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Strong Improvement of Interfacial Properties Can Result from Slight Structural Modifications of Proteins: The Case of Native and Dry-Heated Lysozyme

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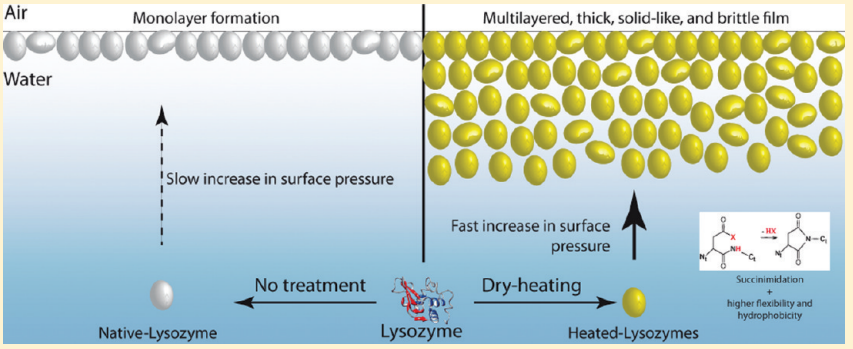
ABSTRACT:



Identification of the key physicochemical parameters of proteins that determine their interfacial properties is still incomplete and represents a real stake challenge, especially for food proteins. Many studies have thus consisted in comparing the interfacial behavior of different proteins, but it is difficult to draw clear conclusions when the molecules are completely different on several levels. Here the adsorption process of a model protein, the hen egg-white lysozyme, and the same protein that underwent a thermal treatment in the dry state, was characterized. The consequences of this treatment have been previously studied: net charge and hydrophobicity increase and lesser protein stability, but no secondary and tertiary structure modification (Desfougères, Y.; Jardin, J.; Lechevalier, V.; Pezennec, S.; Nau, F. *Biomacromolecules* **2011**, *12*, 156−166). The present study shows that these slight modifications dramatically increase the interfacial properties of the protein, since the adsorption to the air−water interface is much faster and more efficient (higher surface pressure). Moreover, a thick and strongly viscoelastic multilayer film is created, while native lysozyme adsorbs in a fragile monolayer film. Another striking result is that completely different behaviors were observed between two molecular species, i.e., native and native-like lysozyme, even though these species could not be distinguished by usual spectroscopic methods. This suggests that the air−water interface could be considered as a useful tool to reveal very subtle differences between protein molecules.

INTRODUCTION

Proteins are biological macromolecules that efficiently stabilize emulsions and foams.[1−3] This ability partly stems from the amphiphilic nature of these molecular species that enable them to adsorb at hydrophobic−hydrophilic interfaces, but it is also related to their propensity to assemble at the interface into a viscoelastic film, and other important parameters such as net charge, surface hydrophobicity, and intrinsic stability probably have to be taken into account to explain the mechanisms of interfacial layer formation, the layer properties, and subsequently the links with foam/emulsion formation and stabilization by polypeptides.[1,4−12] Controlling these events is of particular importance in food technology, particularly when complex ingredients such as egg white are used to modulate the texture of food products. However, studying complex mixtures generally does not allow linking the structure of each protein of the mixture to the interfacial properties. Moreover, besides the structural dissimilarities that make two proteins different, the technological

Received: September 5, 2011
Revised: October 21, 2011

dx.doi.org/10.1021/la203485y | *Langmuir* XXXX, XXX, 000–000

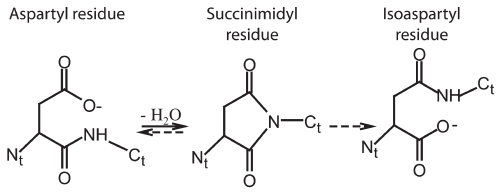


Figure 1. Schematic representation underlying the structural differences between an aspartic residue, a succinimidyl residue, and an isoaspartyl residue. The changes in net charge, hydrophobicity, molecular weight, and intrinsic stability are indicated. The solid arrow indicates the cyclization of an aspartic residue. Dotted arrows show that, upon hydrolysis, a succinimidyl residue can give back an aspartyl residue or an isoaspartyl residue.

treatments applied in an industrial context often lead to structural modifications that are difficult to elucidate.

If one wants to identify protein structural parameters which control the interfacial properties, it is thus highly challenging to compare one protein to another and, consequently, to compare results obtained from one protein to those obtained from another as too many protein features are changed simultaneously. To circumvent these issues, hen egg-white lysozyme (HEWL) was chosen as a model protein, its structure was slightly changed using a dry-heating treatment, and finally its air−water interfacial behavior was analyzed. In this approach, a model system is available, allowing the investigation of both the impact on interfacial properties of small controlled and known changes within a single protein and, at the same time, the effect of heat treatment. Foaming properties of thermally treated lysozyme have already been described.[13] The interfacial properties of the protein (native and dry-heated) are now investigated in detail.

HEWL has been widely used as a model protein because it is a small globular protein (14.3 kDa) that can be easily purified in large amounts. The structure of the molecule has been investigated using high-resolution techniques (NMR and X-ray crystallography),[14,15] and its behavior at the air−water interface has been the subject of previous studies.[12,16−20] HEWL is a rigid molecule that contains four disulfide bridges and has a low surface hydrophobicity. These two parameters are thought to be responsible for the low foaming properties of the protein.[21]

Biochemical studies allowed us to gain precise indications about the structural changes induced by the dry-heating treatment.[22] Dry-heated lysozyme species can be classified into two categories: (1) lysozyme molecules that did not undergo chemical modifications, (2) lysozyme molecules that harbored one to five succinimidyl residues instead of aspartic acid/asparagine residues (Figure 1). The residues that are involved in the degradation process were found to be four aspartic acid residues (Asp18, Asp48, Asp66, Asp101) and one asparagine residue (Asn103). Those succinimidyl residue-containing species do not undergo any secondary or tertiary structure alteration. In other words, all dry-heated molecules possess the same secondary structure content and the same overall conformation, similar to those of the native protein. Strikingly, they have a higher positive net charge and are more hydrophobic, and their intrinsic stability is probably decreased.[22] Moreover, it is noticeable that, when dissolved in a buffer of pH > 6.5, succinimidyl residues are hydrolyzed and lead to the formation of aspartic acid or isoaspartic acid in a ratio of 1:3.5 (Figure 1).[22] This hydrolysis is time and pH dependent. Finally, dry-heating also induces lysozyme dimerization to a small extent (less than 10% of the molecules included in dimers). In the following, different molecular species will be used: the native lysozyme, the dry-heated lysozyme that directly originates from the thermal treatment and then contains all the heated molecules, the native-like lysozyme corresponding to the molecular species that were dry-heated but were not chemically modified, and the succinimide derivatives which are the purified lysozyme molecules that underwent succinimidyl residue formation during dry-heating. It should be pointed out that no standard biochemical and spectroscopic studies enabled differentiation between native-like lysozyme and native lysozyme.[22]

A previous report showed that heating lysozyme in the dry state leads to the formation of molecules with greatly enhanced foaming properties (higher foam density and stability).[13] In the mean time, heated HEWL acquired the property to self-associate in the bulk and form large micrometer-sized aggregates. However, these two phenomena could be separated, and experiments clearly excluded the implication of the large aggregates in the increase of the foaming properties.

The question is now whether it is possible to link the changes in molecular structure to the very different foaming behavior observed between native lysozyme and heated lysozyme. Answering this issue could give important information about the key parameters that define the surface-active properties/foaming properties of proteins. In the present study, the characterization of the interfacial adsorption of lysozyme (native and dry-heated) and the topography of the resulting protein film are first presented; then the conformational changes of the molecules upon adsorption at the air−water interface and the mechanical properties of the interfacial protein layer are described. Finally, the results are discussed in light of our knowledge about the biochemical properties and foaming properties of the different lysozyme species, illustrating how small changes can actually induce significant macroscopic changes, and evidencing the important role of hydrophobicity and the intrinsic stability of proteins in the formation of the interfacial protein film.

