Evidence that the Induction Time in the Surface Pressure Evolution of Lysozyme Solutions Is Caused by a Surface Phase Transition

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Received September 7, 1999. In Final Form: February 25, 2000

Induction periods have been reported in the surface pressure evolution of a wide variety of proteins. In this work, this induction period is shown to be caused by a first-order phase change from a surface gaseous to a liquid-expanded state as the protein lysozyme adsorbs and decreases the mean area per molecule. The evolution of this transition is studied using concomitant fluorescence microscopy and surface pressure measurements. The fluorescent images are obtained by using spread films of the dye NBD-HDA at the air−liquid interface. This dye fluoresces when in contact with hydrophobic moieties and is quenched when in contact with water. The hydrophobic liquid-expanded domains therefore appear bright on a dark background. Lysozyme initially adsorbs to establish a surface gaseous phase, and the interface appears dark. The surface tension is fairly insensitive to changes in area per molecule in this state. Once the adsorbed concentration of lysozyme reaches the surface gaseous binodal, protein interactions drive a first-order phase change. Domains of liquid expanded phase grow at the interface at the expense of the surface gaseous phase. During this phase change, the surface tension remains constant. Only after the interface is covered in the bright, liquid-expanded phase does the surface tension decrease from the clean interface value. These results are discussed in terms of the surface coverage and orientation of lysozyme.

1. Introduction

The dynamic surface tension of a wide variety of protein solutions remains elevated near the clean interface value for a prolonged period of time before it reduces. This has been observed for solutions of globular proteins,[1] for example, lysozyme,[2−7] bovine serum albumin,[6,8−10] human serum albumin,[11] equine myoglobin,[2] cytochrome *C*,[2,7,12] bovine erythrocyte superoxide dismutase,[2] bovine ribonuclease A,[2,6] α-lactalbumin,[6,7] conalbumin,[7] catalase,[7] ovalbumin,[7] pepsin,[7] and trypsin;[7] for solutions of more complex tertiary-structured proteins including immunoglobulin G,[6] xanthine oxidase,[13] and fibronectin;[14] and for the less compact and organized protein *β*-casein.[2,15]

Several interpretations for the induction time have been suggested in the protein literature. For lysozyme, Xu and Damadoran[5] proposed that there is a negative surface excess of protein during the induction time and that the protein adsorbs and the surface pressure increases only after conformational rearrangements have occurred. This is inferred from the elevated surface tensions above that of clean water and the negative surface concentrations that they find from their radio-labeling studies.

Most explanations assume that the adsorbed concentration of the protein is not zero during this time period; indeed, in a number of radio-labeling studies, nonzero surface concentrations have been reported during the induction period for lysozyme (Graham and Phillips[4]) and BSA (Cho et al.[10]), and, more recently, for a variety of proteins, by Sengupta et al.[7]

In a study of the surface pressure evolution of BSA solutions, MacRitchie[9] proposed that the induction period represents a diffusion-controlled adsorption time that ends when the interface is saturated with protein so that the adsorbed protein monolayer creates an energy barrier for further adsorption. Only after this saturation coverage is reached does the surface pressure increase. MacRitchie estimated the saturation concentration for BSA as 7×10^{-8} g/cm^2 on the basis of the area per molecule at lift-off of the surface pressure (defined as $\pi = 0.1$ mN/m) observed in a spread monolayer of BSA. He also showed that the induction time for BSA decreased with the inverse square of the bulk concentration in a manner consistent with the diffusion-controlled adsorption except at the most dilute concentrations, which was faster than could be explained by diffusion and thought to be influenced by natural convection. Sengupta et al.[7] reported induction time and adsorption data that support MacRitchie's hypothesis.

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10.1021/la991179y CCC: $19.00
Published on Web 04/21/2000

Van der Vegt et al.[6] proposed that the proteins do not change the surface pressure until they have changed their conformations; Graham and Phillips[4] suggested a similar mechanism. Ybert and di Meglio[8] hypothesized that throughout the induction time, the protein is adsorbed in a surface gaseous state, and hence the surface pressure changes only slightly with the surface coverage.

Induction periods have also been observed in the dynamic surface tension of several soluble surfactants adsorbing from solution to aqueous−gas interfaces, including *n*-decanol,[16] 7-tetradecyn-6,9 diol,[17] and the *n*-alkyl ether $C_{14}E_1$.[18] In Pollard et al.,[18] the induction period was clearly shown to be caused by a first-order phase change from a surface gaseous (G) phase to a surface liquid-expanded (LE) phase occurring at the interface by concomitant visualization of the evolving domains on the interface and measurement of the surface tension.

