highly interface sensitive but report on nuclear and electron density, respectively, and are therefore indirect methods for probing larger scale conformational changes of interfacial lysozyme.[4,5,7] In contrast, FTIR gives access to protein normal modes, known to be sensitive to protein secondary and tertiary structure, but is much less interface sensitive. Even in reflection-mode FTIR measurements, such as ATR-FTIR, the evanescent IR field typically penetrates hundreds of nanometers to micrometers into the aqueous phase and, therefore, probes protein over a comparatively large interfacial depth.[10] Finally, the changes in surface tension, which were observed upon adsorption of lysozyme to the water–air interface, may result from a variety of molecular level processes. Such processes may include protein accumulation at the interface, changes in molecular orientation, partial unfolding with exposure of hydrophobic residues, lateral rearrangement within the interfacial layer, protein–protein interactions, and possible multilayer formation.

Recent developments in vibrational sum-frequency generation (VSFG) spectroscopy show great promise for resolving the aforementioned challenges. VSFG spectroscopy is a laser-based, second order nonlinear optical technique that is interface specific due to its symmetry selection rules and has been previously demonstrated to be sensitive to fractions of a monolayer at various interfaces.[11–26] Compared with FTIR, VSFG is predominantly sensitive to a molecularly thin interfacial region where inversion symmetry is broken. More recent homodyne, heterodyne-detected, chiral, and simulation-supported VSFG methods provide direct access to biomolecular orientation, interfacial secondary structure, and local aqueous environments at soft interfaces.[27–29] The development of phase-resolved and time-resolved VSFG at aqueous interfaces, including important contributions from Tahara and co-workers, has also highlighted how nonlinear spectroscopy can connect interfacial structure with molecular dynamics in complex environments.[13,30,31] VSFG studies of lysozyme at the air/water (and solid/water) interface have been previously reported.[24,32,33] In those works, the interfacial response of the CH stretching vibration was probed, and the results were consistent with a scenario in which lysozyme denatures.

Earlier VSFG studies established the principal CH-stretch assignments of adsorbed lysozyme and showed that hydrophobic groups become ordered at hydrophobic interfaces. The present study confirms those spectral features but adds two elements. First, the CH stretch spectral response and surface tension as a function of time after lysozyme injection are monitored simultaneously. Second, we probe, at ~30 min after injection, the interfacial response of the amide I vibrational mode, which is sensitive to the secondary structure of proteins. We show that, from a surface tension point of view and consistent with prior studies, lysozyme adsorption can be characterized by three distinct time regimes after subsurface injection: induction (T_1, ~10 min), denaturation/monolayer formation (T_{2a}/T_{2b}, ~10–30/30–47 min), and multilayer adsorption (T_3, T_4, ~47/100 min). Correlation of surface tension with the CH stretch spectral response of interfacial lysozyme suggests that the time period corresponding to monolayer formation is actually composed of two distinct processes: a free and a crowded denaturation time regime, where the latter is the result of steric interactions between neighboring lysozyme molecules. VSFG measurements in the amide I spectral region conclusively establish that, after denaturation, lysozyme at the interface is still partially structured, and both α-helix and β-sheet secondary structures are present.

II. MATERIALS AND METHODS

Egg white lysozyme [Fig. 1(a)] was obtained from Sigma-Aldrich (L6876) and used without further purification. For VSFG measurements in the CH spectral region, the subphase (before Lysozyme injection) was composed of an aqueous sodium phosphate buffer (pH = 7, ionic strength = 0.03M). For measurements in the amide I spectral region, H_2O was replaced by D_2O (Cambridge Isotope Laboratories, Inc., 99.93% purity, used without further purification; pD = 7.4 by the formula pD = pH + 0.4[34]) to avoid spectral interference of the H_2O bending mode with the amide I vibrational mode. The isoelectric point of lysozyme is pH = 11.0; therefore, for all measurements performed in this study, the net charge of lysozyme is positive. Before a VSFG measurement, 19 ml of the subphase buffer solution was placed in a circular home-built Teflon trough (80 mm in diameter). Lysozyme was added to the trough by injecting 1 ml of a 20 mg/ml solution (made with the same solution used for the subphase) into the subphase using a Hamilton microsyringe. The resulting final lysozyme concentration was 1.0 mg ml^{-1} (~70 μM). The surface tension was measured using the Wilhelmy plate method as implemented in a commercial tensiometer (Kibron, Finland). We note that a sudden change in surface tension appears upon addition of the highly concentrated lysozyme solution to the subphase. This is a trivial effect caused by the change in the liquid level but not a real surface tension change and can be eliminated by offsetting or redefining the zero point.