MATERIALS AND METHODS

Materials. Lysozyme hydrochloride powder (lysozyme purity higher than 98% as determined by reversed-phase high-performance liquid chromatography (RP-HPLC); 7.5% water content, water activity 0.31) was a gift from LIOT (Annezin-les-Béthune, France). Thermal treatment in the dry state was performed as described before,[22] i.e., samples were kept for 7 days in an oven equilibrated at 80 °C. All measurements at the air−water interface were performed in 60 mM phosphate buffer, pH 7.0.

Purification of Succinimidyl Residue-Containing Lysozyme and Native-like Lysozyme. The different species of lysozyme generated by dry-heating were separated by cation exchange high-performance liquid chromatography (CE-HPLC).[22] Protein samples were solubilized in 20 mM sodium acetate buffer, pH 5.0, and injected onto an S-HYPERD 10 column (Biosepra) equilibrated in the same buffer. Elution was performed using a NaCl gradient from 0 to 1 M in 44 min, and protein recovery was followed by monitoring absorbance simultaneously at 214 and 280 nm. A Waters HPLC system consisting of a Waters 2695 separation module and a Waters 2487 dual-absorbance

dx.doi.org/10.1021/la203485y |*Langmuir* XXXX, XXX, 000–000

detector was used for this purification procedure. Fractions of interest were collected, dialyzed against water (pH 3.5), and freeze-dried.

Ellipsometry and Surface Pressure Measurements. Ellipsometric measurements were performed using a homemade null ellipsometer using a laser operating at 632.8 nm.[23] The surface pressure was measured with a Wilhelmy plate. The volume of the sample trough made of poly(tetrafluoroethylene) (PTFE) was 8 mL. Just before the experiments, lysozyme samples were prepared in ultrapure water, and the protein concentration was adjusted spectrophotometrically to 10 mg/mL using the extinction coefficient ε of the molecule at 280 nm (ε = 37 750 M^{-1} cm^{-1}).[24] Then 80 μL of this stock solution was injected slowly into the subphase of the pre-equilibrated buffer, giving a final protein concentration of 0.1 mg mL^{-1}. The ellipsometric angle (Δ) and the surface pressure (π) were recorded simultaneously for 8 h. After 15 h, the volume of liquid in the sample trough was adjusted to the initial volume by injecting buffer in the subphase with the help of a peristaltic pump. The ellipsometric angle was then measured again.

Polarization Modulation Infrared Reflection−Absorption Spectroscopy (PM-IRRAS). PM-IRRAS spectra were acquired using a Nicolet Nexus 870 spectrophotometer equipped with a photovoltaic HgCdTe detector cooled at 77 K.[25] For each measurement, 300 spectra were recorded at 8 cm^{-1} resolution. The angle of incidence relative to the normal was 75°. The spectrum of the bare air−water interface was systematically recorded and used as a reference. The preparation and the injection of the samples were the same as described for ellipsometry measurements.

Atomic Force Microscopy (AFM). AFM imaging of Langmuir−Schaefer (LS) films[26] was performed in intermittent contact (IC) mode using a Pico-plus atomic force microscope (Agilent Technologies, Phoenix, AZ) under liquid conditions with a scanner of 100 μm × 100 μm. Topographic images were acquired using ACL SPM probes (AppNano, Santa Clara, CA) at room temperature. These images present the topography of the hydrophilic side of the protein layer.

The protein film was created at the air−liquid interface of an 8 mL glass trough, at the bottom of which two glass spacers were fixed, by injecting 80 μL of a concentrated stock lysozyme solution (10 mg mL^{-1}) as described above. At the end of the protein adsorption kinetics, the interfacial protein layer was collected by lowering horizontally a hydrophobic mica plate at very low speed (2 mm min^{-1}) using a dipper from Nima Technology (England). After contact with the film, the sample was maintained blocked close to the spacers. Then the system was reverted and the trough removed, keeping the sample free and hydrated.

To prepare the hydrophobic mica, a 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine monolayer was transferred by the Langmuir−Blodgett method onto freshly cleaved mica plates at high surface pressure (35 mN m^{-1}). After two days of dehydration, the coated mica could be used.

Representative images were obtained from at least two samples prepared on different days with at least five macroscopically separated areas on each sample.

Interfacial Dilational Rheometry. The mechanical response to compression/dilation of the protein interfaces was studied by an oscillatory drop tensiometer (Tracker, Teclis, France). In the static mode of the tensiometer, a liquid drop of controlled volume was formed at the tip of a syringe: by video analysis, the interfacial tension was measured, solving the Laplace equation describing mechanical equilibrium under capillary and gravity forces. In the dynamic mode, accurate sinusoidal oscillations of the volume were applied, corresponding to controlled compression/dilation of the interfacial area.[27] Then, by monitoring the in-phase and out-of-phase response of the surface tension, the elastic (E'_i) and viscous (E''_i) moduli could be determined. It is worth noting that the response to dilational oscillations mostly reflects the balance between adsorption and desorption dynamical processes at the experimental frequencies. The lysozyme concentration was 1 mg mL^{-1}. The measurements were performed once the surface pressure reached a plateau value.

Interfacial Shear Rheometry. A biconical setup mounted on a rheometer (Anton Paar MCR 301) was used to measure the interfacial shear rheology.[28,29] The interface was sheared between the rotating inner bicone and the outer fixed container wall. All the usual 3D rheological measurements could then be transposed in 2D. In particular, oscillatory measurements such as amplitude or frequency sweeps could be performed. Taking into account the corrections from the bulk,[28,29] the interfacial shear storage (G'_i) and loss (G''_i) moduli were derived as a function of the amplitude or frequency. In practice, 100 mL of a 1 mg mL^{-1} protein solution was required to fill the experimental cup. Once this cup was filled, setting up the instruments required about 15−20 min before the first measurement was run. A shear solicitation mostly reflects the interactions and flows within the interface, since its shape changed but not its area.

RESULTS

Lysozyme Adsorption at the Air−Water Interface. First, the adsorption kinetics of lysozyme species by measuring the ellipsometric angle (Δ) and surface pressure (π) over time were recorded. Parts A and C of Figure 2 show Δ and π evolution during protein adsorption on a time scale of minutes, while parts B and D of Figure 2 show the same results on a time scale of several hours. It is noticeable that native lysozyme underwent a lag phase (3−5 min) before surface pressure started to increase (Figure 2A), consistent with previous results,[30−34] but the same feature was not observed for any of the heated samples. Strikingly, such a difference between native and heated lysozyme at the very beginning of the adsorption was not observed when Δ (which reflects mainly the amount of adsorbed proteins) was considered (Figure 2C). All the samples led to a rapid increase in the ellipsometric angle. At longer times, surface pressure offered the same trend for all samples (Figure 2B). First, π rapidly increased during the first hour of the experiment. Then, the π parameter reached a maximum value that differed according to the samples considered and was 17.1, 23.7, 23.1, and 25.7 mN m^{-1} for native lysozyme, dry-heated lysozyme, native-like species, and succinimide derivatives, respectively. The value obtained for native lysozyme was consistent with previous observations in the same conditions.[35]

Another striking difference between all the samples was the change in the ellipsometric angle as a function of time (Figure 2D). In the case of native lysozyme, Δ first increased rapidly and then more slowly, finally reaching a maximum value of 14° after 8 h. This value can be used to estimate the protein concentration (Γ) at the interface applying the relationship $\Gamma = 0.2\Delta$.[36] That is, the native lysozyme concentration at the interface at equilibrium was evaluated to be around 2.8 mg m^{-2}. This result is consistent with values obtained for other globular proteins forming interfacial monolayers.[37,38] For all the heated lysozyme samples, Δ underwent the same first phase as for native lysozyme and then increased linearly over time to around 16 h (data not shown); the Δ values after 15 h were 14°, 38°, 20°, and 24° for the native lysozyme, the dry-heated sample, the nativelike species, and the succinimidyl residue-containing lysozyme, respectively. The values obtained after 8 and 15 h for the ellipsometric angle and surface pressure are summarized in Table 1.