Brewster angle microscopy (BAM)[19,20] or fluorescence microscopy[21−23] can be used to obtain images of the growing LE phase. In BAM, a beam of polarized light at the Brewster angle for clean water impinges on the aqueous−gas interface; since no p-polarized light is reflected at this angle, a clean interface will appear dark. Adsorbed species alter the index of refraction so that light incident on the adsorbed layers produces a reflection whose intensity depends on the adsorbed concentration. In fluorescence microscopy, the interface is first doped with a small amount of insoluble dye that fluoresces when in contact with hydrophobic moieties but is quenched when in contact with the aqueous phase. In both techniques, as an amphiphile undergoes a G−LE transition, bright domains of the hydrophobic, densely packed LE phase appear in a dark background of G phase. Both of these techniques have been used extensively to image surfactant monolayers (as reviewed in Knobler[24] and Pollard et al.[18]) and have recently been applied to study the surface phase behavior of proteins.[3,25−28]

Henon and Meunier[29] used BAM to study the evolution of LE domains forming as the soluble surfactant sodium octanoate adsorbs at the liquid−gas interface, establishing that a soluble amphiphile can adsorb and undergo a surface phase change. In Pollard et al.,[18] fluorescence microscopy was used to image the interface of $C_{14}E_1$ (CH_3-$(CH_2)_{13}$−O−CH_2CH_2−OH) solutions, and a Wilhelmy plate was used simultaneously to record the surface pressure evolution in a series of experiments that established that the induction period is a result of a first-order phase change occurring as surfactant adsorbs at the interface. When surfactant adsorbs at an initially clean interface, it forms a G phase, and the interface appears dark. As surfactant adsorbs, the area per molecule decreases, and the surface tension decreases slightly. This continues until the gas-phase binodal area per molecule is attained. Thereafter, as more surfactant adsorbs, islands of LE phase (at the liquid-expanded binodal area per molecule) nucleate and grow. For a first-order phase transition, this occurs at constant surface pressure. Once the islands of LE phase grow to cover the entire interface, further adsorption causes a steep rise in the surface pressure (or decrease in the surface tension). The induction period in the dynamic surface tension of $C_{14}E_1$ ends only when the interface is completely covered in the liquid-expanded phase.

On the basis of the work performed on surfactant systems, this mechanism was proposed as the explanation for the induction time that occurs as proteins adsorb at aqueous−gas interfaces by Sundaram et al.[3] In that paper, the surface pressure evolution of lysozyme solutions was studied by both pendant bubble and Wilhelmy plate techniques. The induction time was shown to vary with the inverse square of the bulk concentration at low concentrations, consistent with diffusion-controlled adsorption, and with the inverse bulk concentration at higher concentrations, as is consistent with a kinetically controlled adsorption process. While fluorescent images of coexisting domains at the aqueous−air interface were shown in that work, the relationship between the surface pressure evolution and the evolution of the LE domains at the interface was not established, nor has this relationship been established by others to our knowledge. That is the purpose of this paper.

2. Materials and Methods

Sodium chloride, sodium dihydrogen phosphate and sodium hydrogen phosphate were obtained from Sigma Chemical Company and were of ACS reagent-grade or better. Six-times-recrystallized lysozyme was obtained from Seikagaku Corporation (Falmouth, MA). All reagents were used without further purification. Purified water was obtained from a Millipore Milli-Q 50 Purification System (Bedford, MA) and had a resistivity of not less than 18.0 MΩ cm. This water was used for all cleaning procedures and to make all solutions. For fluorescence microscopy, the interface was doped with 4-hexadecylamino-7-nitrobenz-2-oxa-1,3-diazole (NBD-HDA) at an area per molecule of not less than 2000 Å^2. The dye was purchased from Molecular Probes (Eugene, OR). ACS reagent-grade chloroform was purchased from Aldrich and used both for cleaning and as a spreading solvent for the dye.

A Langmuir trough with dipping well (KSV Instruments) was cleaned by wiping with ethanol, *n*-hexane, and chloroform, in series. It was rinsed copiously, soaked for 12 h, and subsequently rinsed again with water. This cleaning procedure was repeated twice before use in order to eliminate adsorbed protein. A 100 μL syringe (Hamilton) was cleaned thoroughly with chloroform several times. It was then disassembled and cleaned in a water-filled sonication bath for not less than 10 min. A platinum Wilhelmy plate (KSV Instruments) was cleaned by placing it in a small beaker of water and then sonicating for not less than 10 min. The plate was subsequently flame-cleaned immediately prior to use. All glassware was cleaned by soaking in a bath consisting of concentrated sulfuric acid and Nochromix cleaning solution for at least 24 h, followed by rinsing in water.

Fluorescence was excited with a 40 mW argon-ion laser from Ion Laser Technology. The beam was passed through a dichroic sheet polarizer (Melles Griot) before incidence on the interface. Images were obtained using a Karl Zeiss epifluorescence microscope equipped with a 10× objective. A model XC-77 Hamamatsu CCD camera mounted on the microscope was used to visualize the interface. Digitized images were captured with an IBM PC equipped with a PIXCI-SV imaging board and analyzed using XCAP software from Epix. Surface pressure data, obtained using a force transducer (KSV Instruments), were reproducible to within ±0.5 mN/m. Despite the efforts made to reduce evaporation over the time course of our experiments, there was some evaporation from the meniscus on the Wilhelmy plate, which appears as a slight decrease in the surface pressure