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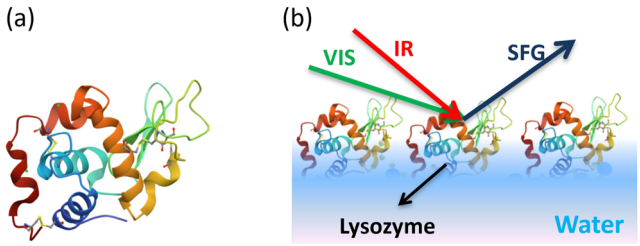


FIG. 1. (a) Cartoon representation of the lysozyme structure obtained from the RCSB Protein Data Bank (PDB ID: [1DPX]).[35] (b) Schematic of the VSFG measurement geometry for lysozyme at the air/water interface. The incident visible (VIS) and infrared (IR) beams generate the sum-frequency (SFG) signal at the interface.

VSFG measurements [Fig. 1(b)] were performed using a setup described in detail elsewhere while rotating the trough at 20 rpm.[36] During the measurement, the Wilhelmy plate remained immersed in the liquid while the trough rotated. The orientation of the plate was optimized to minimize the influence of trough rotation on the surface-tension measurement. For the VSFG setup, in brief, a titanium:sapphire oscillator and a regenerative amplifier (Legend, Coherent Inc., USA) produce a 120 fs laser pulse centered at 800 nm with a repetition rate of 1 kHz. 1 mJ/pulse of the output of the amplifier is used to pump an optical parametric amplifier system (TOPAS, Light Conversion) to generate IR pulses that are tunable between 2.5 and 8 μm. The remaining 0.5 mJ/pulse is spectrally narrowed using an etalon. This spectrally narrowed 800 nm (~25 μJ/pulse, full width at half maximum, fwhm = 15 cm^{-1}) and the IR beam (~6 μJ/pulse at 2800 cm^{-1} and ~3 μJ/pulse at 1640 cm^{-1}, fwhm = 200 cm^{-1}) impinge on the surface at an incidence angle of 40° and 35°, relative to the surface normal, respectively, and are aligned such that they overlap spatially and temporally at the interface. The VSFG signal created by the sample is reflected, focused into a spectrograph (Acton Instruments, USA), and dispersed onto an electron multiplied Charge Coupled Device (emCCD) camera (Newton, Andor, USA). All spectra reported in this study were collected under the ssp polarization condition (s polarized SFG, s polarized visible, and p polarized IR light), at room temperature (22 ± 1 °C). To correct for the non-uniform IR energy as a function of frequency over the bandwidth of the IR pulse, the measured air/water spectra were divided by the non-resonant signal collected from z-cut quartz. The center frequency of the IR pulse is fixed at 2900 cm^{-1} for measurements of the CH stretch spectral region and 1650 cm^{-1} for measurements of the amide I spectral region. The acquisition times for the CH and amide-I regions are 3 and 30 min, respectively, corresponding to 180 000 and 1 800 000 laser pulses at the 1 kHz repetition rate. FTIR transmission spectra of lysozyme solutions were collected on a FTIR spectrometer (Bio-Rad FTS-40A) by sandwiching 1 mg/ml lysozyme in D_2O solution between two CaF_2 windows. The windows were ultrasonically cleaned in acetone and methanol (10 min each) and then in Milli-Q water (15 min) before use. The optical path length was 60 μm and controlled by Teflon spacers. The spectrometer was operated at a resolution of 4 cm^{-1}. 128 interferograms were averaged for each spectrum to enhance the signal-to-noise ratio.