Topography and Thickness of the Interfacial Protein Films. Thanks to the transfer protocol, the face of the film that was in contact with the aqueous phase could be observed. Samples were always kept wet in the same buffer as the one used during the protein adsorption process, thus preventing any potential deformation that could appear upon drying.

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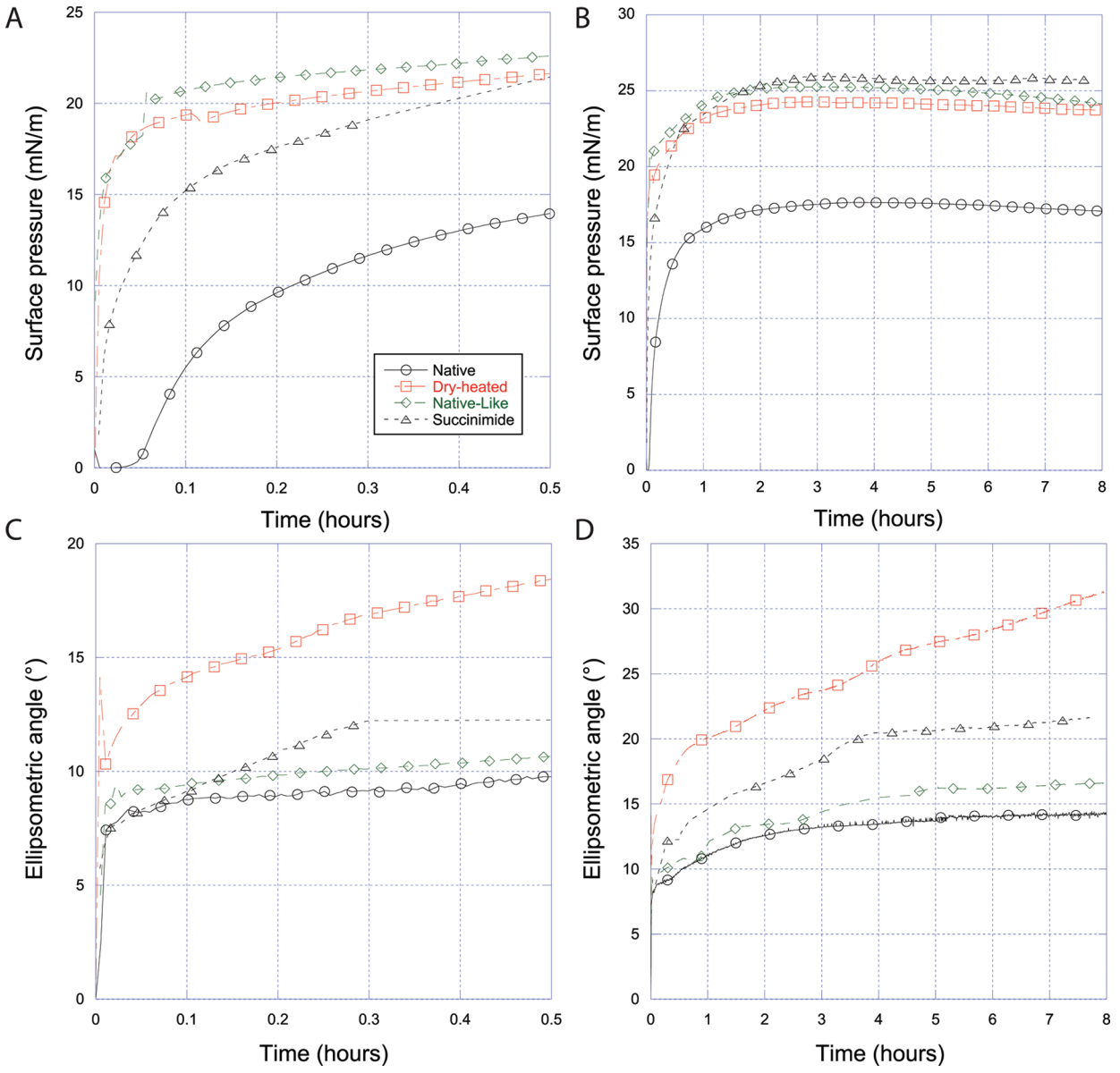

Figure 2. Surface pressure (A, B) and ellipsometric angle (C, D) changes during lysozyme adsorption. The beginning of the kinetics is highlighted in (A) and (C), while the evolution of the two parameters during the overall adsorption process is shown in (B) and (D). Native lysozyme is represented in black, while dry-heated lysozyme, nativelike lysozyme, and succinimide derivatives are shown in red, green, and blue, respectively. All kinetics correspond to a bulk protein concentration of 0.1 mg mL^{-1}.

Table 1. Physical Properties of the Interfacial Films Made of Adsorbed Lysozyme Molecules

	native	dry-heated	native-like	succinimide derivatives
π (mN m^{-1})	17.1	23.7	23.1	25.7
Δ (°), 8 h	14.0	31.2	16.7	22.0
Δ (°), 15 h	14.0	38.0	20.0	24.0
thickness (nm)	2−3	80−100	35−40	70−80

The topography of the films is presented in Figure 3. In the case of native lysozyme, after 15 h, the film appeared smooth (Figure 3A), suggesting a homogeneous single layer of proteins adsorbed to the air−water interface. This result was expected since at low concentrations (below 1 mg mL^{-1}) HEWL sticks to the gas phase with the long axis parallel to the interface, giving an apparent protein film thickness of 3 ± 0.3 nm.[18] On the contrary, for all the treated samples, the surface of the films was heterogeneous with clearly visible nanoscale objects (Figure 3B−D). The nativelike film contained aggregates dispatched within a heterogeneous protein layer. Heated sample and succinimide species produced more homogeneous films with a less random organization. The wormlike structures suggest supramolecular structures interconnected in a macroscopic network. Nevertheless, for these two samples, height variations (<3 nm) were smaller than the size of one lysozyme molecule, indicating that proteins only emerged from the last layer.

To determine the thickness of the films obtained 15 h after protein injection into the subphase, the tip of the atomic force microscope was used to scratch the protein layers until a smooth and rigid surface corresponding to mica was reached. Then the