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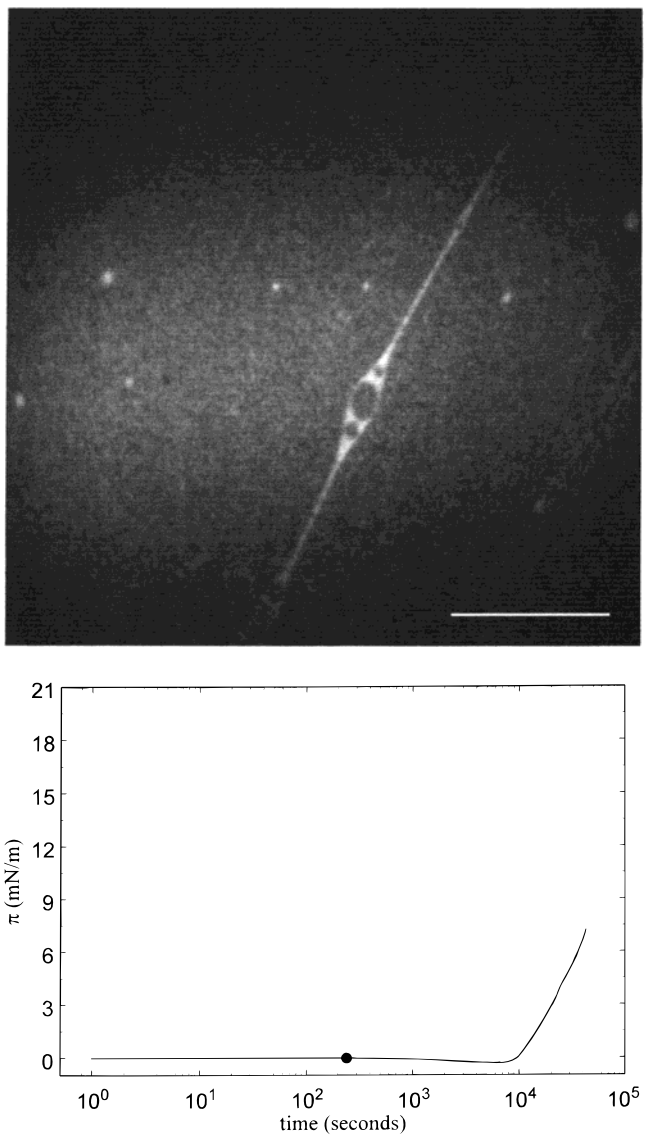

Figure 1. Fluorescence microscopy image 5 min after lysozyme injection. The scale is indicated by the 500 μm bar in the image; all images have the same scale. The surface pressure at the time that the image was acquired is indicated by the bold circle.

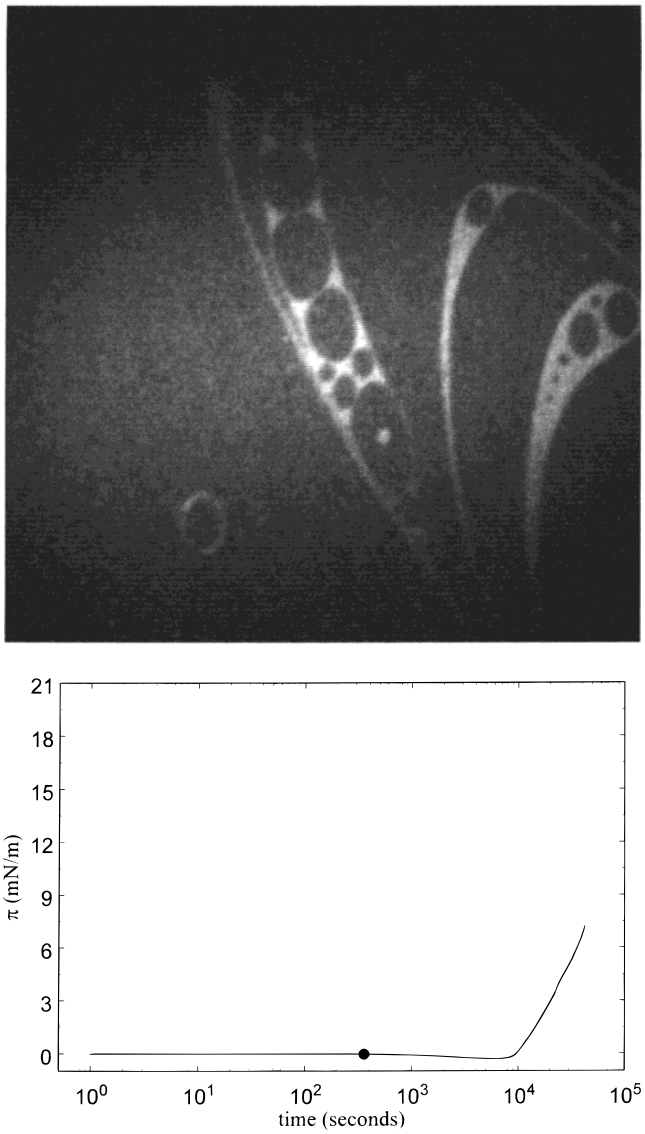

Figure 2. 7 min after lysozyme injection. The surface pressure at the time that the image was acquired is indicated by the bold circle.

evolution of roughly 0.3 mN/m. When the same protein solutions are studied in a tightly sealed environmental chamber, this effect is not observed.