III. DATA ANALYSIS

Because of the coherent nature of the VSFG response, extracting quantitative spectral characteristics requires a lineshape model. We thus follow previous studies[11,14,22,23] and model our observed spectral response as

$$I_{SF} \propto \left|\chi^{(2)}\right|^2 I_{vis} I_{IR} \propto \left|A_{nr} e^{i\phi_{nr}} + \sum_n \frac{A_n}{\omega_n - \omega_{IR} - i\Gamma_n}\right|^2 I_{vis} I_{IR}, \quad (1)$$

where A_{nr} is the amplitude of the non-resonant response, Φ_{nr} is the phase of the non-resonant response, ω_n is the center frequency, A_n is the amplitude, Γ_n is the homogeneous line width (each of the nth resonance), I_{sf} is the sum frequency intensity, I_{ir} is the intensity of the incident infrared, and I_{vis} is the intensity of the incident visible. Equation (1) assumes each of the resonances to be homogeneously broadened. This assumption is justified based on prior studies on the CH response of self-assembled monolayers at solid surfaces[37–40] and various surfactants at the air/water interface.[41]

Prior heterodyne detected VSFG experiments at aqueous interfaces have found the non-resonant phase in such systems to be zero, i.e., the non-resonant response to be real.[22] Using Eq. (1) to fit our series of spectra, we thus set $\Phi_{nr} = 0$ for all measurement analyses. Given three apparent resonances (see data in Fig. 3), we are left with a total of ten parameters to describe each measured spectrum. Within the experimental resolution, employing this model with 10 free parameters results in multiple equally good descriptions of the data (see the supplementary material). Fortunately, prior study on interfacial CH containing systems suggests not all parameters are equally likely to vary. In particular, studies on dodecanol crystals and monolayers of various surfactants as a function of interfacial concentration suggest that the line width of the various CH stretch modes is relatively insensitive to local environment.[38,41] If we make the same assumptions about the CH response in lysozyme and fit our series of 73 measurements with constant linewidths, we can straightforwardly correlate changes in spectral response with the three distinct periods in the surface tension.

IV. RESULTS AND DISCUSSION

A. Surface tension measurement

The surface tension of the air/water interface as a function of time after lysozyme injection is shown in Fig. 2 (black trace). Four distinct regimes are apparent from the data. From 0 to 10 min (T_1), the surface tension is stable at 72.8 mN/m, i.e., the surface tension of bulk water at room temperature (22 ± 1 °C).[42] From 10–~47 min (T_2), it decreases from 72.8 to 67 mN/m. After 47 min (T_3), the rate at which the surface tension decreases accelerates, and in the interval from 47– ~100 min, the surface tension decreases from 67 to 57 mN/m. After ~100 min (T_4), the rate of decrease in surface tension slows again, and the surface tension asymptotically approaches 50 mN/m (long-time data are not shown). Similar multistage surface tension behavior for this system has been observed previously.[9,43–45] The molecular level interpretation of these distinct regimes, particularly the induction period from 0 to 10 min, has varied widely. For example, van der Vegt and coauthors suggested that the induction period reflects transport to the

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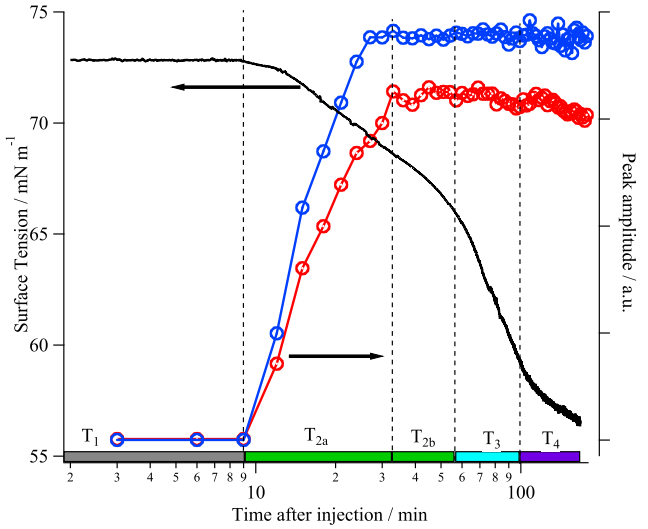


FIG. 2. Black solid trace (left axis): Surface tension of the air/water interface as a function of lysozyme injection time. Circular markers with solid lines (right axis): VSFG peak fitted amplitude (CH_x_s, blue markers) and (CH_x_as, red markers) in Fig. 3 as a function of injection time. Arrows indicate the respective y-axes for the surface-tension trace and the fitted VSFG amplitudes. Colored bars describe the steps for the change in surface tension and VSFG peak intensity of lysozyme at the air/water interface. The resulting final lysozyme concentration was 1.0 mg ml^{-1} (~70 μM).