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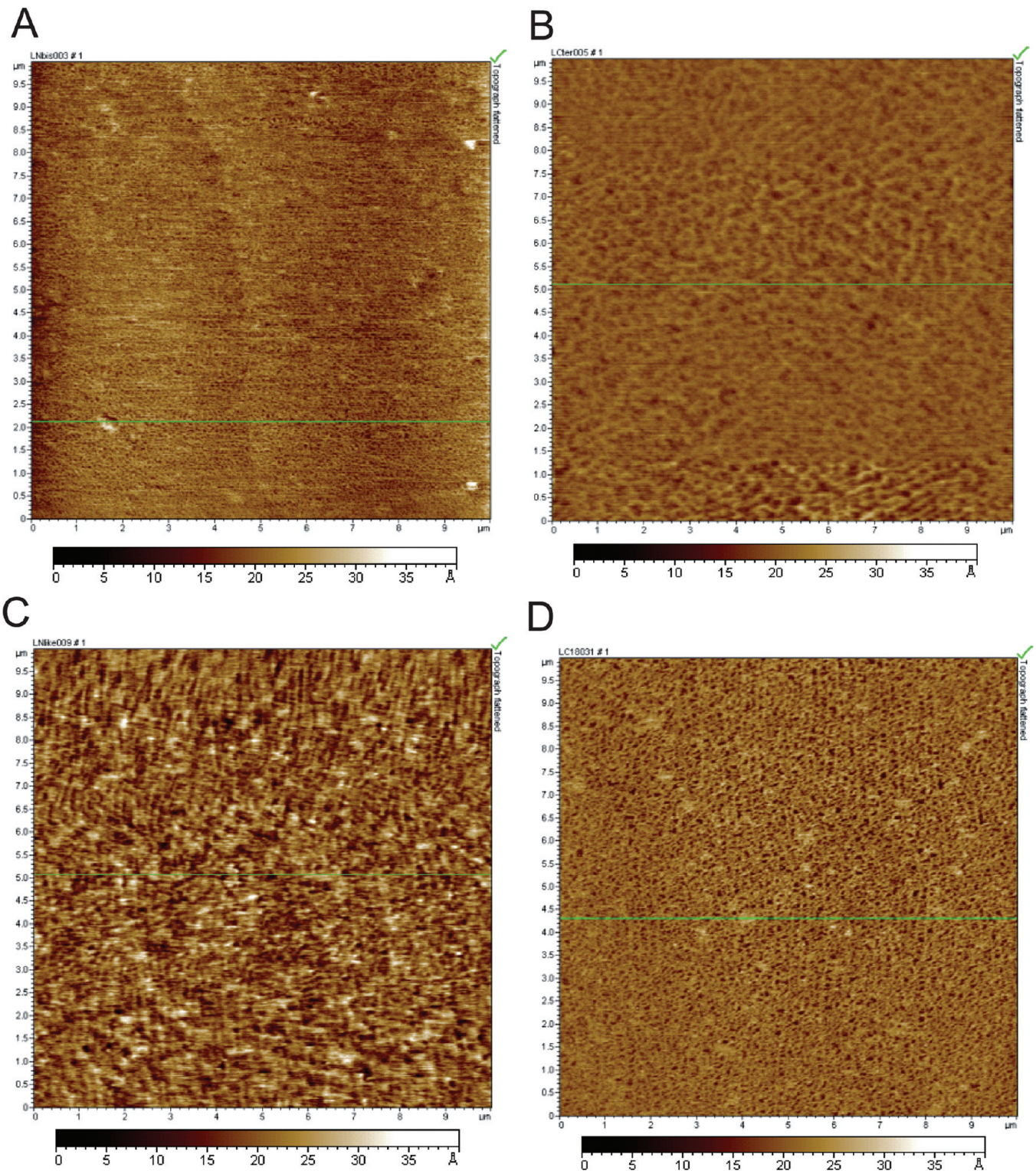

Figure 3. Topography of the interfacial films obtained by atomic force microscopy. The interfacial layers formed with the native lysozyme (A), the dry-heated sample (B), the nativelike lysozyme (C), and the succinimide derivatives (D) were withdrawn 15 h after the beginning of the adsorption process using the Langmuir−Schaeffer technique.

hole obtained by this method was imaged, and the height difference between the top of the film and the mica slide was measured. The results are given in Table 1. These measurements confirmed that native lysozyme adsorbed at the air−water interface in a protein monolayer. Indeed, the film thickness was measured equal to 2−3 nm, meaning values consistent with the dimensions of a lysozyme molecule ($3.0 \times 3.0 \times 4.5$ nm^3).[39] The value obtained for dry-heated sample (containing all the molecular species) reached 80−100 nm, corresponding to more than 25−35 lysozyme molecules piled below the air−water

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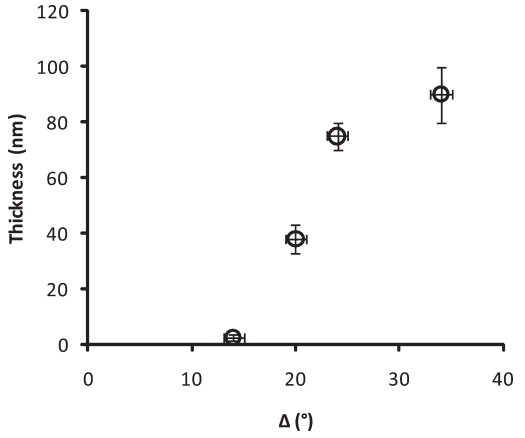

Figure 4. Interfacial film thickness vs ellipsometric angle (Δ). The interfacial film thickness was obtained by AFM and the ellipsometric angle by null ellipsometry. The two parameters were measured 15 h after the protein injection into the subphase. The values plotted are the mean values (and standard deviations) of two independent experiments. Each point corresponds to a lysozyme species.

interface. The thickness of the films made of nativelike molecules and succinimide derivatives were 35−40 and 70−80 nm, respectively. When the thickness of the films measured after 15 h is represented as a function of the ellipsometric angle measured after the same duration (Figure 4), the correlation between these two parameters can be experimentally established. It is noticeable that, though a linear relationship is usually run for translating the ellipsometric angle into surface concentrations in the case of monolayers,[30] a linear dependence for multilayers of thickness between 3 and 80 nm is still valid, but with another slope.

Protein Conformation and Interactions at the Air−Water Interface. To investigate potential changes in the protein conformation at the air−water interface and/or protein−protein interactions in the interfacial films, we focused on the two samples that exhibited the most different behaviors, i.e., native lysozyme (monolayer) and the dry-heated sample (the thickest film). The films have been characterized by time-lapse PM-IRRAS; the spectra obtained are presented in Figure 5. The intensity increased as a function of time for both samples, reflecting the adsorption of the molecules to the air−water interface. However, the final values of intensity are much higher for the dry-heated sample compared to the native one, consistent with a larger amount of proteins adsorbed under the gas phase in the former case, as already shown by ellipsometric angle measurements.

The dry-heated sample exhibited major peaks at 1657 and 1538 cm^{-1} corresponding to amide I and amide II bands, respectively. This is consistent with a major helix content in the proteins.[40] The interpretation of the spectra obtained from native lysozyme films was more complicated since the global intensity was lower than that for heated proteins. However, the same major peaks as above can be attributed to high helix content, but two other peaks were detected at 1682 and 1625 cm^{-1}, which increased over time (Figure 5A). These observations suggest that intermolecular β-sheet formation occurred. On the other hand, these peaks were not detected in the dry-heated lysozyme films (Figure 5B).

Mechanical Properties of the Interfacial Protein Films. Native and dry-heated lysozymes, exhibiting the most different behaviors, were studied.

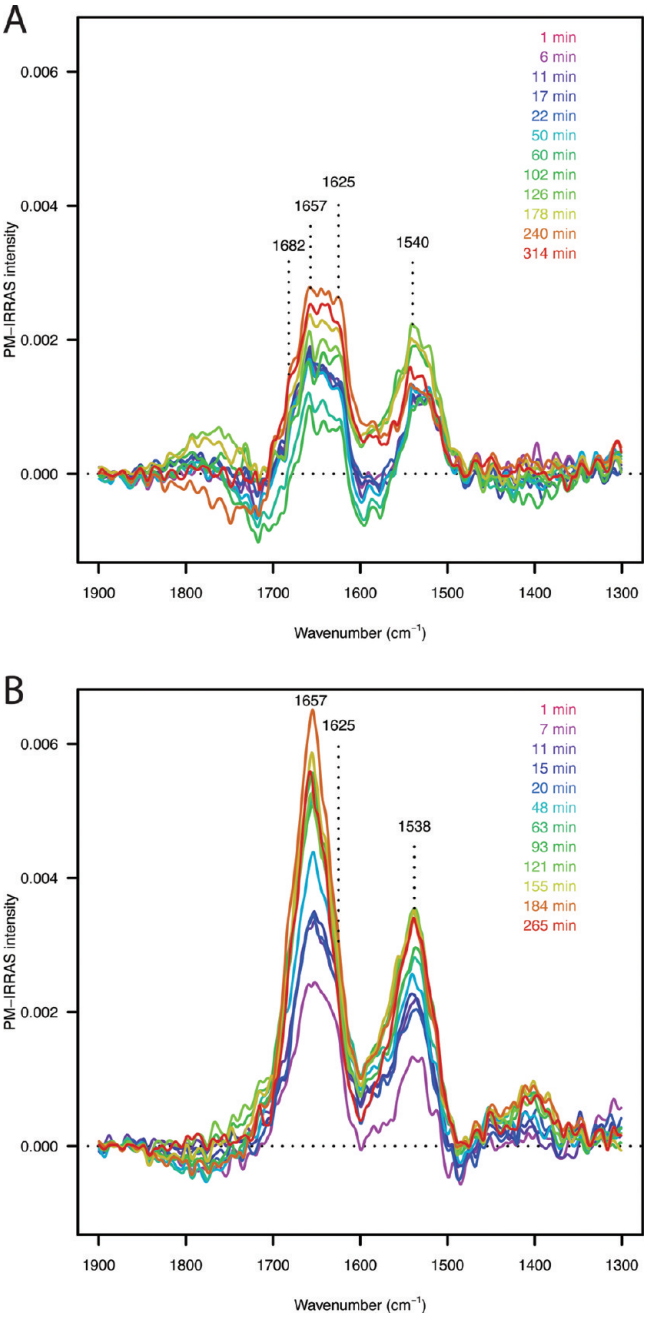

Figure 5. PM-IRRAS spectra of native (A) and dry-heated (B) lysozyme recorded during protein adsorption at a bulk concentration of 0.1 mg mL^{-1}.