In a typical experiment, a fresh aqueous−air interface was created with pH 7.4 phosphate-buffered saline of ionic strength 0.17, accounting for both the NaCl and the buffer salts. This interface was doped with a small amount of dye and examined for impurities. If any contaminants were evident, they were removed by aspirating the interface and repeating the application of dye. Lysozyme was introduced into the subphase by injection with a Hamilton syringe; 200 μL of 1.0 wt % lysozyme solution was injected into the subphase of the dipping trough, of volume of roughly 300 mL. (The variability in the subphase volume depends on the height of the meniscus that forms above the trough.) The final bulk concentration is therefore approximately 7×10^{-4} wt %. No corrections for loss by adsorption to the sides of our Teflon trough were made, so this concentration should be considered as an upper bound. The experiment was repeated several times ($N > 10$).

3. Results

Typical results are reported sequentially in figures 1−6. The scale, which is the same for each image, is indicated by the bar, which indicates a 500 μm length in Figure 1. In each figure, a fluorescent image at a specified time after protein injection is reported; the surface pressure at the time that the image is acquired is indicated by a bold circle on the graph portion of the figure. Before protein is introduced, the interface is free of fluorescing domains.

The growth of the LE phase in the dark surface gaseous monolayer is apparent as time passes and adsorption proceeds; the surface pressure remains fixed throughout this process. In Figure 1, 5 min after the protein is introduced, the LE phase is visible as bright spots (i.e., 2-D liquid drops) and striations in the dark G phase. In Figure 2, 7 min after lysozyme injection, the two-dimensional LE "drops" merge to form striations in the dark G phase. In Figure 3, after 31 min have elapsed, the dark G phase domains, which appear as two-dimensional "bubbles", coalesce with LE phase entrapped between them. In Figure 4, 42 min after lysozyme injection, bright LE striations are apparent, which merge together with time. In Figure 5, the surface pressure remains constant 127 min after lysozyme injection; the interface is covered with a bright LE monolayer with defects (none of which were captured in this image.) Finally, in Figure 6, after 168 min have elapsed, the interface is covered with a bright LE monolayer. The surface pressure begins increasing as

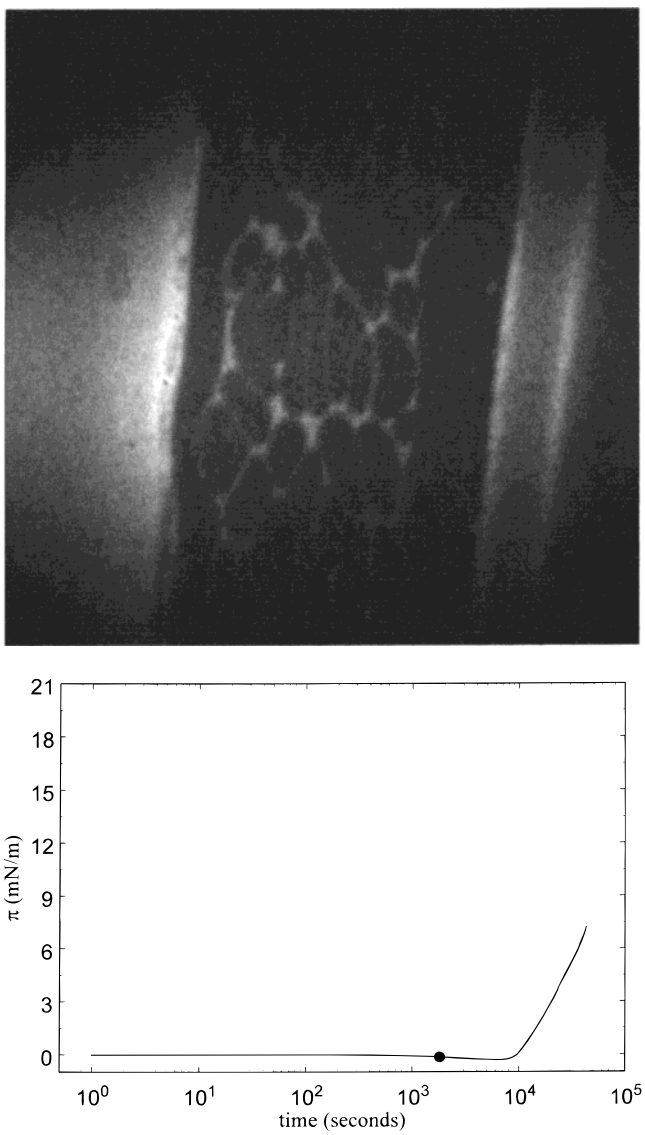

Figure 3. 31 min after lysozyme injection. The surface pressure at the time that the image was acquired is indicated by the bold circle.