interface during this time.[46] However, by combining surface tension measurements (Wilhelmy plate method) and fluorescence microscopy, Sundaram *et al.* have demonstrated that changes in surface tension lag changes in surface mass.[43] Because fluorescence microscopy allows direct imaging of the interface, the authors were also able to conclude that the end of the induction period was the result of a transition from a two dimensional gas to a liquid expanded phase for the interfacial molecules.[43] Other authors, using radiolabeling methods to measure the interfacial mass in combination with surface tension measurement, have reached similar conclusions.[8,9] A simple estimate (see the supplementary material) suggests that in our experimental geometry lysozyme should reach the interface, via diffusion, in less than 1 min after its subsurface injection. Because no change in surface tension is apparent until ten minutes after injection (i.e., the T_1/T_2 boundary), we expect that for our system the end of the induction period reflects the initiation of structural change in protein molecules already present at the interface. This expectation is consistent with our VSFG results (discussed below).

For the discussion below, we subdivide T_2 into T_{2a} and T_{2b}. During T_{2a} (~10–30 min), the CH-stretch amplitudes rise strongly as the surface tension decreases; during T_{2b} (~30–47 min), the CH amplitudes remain approximately constant while the surface tension continues to decrease. Following the induction period, the next major surface tension regime change appears at ~47 min after injection and 67 mN/m (i.e., the T_{2b}/T_3 boundary). This point of inflection in the rate of surface tension change has been observed previously and interpreted as the result of crowding effects (where the adsorbing protein molecules are prevented from unfolding by the pressure of the molecules already in the film) inhibiting denaturation as the surface approaches lysozyme saturation[8,47] or the result of multilayer or aggregate formation.[44] Given the complexity of lysozyme itself and the resulting complexity of denaturation and aggregation processes, it is perhaps not surprising that attempts to quantitatively model surface tension changes following adsorption using simple analytical models have substantiated these contradictory physical pictures.[43–45] Clearly, molecular level insight into lysozyme structure during adsorption (following subphase injection) is required to constrain such modeling efforts.

B. Kinetic VSFG measurements in the CH-stretch region

To gain structural insight into interfacial lysozyme as a function of time after subphase injection, we monitored the VSFG spectral response in the CH stretch window, between IR frequencies of 2750 and 3050 cm^{-1}. As shown in Fig. 3, at the beginning of the injection, the interfacial spectral response (trace 1) is featureless. Ten minutes after injection, resonances centered at 2867 and 2935 cm^{-1} appear. Traces 2 through 4 of Fig. 3 indicate a series of changes take place in the time from 10 to 30 min after lysozyme injection: the 2867 and 2935 cm^{-1} peaks increase in intensity and undergo a red shift while an additional resonance appears at 2906 cm^{-1}. At later points (i.e., more than 30 min after injection), the changes in the spectral response slow dramatically.

Following prior study, we assign the apparent peak at 2867 cm^{-1} to the mixture of CH_3 and/or CH_2 symmetric stretch, at 2906 cm^{-1} to the Fermi resonance of CH_3 and/or CH_2 vibration mode (a coupled vibration of the symmetric stretch and bend overtone), and at 2935 cm^{-1} to the asymmetric stretch modes of CH_3 and/or CH_2.[32,33] We attribute the broad, low amplitude response