Dilational Viscoelasticity. Figure 6 gives the elastic and viscous dilational moduli for native and heated samples. The results are plotted as a function of the excitation frequency and for a fixed oscillation amplitude of 1%. For both samples, high elastic moduli E'_i were measured, even at low frequencies, and almost no dependence was found on frequency. When comparing native to heated protein layers, the latter is significantly more elastic, with E'_i reaching strikingly high values (higher than 100 mN m^{-1}). The viscous component E''_i was about 5−7 times smaller than the elastic component E'_i for both samples and within a more usual range of 10−15 mN m^{-1}.

It must be pointed out that the heated sample led to problems of measurements related to the high measured values. Actually,

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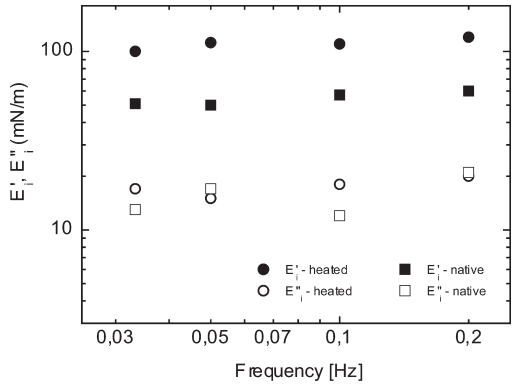

Figure 6. Elastic and viscous moduli (E'_i, filled symbols; E''_i, open symbols) in compression/dilation as a function of the frequency for a fixed strain amplitude of 1% for native lysozyme (circles) and dry-heated lysozyme (squares). The measurements were performed once the surface pressure reached a plateau value.

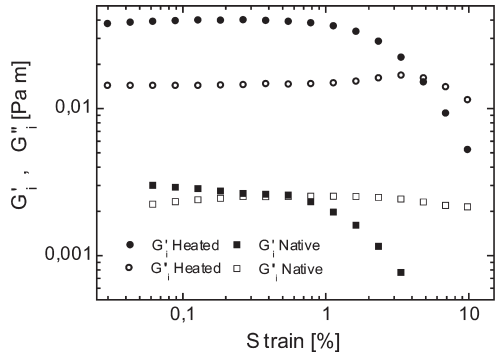

Figure 7. Elastic and viscous shear moduli (G'_i, filled symbols; G''_i, open symbols) as a function of strain for a fixed frequency of 1 Hz. For the heated sample (circles), the measurements were performed at the earliest possible age (17 min); for the native sample (squares), we have plotted the measurements performed at 37 min, as no signals were detected during the first 30 min.

the interfacial layer was so solid-like that it became brittle: In some cases, during area compression and already for deformation amplitudes as low as 5%, the elastic modulus decreased during the oscillations, meaning that the layer was damaged with irreversible loss of adsorbed materials toward the bulk. Thus, the heated protein interface was extremely elastic, but this was associated with a low fracture threshold. In that respect, the data shown in Figure 6 have been collected for a 1% amplitude.

Shear Viscoelasticity. For native and heated samples, the interfacial storage (G'_i) and loss (G''_i) moduli have been measured as a function of strain at fixed frequency (Figure 7). The measurements corresponded to an age of 17 min for the heated sample and 37 min for the native one. For the native sample, it turns out that about 25 min was required to detect a response to shear, in particular to measure an elastic contribution (G'_i). A large quantitative difference was observed at these early layer ages between the two samples and for both moduli. For the heated protein layer, the moduli already showed, at this earliest age of 17 min, a specific dependence on the strain amplitude: In the range of low strain values, G'_i was higher than G''_i and both quantities were constant. As strain increased, G'_i decreased and finally became smaller than G''_i; at the crossing point, a small

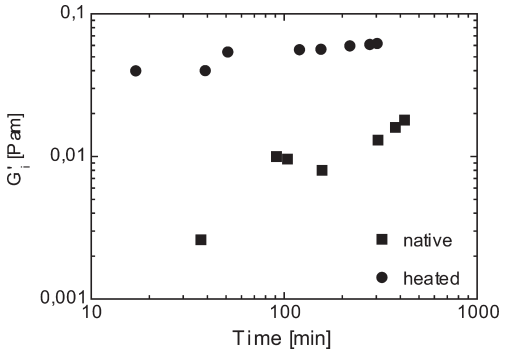

Figure 8. Evolution of the elastic modulus G'_i with the interface age for the two samples: heated sample (squares) and native sample (circles). The frequency was fixed at 1 Hz and the amplitude at 1%.

maximum in G''_i was observed. In the range of the highest strains, the decrease of the two moduli could be adjusted by power laws, the exponent being −0.8 for G'_i and −1.7 for G''_i.

The mechanical properties of the interfacial film could be measured during the sample aging. For the native sample, both moduli continuously increase, and eventually the qualitative features seen for the heated sample fully develop (i.e., a flat region of the modulus on a larger and larger range of low strain with $G'_i > G''_i$ and drastic falls at high strains with $G''_i > G'_i$). Figure 8 shows how the elastic moduli G'_i at an amplitude of 1% evolved over 500 min: The increase rate was faster for the native protein layer modulus, but this modulus always remained much lower than that for the heated protein layer.

DISCUSSION

The interfacial behavior of well-characterized varieties of the same protein and the properties of the resulting film are described. Here, the results obtained at different scales, from the molecular to the macroscopic point of view, are discussed to try to rationalize the behavior of the different lysozyme species and to highlight the key parameters that are of main importance for protein tensioactivity and film formation.

As a major conclusion, the present study highlights the strong effect of slight chemical modifications of the protein on the interfacial behavior for a model globular protein, i.e., HEWL. With the native protein, the ellipsometric angle immediately increases as soon as subphase injection occurs, while the surface pressure requires a lag phase of several minutes under our conditions. This discrepancy between these two parameters suggests either that a minimal amount of protein molecules is required at the interface for the surface pressure increase or that the required rearrangements at the interface are not immediate after native protein adsorption. On the contrary, dry-heated lysozyme species readily adsorb and are surface active after subphase injection, as indicated by surface pressure and ellipsometry measurements. The immediate and higher increase of the surface pressure could be attributed to an enhanced surface hydrophobicity and a probable enhanced flexibility of dry-heated lysozyme[22] that could favor protein adsorption and intermolecular interactions inside the air−water interface. It should be mentioned that faster adsorption kinetics of the dry-heated lysozyme cannot be due to electrostatic effects, since succinimidyl residue formation results in a net charge increase that should even reduce protein adsorption because of stronger protein−protein

dx.doi.org/10.1021/la203485y |*Langmuir* XXXX, XXX, 000–000

repulsions. Moreover, the stronger ellipsometric angle increase could be linked to the decreased solubility of dry-heated lysozyme molecules. Indeed, a protein concentration gradient is expected underneath the air−water interface, and dry-heated lysozyme aggregation is known as a concentration-dependent process.[13] It could then be hypothesized that dry-heated protein acquired the ability to accumulate at the air−water interface, probably making a multilayer film. Interestingly, multilayer formation has only been reported for pure native HEWL solution above a protein concentration of 1.43 mg mL^{-1} [19] and when mixed with a concentrated (>3 M) chaotropic reagent (guanidinium hydrochloride).[41] In the latter case, the ability of HEWL to form protein multilayers stems from the initial decrease in intrinsic stability generated by incubation in the presence of a chaotropic reagent. In this case, dry-heated lysozyme has a decreased intrinsic stability since it is prone to aggregation under certain physicochemical conditions.[13] This suggests that intrinsic stability probably plays an important role in protein aggregation at the air−water interface.