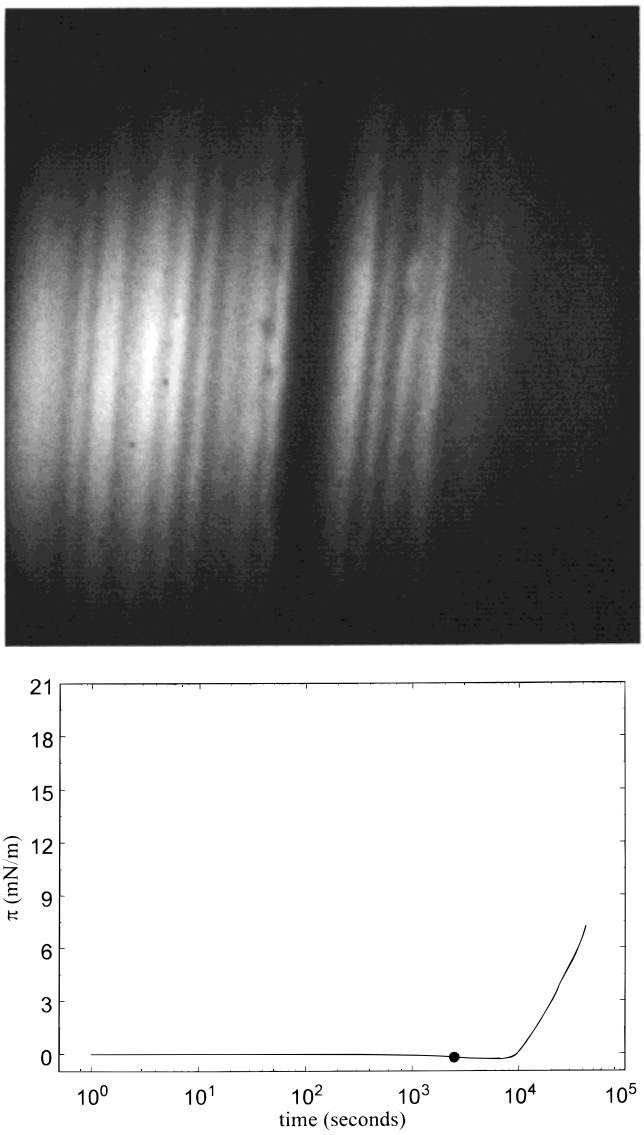

Figure 4. 42 min after lysozyme injection. The surface pressure at the time that the image was acquired is indicated by the bold circle.

adsorption occurs in the LE state. As further adsorption proceeds, the interface remains bright as is shown in Figure 6, and the surface pressure rises. The stages of the growth of the domains at the interface are reproducible, although the exact time at which they occur varies slightly, particularly for the earliest features formed, because of the convection setup when injecting the protein.

The time-evolving fluorescence images and the surface pressure data correspond closely to those reported for simple surfactants that undergo G−LE transitions as they adsorb at liquid−gas interfaces. These data corroborate our hypothesis that the induction period apparent in surface tension evolution of proteins solutions can be explained as a first-order phase transition occurring in the interface.

4. Discussion

Proteins adsorb at interfaces in order to remove their exposed hydrophobic moieties from solution. It is widely accepted that they also change their conformation upon adsorption and that adsorption is irreversible. Because of this, no true equilibrium state is established either with the bulk or at the interface as the unfolding proceeds; the dynamic surface tension thus continues to decrease over prolonged periods of time for protein solutions. In this manner, protein adsorption differs strongly from the adsorption of simple surfactants comprised of hydrocarbon tails and polar headgroups, which can desorb and eventually establish equilibrium with the bulk solution.

The argument that the induction period in the surface tension indicates a first-order phase change in an adsorbed protein monolayer does not rely on long-time equilibrium within the interface or with the bulk solution. Rather, it requires that there be a rapid adjustment of the surface pressure to the instantaneous surface concentration of adsorbed protein and that the protein self-assemble into a disordered adsorbed liquid state.

The formation of the surface LE state may be driven by attractive interactions between the adsorbed proteins. While the interactions in the interface have not been characterized to our knowledge, the second virial coefficients reported for lysozyme in bulk solution at this pH and ionic strength are attractive.[30,31] For lysozyme, adsorption data, surface pressure isotherms, and data from neutron and X-ray reflectivity experiments indicate that lysozyme does not unfold rapidly to become an uncoiled polymeric structure on adsorption. Rather, these data