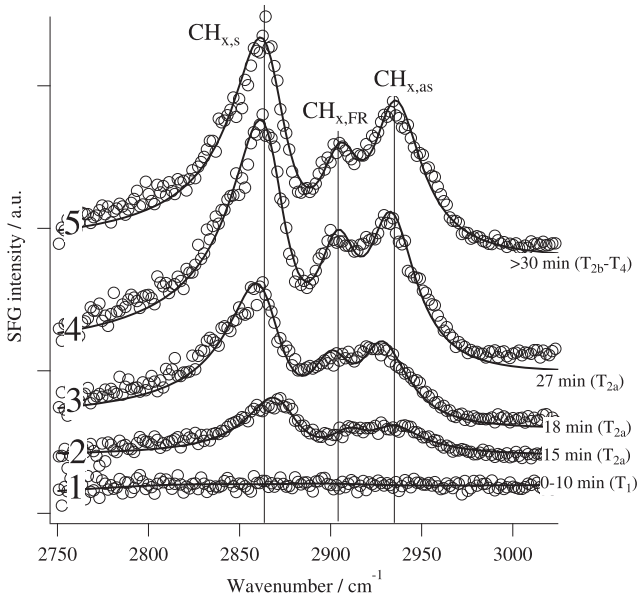


FIG. 3. VSFG spectra of lysozyme at different times after injection in ssp (SFG, VIS, and IR) polarization combinations. Open circular markers are experimental data; solid traces are fits according to Eq. (1). The three main resonances are indicated. The resulting final lysozyme concentration was 1.0 mg ml^{-1} (~70 μM).

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from 0 to 10 min after lysozyme injection to either the non-resonant response or the low frequency tail of the OH stretch response of H_2O (centered near 3200 cm^{-1} and outside of our spectral window). Both the assignments of these peaks and the qualitative spectral response follow those reported previously.[32,33]

To quantitatively monitor the change of each peak as a function of time, we have fitted all the spectra according to Eq. (1), as described in Sec. III. In T_1 (from 0 to 10 min in our experimental setup), neither a change in surface tension nor a CH response from interfacial lysozyme is detected. As mentioned earlier, prior radiolabel and fluorescence imaging studies have found lysozyme at the air/water interface during this period. The combined literature and present data are consistent with lysozyme being present at or near the interface during T_1 while retaining a largely native and/or orientationally disordered configuration. Such a population can yield little net CH-VSFG because oppositely oriented or isotropically distributed CH oscillators cancel in the macroscopic $\chi^{(2)}$. Our data therefore establish the absence of detectable net CH ordering during T1, not the absence of lysozyme from the interfacial region. After T_1, the interaction with the hydrophobic surface drives the partial denaturation of the protein. This denaturation results in an increased orientation of hydrophobic CH groups with respect to the water surface, presumably pointing out toward the vapor phase, and promotes the direct contact of lysozyme with the surface where it reduces the surface tension. Going from randomly oriented to oriented CH groups of the protein is expected to lead to increases in the CH_x response in the respective VSFG spectra, as we find in period T_{2a} (see Fig. 2). With increasing lysozyme denaturation, the density of these hydrophobic groups pointing toward the vapor phase increases. Prior studies of similar systems, e.g., monitoring of the interfacial CH stretch spectral response as a function of time during attachment of an alkane thiol self-assembled monolayer on gold, have found that an increasing chain density can lead to a shift of the CH stretch mode to lower frequencies, presumably because of favorable CH/CH interaction.[38] Our data (Fig. 3) show similar effects.

As lysozyme surface density increases, steric constraints on denaturation are expected to occur: at some point, additional lysozymes cannot be accommodated. Such a surface saturation would be expected to appear in the CH spectral response as a saturation in amplitude of both the symmetric and asymmetric modes. If, at this point, lysozymes already present at the interface have not reached their equilibrium conformation, we expect continued structural reorganization. If this continued structural reorganization involves any hydrophobic moieties in lysozyme, it would be apparent in the CH spectral response as a changing relative intensity of the symmetric and asymmetric modes. The inspection of the data (see Fig. 2) clarifies that after T_{2a}, both the absolute and relative intensities of the CH_x symmetric and asymmetric stretch modes remain constant while surface tension continues to decrease. These observations are thus consistent with a scenario in which during T_{2b} interfacial lysozyme molecules continue to structurally evolve in such a manner so as to optimize favorable lysozyme/lysozyme interaction but leave hydrophobic portions of the protein unperturbed. After the T_{2b} period, there are no distinctive features in the interfacial CH spectral response; however, a clear change in the slope of surface tension as a function of time after injection occurs at ~47 min (i.e., the T_{2b}/T_3 boundary). In earlier publications, this change in slope has been observed as well and attributed to the beginning of multilayer formation (i.e., a second lysozyme layer adsorbing to the first, denatured layer) in T_3.[48] Our CH observations, i.e., no change in both amplitude and center frequency, are consistent with this scenario provided that lysozyme in any subsequent adsorbed layers beyond the first has a conformation, presumably near its native state, in which all internal CH stretch modes cancel. The sequence of events suggested by our surface tension and VSFG data is consistent with prior x-ray diffraction data, suggesting lysozyme secondary structure changes in T_2, as well as enzymatic assays, that have found that any lysozyme present in multilayers retains a sufficiently native state structure to preserve its biological activity.[7,48] Longer term surface tension changes, the T_4 regime, are consistent with an insensitivity of measured surface tension beyond a critical number of multilayers. It should be noted that VSFG does not have a fixed sensitivity corresponding to a defined number of molecular layers. Additional layers can contribute if they retain net noncentrosymmetric orientational order, whereas layers that are approximately isotropic or whose coherent responses cancel may contribute only weakly or remain undetected. The absence of a further increase in CH amplitude after T_{2b} therefore constrains the net CH ordering of subsequently adsorbed material but does not by itself demonstrate that those layers are absent or fully native.