One additional hypothesis would be that only a small fraction of the modified species (the most surface-active ones) could adsorb first at the air−water interface, and the subsequent adsorption of less modified species could thus be facilitated by a synergistic effect. In such a model, the adsorption of the most surface-active molecules would be sufficient to substantially change the surface properties. The rapid increase in surface pressure would prevent structural changes in native lysozyme adsorbed at the air−water interface, as discussed below. The potential synergy between the different molecular species is supported by two recent studies on the interfacial and foaming properties of lysozyme/ovalbumin mixtures.[42,43] It was shown that the addition of small amounts of ovalbumin drastically changed the behavior of lysozyme and vice versa. This assumption would explain why the thickest interfacial protein film is obtained with the dry-heated sample which contains a mixture of different species (Figure 2D). Nevertheless, it should be pointed out that surface activity does not seem to depend on the film thickness (Figure 2B).

The combination of ellipsometry and atomic force microscopy permitted the relationship that exists between the value of the ellipsometric angle and the "effective" film thickness to be experimentally described (Figure 4). When the air−water interface is protein-free, the film thickness and ellipsometric angle are obviously null. When native lysozyme adsorbs to the interface and makes a single monolayer, the ellipsometric angle jumps to 14°, a value that is characteristic of native HEWL. By comparison, a monolayer of ovalbumin adsorbed at the air−water interface results in an ellipsometric angle equal to 10°,[43] whereas the film thickness is expected to be higher than that for HEWL considering the dimensions of ovalbumin ($7.0 \times 4.5 \times 5.0$ nm^3). Then these values suggest that packing of HEWL at the interface could be higher than that for ovalbumin. This is consistent with previous results showing that less dense layers were obtained when higher molecular weight proteins were adsorbed on solid interfaces.[44] When higher amounts of HEWL adsorb under the gas phase, the ellipsometric angle increases accordingly, giving a linear relationship between Δ and the film thickness, until Δ reaches a value close to 25°. Although the ellipsometric signal is more complicated to interpret when multiple layers are formed, with no clear-cut limits between two adjacent layers, as is probably the case in the present study, the experimental results demonstrate that a linear relationship applies in a thickness range much larger than that expected. However, this linear relationship no longer applies at angles above 25°; the film thickness is then lower than the value expected from the relationship established for lower angle values. One explanation could be that the interfacial film transferred on a mica plate is very flexible, while the AFM tip is rigid. The contact of the tip on the film could then flatten the interface, thus giving a thickness value lower than the real one; the film thickness would then be underestimated. Another explanation of the results is that the ellipsometric response is periodic, and at higher thickness values the ellipsometric signal could start to decrease.

One striking result is that the interfacial behavior of native-like lysozyme differs significantly from that of native protein. Native-like lysozyme is a molecular species that underwent dry-heating treatment, but with no detectable chemical modification. Moreover, no secondary or tertiary structure modifications could be detected by circular dichroism or steady-state fluorescence measurements. Nevertheless, its surface hydrophobicity was slightly higher compared to that of the native lysozyme, and its intrinsic stability was slightly lower than that of the native protein.[22] It is then surprising how such small physicochemical modifications can lead to a very different behavior at the air−water interface, and it seems that study of the adsorption at the air−water interface reveals somehow the modifications induced by dry-heating, whereas these slight modifications could not be observed by usual spectroscopic methods. This also suggests that surface hydrophobicity and intrinsic stability could be key parameters for protein interfacial behavior.

The PM-IRRAS results first show that much higher intensities are obtained with dry-heated lysozyme, thus corroborating the results from ellipsometry and AFM: Adsorption of dry-heated protein results in much thicker interfacial films than in the case of the native lysozyme. PM-IRRAS also suggests that native HEWL undergoes structural modifications upon adsorption to the air−water interface. At the beginning of the adsorption process, a majority of helices are detected at the interface, consistent with the secondary structure content of native protein: 38% α-helices, 10% β-sheets. As a function of time, the proportion of β-sheets increases as reported by other authors,[17,45−47] suggesting changes in the overall HEWL conformation and establishment of intermolecular interactions. These results feed the discussion about the potential rearrangement of lysozyme at the air−water interface. Indeed, this is still a matter of debate in the literature, and many authors have reported no structural change of lysozyme upon adsorption,[18,39,41,48,49] while others clearly detected structural modifications.[17,45,46,50] These discrepancies could arise from different buffer compositions and different methods/techniques used. The present study is clearly in favor of a rearrangement of the native lysozyme when adsorbed at the air−water interface in our conditions. However, strikingly, such observations are not obtained for the dry-heated lysozyme. Since dry-heating has been shown to decrease the structural stability of lysozyme, at least in solution,[22] one could have expected more pronounced conformational changes upon adsorption at the air−water interface and film aging than for the native form. With dry-heated lysozyme, in contrast, the signal corresponding to α-helices is always prominent all along the experiment, and the signal characteristic of β-sheets remains hardly detectable, suggesting that the conformation of the adsorbed dry-heated lysozyme remains quite similar to the conformation of lysozyme in solution (data not shown). Considering the multilayer adsorption of the dry-heated lysozyme, resulting in a thick interfacial film and a high surface concentration, it could be hypothesized

that the overall PM-IRRAS signal is dominated by molecules of "deep" layers, protected from the destabilizing contact with the gas phase. It seems unlikely, since (i) the protein concentration probably decreases rapidly when the distance to the interface increases, as suggested by the relationship observed between thickness and the ellipsometric angle Δ, and (ii) the polarity of the protein environment in deep, hydrated layers would also reduce the specific PM-IRRAS intensity. Alternatively, considering the higher surface pressure obtained with the dry-heated form of the protein, we may propose that conformational changes are hindered at high values of surface pressure. Such effects of surface pressure on the extent of protein denaturation at the gas−water interface have already been described.[51,52] In addition, the lag phase observed in the kinetics of surface pressure increases upon adsorption of native lysozyme, in contrast with the dry-heated form, could also partly account for the higher extent of conformational changes during protein adsorption at low pressure. However, it seems difficult to identify clearly the nature of structural changes which confer to lysozyme the ability to self-associate into a thick, multilayer interfacial film and dramatically increase its ability to lower the surface tension.

Interfacial rheologies of native and heated protein layers are significantly different, both under compression and in shear. Thus, dry-heating modifies not only the structure of the films, but also the dynamics of the interfaces.

When compared to other similar studies on proteins, qualitatively and quantitatively speaking, native lysozyme behaves similarly to many other systems. The range of compression and shear moduli is actually within the known and expected values.[53−55] In addition, it is usual that the elastic shear response arises at long times, as it has often been reported that an interfacial network slowly develops. The constant elastic modulus E'_i also indicates that there is no rapid exchange and equilibration between the bulk and the interface, as expected for proteins.

In that respect, the different results found for the heated protein layer are much more surprising, since they reveal an outstanding solid-like and cohesive interfacial layer. This is actually associated with a high brittleness: As noted in the Results, the rigidity to compression is so high that the layer can irreversibly break into pieces, rather than being compressed. Concerning the shear response, the shapes of G'_i and G''_i versus strain indicate that the interface undergoes a yielding transition when strain increases; these curves are indeed very similar to what is found in 3D for disordered and soft glassy materials.[56] This suggests that the interfacial layer can be considered as a highly disordered and amorphous solid, which is also consistent with a fast adsorption process.