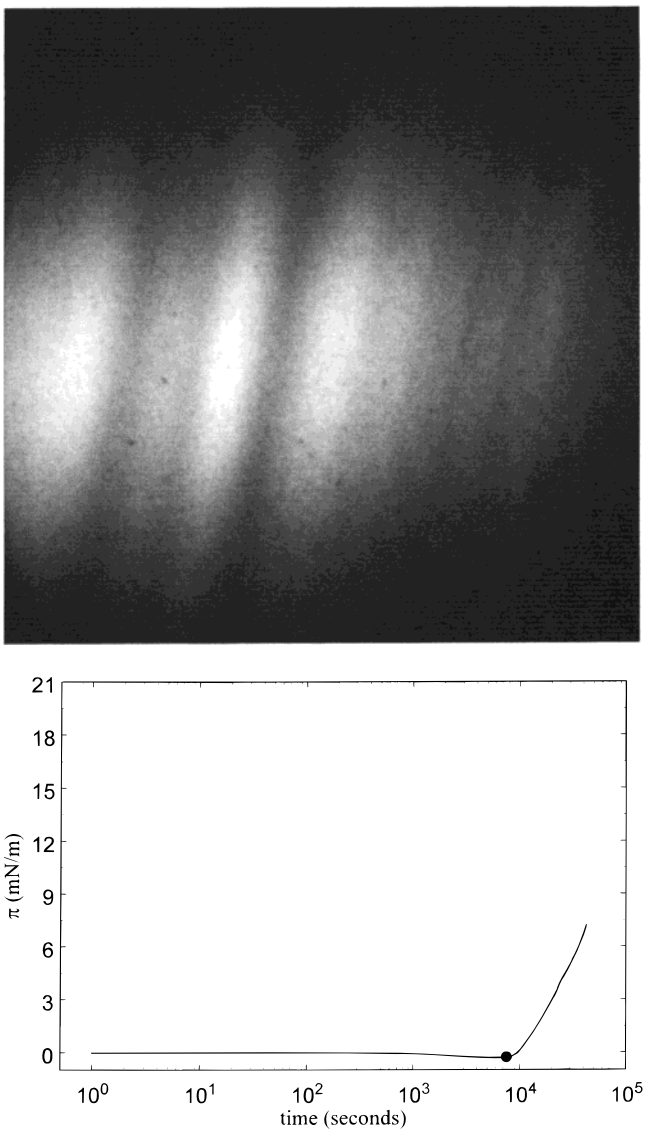

Figure 5. 127 min after lysozyme injection. The surface pressure at the time that the image was acquired is indicated by the bold circle.

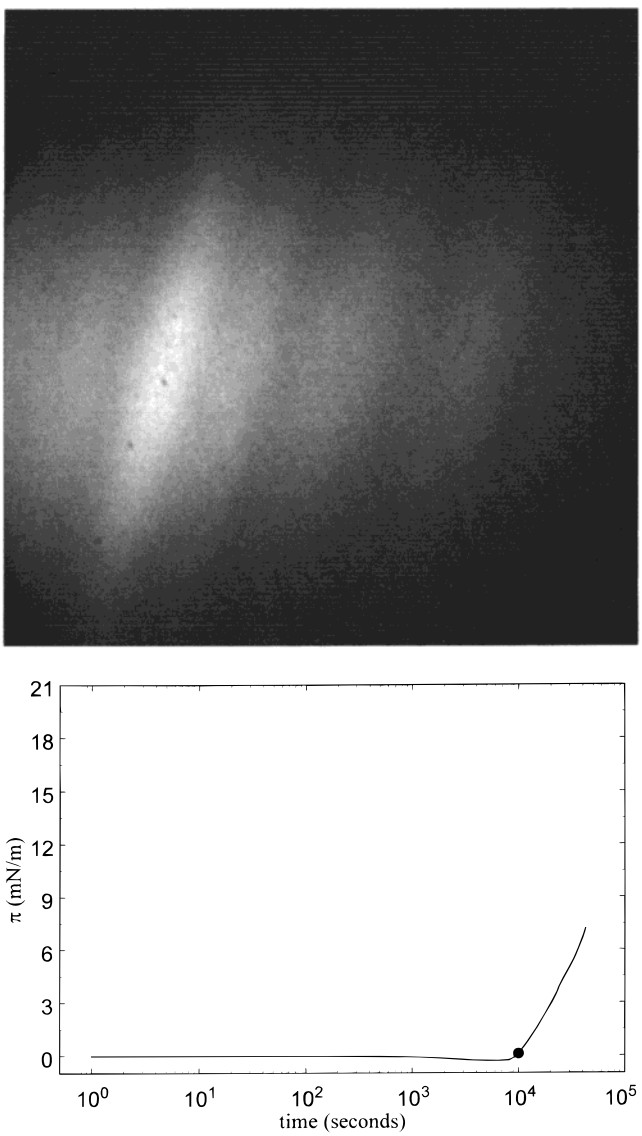

Figure 6. 168 min after lysozyme injection. The surface pressure at the time that the image was acquired is indicated by the bold circle. At later times, the interface appears the same as the surface pressure rises.

suggest that lysozyme adsorbs to form layer thicknesses consistent with its crystallographic dimensions and that it organizes to form a surface gaseous, liquid-expanded, and possibly a liquid-condensed state.

Despite the lack of a true equilibrium state, there have been many studies of protein adsorption in which "isotherms" have been reported for protein surface concentrations Γ and surface pressures π as a function of bulk concentration C. These data rely on the observation that Γ (obtained by radio-labeling techniques) approaches a plateau at long times (usually truncated at 24 h unless otherwise noted). A few of the lysozyme studies are discussed below.