Our simultaneous measurements of surface tension and the CH stretch spectral response reveal qualitative changes in the mechanisms of lysozyme interfacial reorganization not accessible through surface tension measurement alone. Prior attempts to analytically model surface tension change at the air/water interface following subsurface lysozyme injection generally include either mass transfer (via diffusion) and hard sphere[49] rotation or structural reorganization.[43–45] This study clearly demonstrates that both factors need to be considered in any such modeling efforts. To go beyond this insight—to quantify the relationship between small changes in CH_x spectral response and structural changes—is extremely challenging. Such an effort would require the explicit description of the spectral response of all CH groups in the protein, their relative orientation, and how their relative orientation changes in time, and it is beyond the scope of this study. Because of its sensitivity to the local environment, gaining insight into protein structure from the amide I spectral response is generally substantially more straightforward than for the CH.[20] To gain such insight, then, we next investigate the VSFG amide I response of lysozyme.

C. VSFG measurements in the amide I region

Characterizing the amide I spectral response of interfacial lysozyme is considerably more challenging than the CH response.[20,50,51] On the one hand, in our setup, the generation of infrared light near 1600 cm^{-1} is substantially less efficient than that at 3000 cm^{-1}, resulting in a substantially worse signal-to-noise ratio in the amide I region than for the CH stretch. As longer integration times are required, we are thus unable to perform the same sort of time resolved measurements as for the CH spectral region. On the other hand, the amide I response is more complex than the response of the CH-stretching vibrations; a broad peak spectral response measured between 1620 and 1690 cm^{-1} for lysozyme

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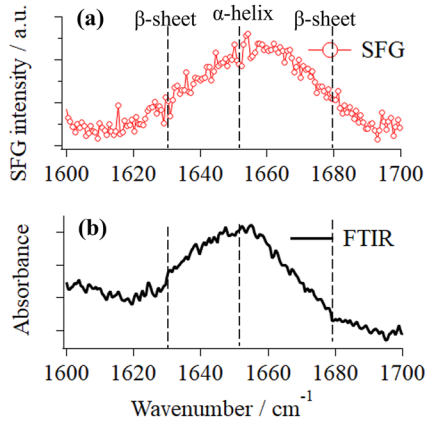


FIG. 4. (a) VSFG spectrum of lysozyme at the air/water interface in the equilibrium state in ssp (SFG, VIS, and IR) polarization combinations. (b) FTIR spectrum of lysozyme in bulk solution under the same experimental conditions as the VSFG measurement.

contains both alpha-helix and beta sheet structures.[20,25,28,50] Clearly, our observations, shown in Fig. 4, cannot resolve this sort of complexity, so we thus restrict ourselves to describing qualitative trends in the data.