One can also wonder how these interfacial results might be correlated to foam capacity and foam stability. Though it is tempting to draw links between interfacial properties and foams, one always has to remain careful, especially as time and length scales are different, and other effects due to confinement, packing of the bubbles, and foaming processes can play important roles. Keeping these warnings in mind, we can however predict that the heated lysozyme would produce a foam with high stability; especially, slower drainage and/or coarsening could be expected, as a consequence of high viscoelasticity of the protein interface.[57] However, the interfacial moduli measured with the heated lysozyme might also predict a low foamability (or foaming efficiency). Indeed, such very high elastic values (higher than 100 mN m^{-1}) mean that the molecules are strongly adsorbed and do not easily desorb: This generally occurs either for insoluble molecules deposited over an interface or for molecules that require high energy input to adsorb at the interface (such as solid particles).[58] In other words, the disadvantage of high interfacial elasticity is that the interfacial layer is generally not spontaneously formed. As a matter of fact, the dry-heated lysozyme is actually a good counterexample of what is usually accepted: This complex mixture of different molecular species of lysozyme is soluble, it easily and spontaneously adsorbs at the air−water interface, and because of this complexity, interactions and structural changes occur once at the interface, for finally inducing very low desorption. Therefore, not only do these proteins satisfactorily foam, but also the foam stability is quite good. However, the differences observed between the interfaces created with either native or heated lysozyme are probably not sufficient to fully explain the already reported differences in foam stability between these proteins. As often observed, other effects due to confinement in the foam channels (the so-called "Plateau borders") would probably be important, as shown by preliminary experiments where the bubble size has been varied (data not shown).

CONCLUSIONS

This work shows how very small structural changes can strongly affect the air−water interfacial behavior of a model protein. Indeed, under the experimental conditions, native lysozyme adsorbs at the air−water interface, producing a single molecule layer with mechanical properties similar to those of other globular protein systems. In contrast, dry-heating of hen egg-white lysozyme leads to the formation of protein species that have the ability to accumulate under the air−water interface, making multilayers of polypeptides without changes in their overall conformation. Moreover, the resulting film is solid-like and has mechanical properties similar to those of highly disordered and amorphous solids. Collectively, the results are in good agreement with our previous observations, which highlighted the increase of foam stability after dry-heating of lysozyme. The resulting molecules indeed adsorb more rapidly to the air−water interface and produce highly viscoelastic protein layers. The expected low foaming efficiency associated with high elasticity values is not observed, probably due to the dynamic rearrangement of proteins at the air−water interface that promotes the transition from a soluble to an insoluble state.

ACKNOWLEDGMENT

Y.D. was supported by a Ph.D. fellowship from the Région Bretagne.

REFERENCES

(1) Dickinson, E. Proteins at interfaces and in emulsions: Stability, rheology and interactions. *J. Chem. Soc., Faraday Trans.* **1998**, *94* (12), 1657–1669.

(2) Murray, B. S. Stabilization of bubbles and foams. *Curr. Opin. Colloid Interface Sci.* **2007**, *12* (4−5), 232–241.

(3) Murray, B. S.; Ettelaie, R. Foam stability: Proteins and nanoparticles. *Curr. Opin. Colloid Interface Sci.* **2004**, *9* (5), 314–320.

(4) Wierenga, P. A.; Meinders, M. B. J.; Egmond, M. R.; Voragen, A. G. J.; De Jongh, H. H. Protein exposed hydrophobicity reduces the kinetic barrier for adsoption of ovalbumin to the air−water interface. *Langmuir* **2003**, *19*, 8964–8970.

(5) Sengupta, T.; Razumovsky, L.; Damodaran, S. Energetics of protein−interface interactions and its effect on protein adsorption. *Langmuir* **1999**, *15* (20), 6991–7001.