Graham and Phillips[32] report that the surface concentration of 1-C^{14} acetyl lysozyme rises monotonically with the bulk concentration at $I = 0.1$ M and pH = 7. Hunter and collaborators[33] performed adsorption experiments with reductively methylated lysozyme under similar conditions and made an effort to correct C for bulk depletion effects caused by adsorption to the sides of the cuvettes. Noting that lysozyme is a hard, ellipsoidal protein with crystallographic dimensions[34] of 30 × 30 × 45 Å, they identified three regions in the $\Gamma - C$ curve. At low concentrations (less than roughly 10^{-5} wt %), the protein is inferred to adsorb in a side-on orientation. A plateau between 2×10^{-6} and 10^{-5} wt % in the isotherm corresponds to a mean area per molecule of 1530 Å^2, which compares roughly to 1350 Å^2, the area occupied by lysozyme in the side-on state. At higher concentrations (between 10^{-5} and 10^{-3} wt %), the surface concentration increases monotonically, approaching 2.7 mg/m^2, roughly 900 Å^2, the area occupied by lysozyme in the end-on state. Finally, for higher concentrations, multilayers of lysozyme form at the interface.

Other authors also report a reorientation of lysozyme at the interface, although the bulk concentrations at which

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the reorientation occurs do not agree with Hunter's data. Recently, Lu et al.[35,36] studied lysozyme adsorption using neutron reflectivity, recording their data between 1 and 8 h after the interface is exposed to the lysozyme solution. At a pH of 7.0 and $I = 0.02$, the protein was reported to be adsorbed in a side-on state for C between 9×10^{-5} and 10^{-2} wt %, to adsorb in an end-on configuration at 0.1 wt %, and to form multilayers at 0.4 wt %. These authors used a one-layer model to find the protein layer thickness and found no evidence of extensive unfolding that would change the protein layer thickness. The trends reported by Lu et al. are corroborated by Postel[37] et al., who used X-ray reflectivity to measure the surface concentrations of lysozyme after 24 h periods at pH 6.9, $I = 0.1$ M, and found that the protein remains side-on and roughly constant for 7.6×10^{-4} to 10^{-2} wt %. Postel et al. used similar conditions as did Hunter et al. but did not correct their bulk concentration for depletion by adsorption to the interface or sides of their container. In conclusion, several studies have indicated that lysozyme adsorbs to form monolayers of either side-on or end-on orientation. However, there is a lack of agreement on the bulk concentration at which this reorientation occurs. This complicates the interpretation of the phase transition discussed in this paper in terms of the orientation of the adsorbed globular protein.

In Sundaram et al.,[3] it was suggested that the G−LE transition corresponded to the side-on to end-on reorientation at the interface. This was supported by a diffusion-controlled mass-transfer argument. The surface concentration that could adsorb by a diffusion-controlled mechanism during the induction time at dilute bulk concentrations corresponded with a surface coverage of 2.7 mg/m^2 (end-on adsorption) using a diffusivity D of 1.5×10^{-6} cm^2/s. However, a reduction in the diffusion coefficient by a factor of 2.4 would predict a surface coverage that is consistent with side-on adsorption at the end of the induction time. Because of the uncertainty in the diffusion coefficient for lysozyme near interfaces, and the uncertainty in the bulk concentrations at which the reorientations at the interface occur, this argument concerning the orientation of lysozyme at the end of the G−LE transition is not definitive.

Interesting trends that suggest that the induction period is related to a dilute side-on concentration and that there are other phase transitions in the adsorbed lysozyme emerge when the π vs $1/\Gamma$ data presented in Figure 4 of Graham and Phillips[38] is considered. The liftoff concentration at which the surface pressure departs appreciably from zero, 0.55 mg/m^2, corresponds to roughly $^1/_3$ of the side-on close-packed concentration; (this is typical of a liftoff surface concentration in lipid monolayers, which range, from roughly $^1/_3$ to $^1/_2$ of the close-packed surface concentration).[39] The surface pressure rises monotonically as the area/molecule is reduced until the close-packed side-on concentration is reached, 1.7 mg/m^2. For 1.7 mg/m^2 < Γ < 3.0 mg/m^2 (roughly corresponding to end-on adsorption), the surface pressure remains fixed at 8 mN/m. This suggests that there is a second phase change from a liquid-expanded to a liquid-condensed phase (or a liquid crystalline phase, referred to as LC) in the adsorbed monolayer of lysozyme that corresponds to the side-on to end-on transition. Further increases in Γ in the end-on packed monolayer cause the surface pressure to rise steeply. If the surface concentration at which the surface pressure lifts off is interpreted as the LE binodal concentration for the G−LE transition, the induction period for lysozyme would end when the surface concentration had approached this value. The surface tension would then decrease with time.