We compare in Fig. 4 the amide I VSFG spectrum from the T_{2b} regime to the FT-IR spectrum of lysozyme measured in bulk solution. There was no difference between the amide I VSFG response for this system measured at 30 min and 12 h after lysozyme injection (data not shown). Because the frequency of the bending mode of H_2O overlaps strongly with that of the amide I mode, for both these measurements H_2O was replaced with D_2O. The VSFG response shows a broad peak centered at 1655 cm^{-1} with two shoulders at 1630 and 1680 cm^{-1}. While the overall spectral shape does not differ dramatically from that of the FTIR spectrum, the peak maximum of the latter is centered at 1650 cm^{-1} and exhibits a much weaker high-frequency shoulder. The amide I response is known to consist principally of the C=O stretch in the peptide bond but to change frequencies as a function of secondary structure.[20] Following prior study, we assign the 1650 cm^{-1} response to α-helices and the two shoulders at 1631 and 1680 cm^{-1} to β-sheet structures.[6,52] With this assignment, and consistent with prior study, our measurement suggests that in bulk solution, the amide I spectral response is dominated by α-helices,[6,52] while at the surface both α-helices and β-sheets contribute. However, because the VSFG response is coherent and strongly dependent on molecular orientation, these differences should not be overinterpreted in terms of changes in secondary-structure populations. Rather, they suggest that the different secondary-structure elements of lysozyme contribute with different orientational weights at the interface.[24,27]

V. SUMMARY AND CONCLUSIONS

In summary, we have studied the kinetics and structure of lysozyme adsorbed at the air/water interface using *in situ* VSFG spectroscopy and surface tension measurements. We can directly relate changes in surface tension to changes in molecular conformation: the decrease in surface tension is directly due to the adsorption-induced unfolding of lysozyme at the interface. By monitoring the interfacial CH spectral response, we are able to distinguish a step in the denaturation not apparent in the surface tension measurements alone, a period characterized by lysozyme hydrophobic moiety orientation being relatively constant but lysozyme area per molecule continuing to increase, presumably as a result of favorable lysozyme/lysozyme interaction. Prior studies have disagreed on the structural significance of the change in surface tension slope as detected at 47 min after lysozyme injection (i.e., the T_{2b}/T_3 transition). Some authors have attributed this change to the beginning of a crowded denaturation regime, others to the formation of lysozyme multilayers.[7,48] Our spectroscopic results are consistent with the latter explanation—crowded denaturation occurs during T_{2b}; this change in surface tension as a function of time after injection is due to multilayer formation. Finally, direct measurements of the interfacial lysozyme amide I response demonstrate that there are more β-sheet vibrational features at the interface than in the bulk solution.

Taken together, these observations shed significant light on this prototypical interfacial protein system as well as point the way toward significant possible methodological improvements. Clearly, there is yet more information to be gained by similar measurements that have both increased signal-to-noise and yielded additional spectroscopic observables (as might be expected from phase-resolved heterodyne-detected VSFG, simulation-supported spectral analysis, and two-dimensional VSFG spectroscopy). Additional measurements on the effects of pH values and ionic strength, as well as the chemicals, on the denaturation of lysozyme at the interface are desired.

Between individual amino acids and structurally complex proteins, short peptides offer particularly useful model systems for future studies. Their reduced structural complexity permits more direct relationships between sequence, interfacial folding, side-chain orientation, and SFG spectral response, while biologically active peptides demonstrate that such systems can nevertheless retain functional relevance.[24,27]

Brewster-angle microscopy (BAM) could provide complementary information on the lateral organization of the adsorbed protein layer. For lysozyme at the air/water interface, Nath *et al.*[53] observed relatively homogeneous films at low surface pressure and stripe-like structures upon compression, demonstrating the sensitivity of BAM to mesoscale morphological changes accompanying interfacial reorganization. Combining BAM with VSFG would therefore be particularly valuable, as BAM probes lateral morphology whereas VSFG reports molecular-level ordering. Such a combination is possible in principle, although implementation in the present rotating-trough geometry would require modification of the setup.

SUPPLEMENTARY MATERIAL

The supplementary material provides additional details on the SFG spectral fitting procedure, including the parameter ambiguity in the CH-stretch region and the constraints used in the analysis. It also presents simulations of the orientation-dependent CH_3 and CH_2

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stretch intensities, as well as an estimate of the diffusion-controlled adsorption lag time for lysozyme at the air/water interface.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Yujin Tong: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Methodology (lead); Project administration (equal); Resources (equal); Validation (lead); Writing – original draft (lead); Writing – review & editing (lead). **R. Kramer Campen**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Methodology (equal); Project administration (equal); Resources (equal); Validation (equal); Writing – original draft (equal); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available within the article and its supplementary material.

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