(6) Song, K. B.; Damodaran, S. Influence of electrostatic forces on the adsorption of succinylated β-lactoglobulin at the air−water interface. *Langmuir* **1991**, *7*, 2737–2742.
(7) Wierenga, P. A.; Meinders, M. B.; Egmond, M. R.; Voragen, A. G.; de Jongh, H. H. Quantitative description of the relation between protein net charge and protein adsorption to air−water interfaces. *J. Phys. Chem. B* **2005**, *109* (35), 16946–52.
(8) de Jongh, H. H.; Kosters, H. A.; Kudryashova, E.; Meinders, M. B.; Trofimova, D.; Wierenga, P. A. Protein adsorption at air−water interfaces: A combination of details. *Biopolymers* **2004**, *74* (1−2), 131–5.
(9) Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces: II. Adsorption isotherms. *J. Colloid Interface Sci.* **1979**, *70* (3), 415–426.
(10) Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces: III. Molecular structures of adsorbed films. *J. Colloid Interface Sci.* **1979**, *70* (3), 427–439.
(11) Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces: I. Kinetics of adsorption and surface denaturation. *J. Colloid Interface Sci.* **1979**, *70* (3), 403–414.
(12) Perriman, A. W.; White, J. W. Kinetics of adsorption of lysozyme at the air−water interface and the role of protein charge. *Physica B (Amsterdam)* **2006**, *385−386* (Part 1), 716–718.
(13) Desfougères, Y.; Lechevalier, V.; Pezennec, S.; Artzner, F.; Nau, F. Dry-heating makes hen egg white lysozyme an efficient foaming agent and enables its bulk aggregation. *J. Agric. Food Chem.* **2008**, *56* (13), 5120–5128.
(14) Diamond, R. Real-space refinement of the structure of hen egg-white lysozyme. *J. Mol. Biol.* **1974**, *82* (3), 371–391.
(15) Schwalbe, H.; Grimshaw, S. B.; Spencer, A.; Buck, M.; Boyd, J.; Dobson, C. M.; Redfield, C.; Smith, L. J. A refined solution structure of hen lysozyme determined using residual dipolar coupling data. *Protein Sci.* **2001**, *10* (4), 677–688.
(16) Hunter, J. R.; Kilpatrick, P. K.; Carbonell, R. G. Lysozyme adsorption at the air/water interface. *J. Colloid Interface Sci.* **1990**, *137* (2), 462–482.
(17) Postel, C.; Abillon, O.; Desbat, B. Structure and denaturation of adsorbed lysozyme at the air−water interface. *J. Colloid Interface Sci.* **2003**, *266* (1), 74–81.
(18) Lu, J. R.; Su, T. J.; Thomas, R. K.; Penfold, J.; Webser, J. Structural conformation of lysozyme layers at the air/water interface studied by neutron reflection. *J. Chem. Soc., Faraday Trans.* **1998**, *94*, 3279–3287.
(19) Alahverdjieva, V. S.; Grigoriev, D. O.; Ferri, J. K.; Fainerman, V. B.; Aksenenko, E. V.; Leser, M. E.; Michel, M.; Miller, R. Adsorption behaviour of hen egg-white lysozyme at the air/water interface. *Colloids Surf., A* **2008**, *323* (1−3), 167–174.
(20) Malcolm, A. S.; Dexter, A. F.; Middelberg, A. P. J. Mechanical properties of interfacial films formed by lysozyme self-assembly at the air/water interface. *Langmuir* **2006**, *22* (21), 8897–8905.
(21) Townsend, A. A.; Nakai, H. Relationships between hydrophobicity and foaming characteristics of food proteins. *J. Food Sci.* **1983**, *48* (2), 588–594.
(22) Desfougères, Y.; Jardin, J.; Lechevalier, V.; Pezennec, S.; Nau, F. Succinimidyl residue formation in hen egg-white lysozyme favors the formation of intermolecular covalent bonds without affecting its tertiary structure. *Biomacromolecules* **2011**, *12* (1), 156–66.
(23) Berge, B.; Renault, A. *Ellipsometry Study of 2D Crystallization of 1-Alcohol Monolayers at the Water Surface*; EDP Sciences: Les Ulis, France, 1993; Vol. 21.
(24) Aune, K. C.; Tanford, C. Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. I. Dependence on pH at 25°. *Biochemistry* **1969**, *8* (11), 4579–85.
(25) Blaudez, D.; Turlet, J. M.; Dufourcq, J.; Bard, D.; Buffeteau, T.; Desbat, B. Investigations at the air/water interface using polarization modulation IR spectroscopy. *J. Chem. Soc., Faraday Trans.* **1996**, *92* (4), 525–530.
(26) Langmuir, I.; Schaefer, V. J. Activities of urease and pepsin monolayers. *J. Am. Chem. Soc.* **1938**, *60*, 1351–1360.
(27) Benjamins, J.; Cagna, A.; Lucassen-Reynders, E. H. Viscoelastic properties of triacylglycerol/water interfaces covered by proteins. *Colloids Surf., A* **1996**, *114*, 245–254.
(28) Erni, P.; Fischer, P.; Heyer, P.; Windhab, E. J.; Kusnezov, V.; Lauger, J. Rheology of gas/liquid and liquid/liquid interfaces with aqueous and biopolymer subphases. In *Mesophases, Polymers, and Particles*; Lagaly, G.; Richtering, W., Eds.; Springer-Verlag: Berlin, 2004; Vol. 129, pp 16−23.
(29) Erni, P.; Fischer, P.; Windhab, E. J.; Kusnezov, V.; Stettin, H.; Lauger, J. Stress- and strain-controlled measurements of interfacial shear viscosity and viscoelasticity at liquid/liquid and gas/liquid interfaces. *Rev. Sci. Instrum.* **2003**, *74* (11), 4916–4924.
(30) de Feijter, J. A.; Benjamins, J. *Adsorption Kinetics of Protein at the Air−Water Interface*; Royal Society of Chemistry: London, 1987.
(31) Xu, S.; Damodaran, S. The role of chemical potential in the adsorption of lysozyme at the air−water interface. *Langmuir* **1992**, *8* (8), 2021–2027.
(32) Xu, S.; Damodaran, S. Comparative adsorption of native and denatured egg-white, human, and T4 phage lysozymes at the air−water interface. *J. Colloid Interface Sci.* **1993**, *159* (1), 124–133.
(33) Sengupta, T.; Damodaran, S. Role of dispersion interactions in the adsorption of proteins at oil−water and air−water interfaces. *Langmuir* **1998**, *14* (22), 6457–6469.
(34) Sundaram, S.; Ferri, J. K.; Vollhardt, D.; Stebe, K. J. Surface phase behavior and surface tension evolution for lysozyme adsorption onto clean interfaces and into DPPC monolayers: Theory and experiment. *Langmuir* **1998**, *14* (5), 1208–1218.
(35) Chang, S.-H.; Chen, L.-Y.; Chen, W.-Y. The effects of denaturants on protein conformation and behavior at air/solution interface. *Colloids Surf., B* **2005**, *41* (1), 1–6.
(36) de Feijter, J. A.; Benjamins, J.; Veer, F. A. Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air−water interface. *Biopolymers* **1978**, *17* (7), 1759–1772.
(37) Croguennec, T.; Renault, A.; Bouhallab, S.; Pezennec, S. Interfacial and foaming properties of sulfydryl-modified bovine beta-lactoglobulin. *J. Colloid Interface Sci.* **2006**, *302* (1), 32–9.
(38) Croguennec, T.; Renault, A.; Beaufils, S.; Dubois, J. J.; Pezennec, S. Interfacial properties of heat-treated ovalbumin. *J. Colloid Interface Sci.* **2007**, *315* (2), 627–36.
(39) Lu, J. R.; Su, T. J.; Howlin, B. J. The effect of solution pH on the structural conformation of lysozyme layers adsorbed on the surface of water. *J. Phys. Chem. B* **1999**, *103* (28), 5903–5909.
(40) Barth, A. Infrared spectroscopy of proteins. *Biochim. Biophys. Acta, Bioenerg.* **2007**, *1767* (9), 1073–1101.
(41) Perriman, A. W.; Henderson, M. J.; Evenhuis, C. R.; McGillivray, D. J.; White, J. W. Effect of the air−water interface on the structure of lysozyme in the presence of guanidinium chloride. *J. Phys. Chem. B* **2008**, *112* (31), 9532–9539.
(42) Le Floch-Fouéré, C.; Beaufils, S.; Lechevalier, V.; Nau, F.; Pézolet, M.; Renault, A.; Pezennec, S. Sequential adsorption of egg-white proteins at the air−water interface suggests a stratified organization of the interfacial film. *Food Hydrocolloids* **2010**, *24* (4), 275–284.
(43) Le Floch-Fouéré, C.; Pezennec, S.; Lechevalier, V.; Beaufils, S.; Desbat, B.; Pézolet, M.; Renault, A. Synergy between ovalbumin and lysozyme leads to non-additive interfacial and foaming properties of mixtures. *Food Hydrocolloids* **2009**, *23* (2), 352–365.
(44) Voros, J. The density and refractive index of adsorbing protein layers. *Biophys. J.* **2004**, *87* (1), 553–61.
(45) Thakur, G.; Leblanc, R. M. Conformation of lysozyme Langmuir monolayer studied by infrared reflection absorption spectroscopy. *Langmuir* **2009**, *25* (5), 2842–9.
(46) Damodaran, S. In situ measurement of conformational changes in proteins at liquid interfaces by circular dichroism spectroscopy. *Anal. Bioanal. Chem.* **2003**, *376* (2), 182–8.
(47) Lad, M. D.; Birembaut, F.; Matthew, J. M.; Frazier, R. A.; Green, R. J. The adsorbed conformation of globular proteins at the air/water interface. *Phys. Chem. Chem. Phys.* **2006**, *8* (18), 2179–86.

(48) Min, D. J.; Winterton, L.; Andrade, J. D. Behavior of model proteins, pretreated in urea and/or dithiothreitol, at air/solution interfaces. *J. Colloid Interface Sci.* **1998**, *197* (1), 43–47.

(49) Yampolskaya, G. P.; Tarasevich, B. N.; Elenskii, A. A. Secondary structure of globular proteins in adsorption layers at the solution−air interface by the data of fourier transform IR spectroscopy. *Colloid J.* **2005**, *67* (3), 385–391.

(50) Yano, Y. F.; Uruga, T.; Tanida, H.; Toyokawa, H.; Terada, Y.; Takagaki, M.; Yamada, H. Driving force behind adsorption-induced protein unfolding: A time-resolved X-ray reflectivity study on lysozyme adsorbed at an air/water interface. *Langmuir* **2009**, *25* (1), 32–5.

(51) Gallant, J.; Desbat, B.; Vaknin, D.; Salesse, C. Polarization-modulated infrared spectroscopy and x-ray reflectivity of photosystem II core complex at the gas−water interface. *Biophys. J.* **1998**, *75*, 2888–2899.

(52) Lavoie, H.; Gallant, J.; Grandbois, M.; Blaudez, D.; Desbat, B.; Boucher, F.; Salesse, C. The behavior of membrane proteins in monolayers at the gas−water interface: Comparison between photosystem II, rhodopsin and bacteriorhodopsin. *Mater. Sci. Eng., C* **1999**, *10* (1−2), 147–154.

(53) Martin, A. H.; Cohen Stuart, M. A.; Bos, M. A.; van Vliet, T. Correlation between mechanical behavior of protein films at the air/water interface and intrinsic stability of protein molecules. *Langmuir* **2005**, *21* (9), 4083–9.

(54) Wang, Z.; Narsimhan, G. Interfacial dilatational elasticity and viscosity of β-lactoglobulin at air−water interface using pulsating bubble tensiometry. *Langmuir* **2005**, *21* (10), 4482–4489.

(55) Jung, J.-M.; Gunes, D. Z.; Mezzenga, R. Interfacial activity and interfacial shear rheology of native β-lactoglobulin monomers and their heat-induced fibers. *Langmuir* **2010**, *26* (19), 15366–15375.

(56) Coussot, P. Rheophysics of pastes: A review of microscopic modelling approaches. *Soft Matter* **2007**, *3* (5), 528–540.

(57) Saint-Jalmes, A. Physical chemistry in foam drainage and coarsening. *Soft Matter* **2006**, *2*, 836–849.

(58) Tcholakova, S.; Denkov, N. D.; Lips, A. Comparison of solid particles, globular proteins and surfactants as emulsifiers. *Phys. Chem. Chem. Phys.* **2008**, *10* (12), 1608–1627.