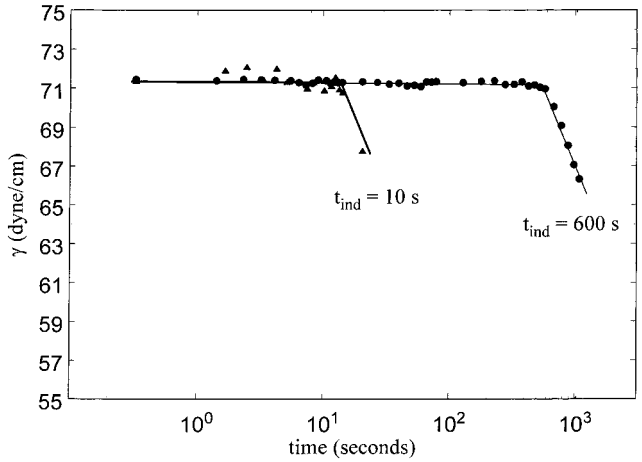

Figure 7. Surface tension evolution of lysozyme solutions at pH = 7.4 and $I = 0.17$ by the pendant bubble technique. The circles and triangles are 5.13×10^{-2} and 0.59 wt % lysozyme, respectively.

It has been suggested that the induction period is a characteristic time required for a protein to undergo structural rearrangements at the interface. However, the induction time can be varied by changing the bulk concentration of protein, an unlikely control parameter for an unfolding event. In pendant bubble experiments performed in our laboratory and reported in Figure 7, the induction period decreased strongly with the bulk concentration of lysozyme. At high concentrations, the interface rapidly becomes crowded with protein, reducing the space available for the protein to unfold, yet the surface tension drops at very short times. For example, for concentrations of 0.59 wt %, the induction time was only 10 s. While this evolution was too rapid to acquire images such as those presented in this paper, they do provide supporting evidence that the induction period is related to the time required for a given amount of protein to adsorb.

Heats of denaturation of 150 kJ/mol or greater have been reported for several of the globular proteins listed in the Introduction. (These heats of denaturation were reported for a variety of conditions for lysozyme,[40−42] ovalbumin,[43] ribonuclease A,[40,42] enquine myoglobin,[42] cytochrome C,[40,42] α-lactalbumin,[40,44] BSA,[45−47] HSA,[48] immunoglobulin G,[49] bovine superoxide dismutase,[50]

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trypsin inhibitor,[51] catalase I,[52] and various individual globular domains of fibronectin.[53,54]) In general, researchers who ascribe to MacRitchie's hypothesis associate the induction period with the time required to attain roughly 50% coverage of adsorbed, globular proteins. These proteins, like lysozyme, may adsorb without significant loss of their globular domains, have attractive interactions at the interface, and thus undergo surface phase transitions as they adsorb at the interface.

There is at least one protein, casein, which exhibits an induction time and is not highly structured in solution. However, it, too, may undergo a G−LE phase transition. Two studies have reported an induction time for this protein. Serrien et al.[15] report an induction period of 250 s at 0.066 g/L and pH 4.9. Tripp et al.[2] used a pendant drop technique and report an induction time of 1 min for β-casein at concentrations of 0.01 g/lat pH 7.4. (Graham and Phillips[4] also studied the adsorption of this protein but reported their first data point roughly 15 min after the interface is created, too late to capture the induction times reported in the other two studies.) A recent study of spread monolayers of β-casein[26] shows distinct regions in a $\pi-A$ graph with a liftoff area/molecule ranging from 0.6 to 0.35 m^2/mg at pH 4.5, which increases to roughly 0.75 m^2/mg at pH 7; whether this liftoff at fairly elevated surface concentrations indicates a G−LE phase transition remains an open issue. It is known that polymeric amphiphiles can undergo G−LE transitions.[55] The unfolded protein would occupy a larger area/molecule for a given adsorbed mass and therefore crowd the interface at far lower surface concentrations, and could therefore pass rapidly through the phase transition to create the short induction periods reported.

5. Conclusions

Induction periods in the surface pressure evolution are commonly observed at the aqueous−air interfaces of protein solutions. In this work, fluorescent images of the liquid-expanded (LE) phase formed by lysozyme at the air−liquid interface and concomitant surface pressure data are presented that show that the induction period in the surface tension of lysozyme solutions is caused by a first-order G−LE phase transition. As lysozyme adsorbs, it first forms the G phase. At the G phase binodal, the LE phase nucleates and grows with time until it covers the interface. During this time, the surface pressure remains constant. The surface pressure rises only after the entire interface is covered with the LE phase.

This interpretation of the induction period supports MacRitchie's hypothesis that the induction period ends when the interface reaches a given saturation indicated by the surface concentration at which the surface pressure lifts off. The phase change argument presented here implies that the liftoff surface concentration is equal to the LE binodal for the G−LE phase transition.

The relationships between this observation, the orientation of lysozyme, and lysozyme adsorption data from the literature are discussed.

Acknowledgment. The authors acknowledge the contributions of S. S. Datwani in obtaining the pendant bubble data; J.S.E. acknowledges the support of the Material Initiative Fellowship from the Whiting School of Engineering and the support of a NASA GSRP fellowship. The authors acknowledge helpful comments by a reviewer.

LA991179Y

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