Reversible self-association of ovalbumin at air–water interfaces and the consequences for the exerted surface pressure

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Abstract

In this study the relation between the ability of protein self-association and the surface properties at air–water interfaces is investigated using a combination of spectroscopic techniques. Three forms of chicken egg ovalbumin were obtained with different self-associating behavior: native ovalbumin, heat-treated ovalbumin—being a cluster of 12–16 predominantly noncovalently bound proteins, and succinylated ovalbumin, as a form with diminished aggregation properties due to increased electrostatic repulsion. While the bulk diffusion of aggregated protein is clearly slower compared to monomeric protein, the efficiency of transport to the interface is increased, just like the efficiency of sticking to rather than bouncing from the interface. On a timescale of hours, the aggregated protein dissociates and adopts a conformation comparable to that of native protein adsorbed to the interface. The exerted surface pressure is higher for aggregated material, most probably because the deformability of the particle is smaller. Aggregated protein has a lower ability to desorb from the interface upon compression of the surface layer, resulting in a steadily increasing surface pressure upon reducing the available area for the surface layer. This observation is opposite to what is observed for succinylated protein that may desorb more easily and thereby suppresses the buildup of a surface pressure. Generally, this work demonstrates that modulating the ability of proteins to self-associate offers a tool to control the rheological properties of interfaces.

Keywords: ovalbumin; aggregation; air–water interface; IRRAS; FCS

Proteins in an aqueous environment are in a continuous balance between self-association, driven by hydrophobic forces, and dissolution to accommodate for the electrostatic repulsions and loss of entropy upon self-association. The introduction of an interface in the system might shift this balance, due to a locally altered dielectric constant or the high concentration of proteins that may accumulate at interfaces (Meinders et al. 2001; Meinders and de Jongh 2002;

Kudryashova et al. 2003). Whereas, for example, a lipid membrane might exert electrostatic attractions to favor accumulation at the interface, an air–water or oil–water interface can only yield dehydration of parts of the protein and thereby promote protein–protein interactions.

Recently it was shown that at air–water interfaces, highly concentrated surface layers are formed with local protein concentrations up to 150–250 mg/mL (Kudryashova et al. 2003). Concentrating proteins to such high values, as can be found, for example, in red blood cells or in inclusion bodies, without inducing protein aggregation is not easily accomplished in vitro. From the generally accepted view on chaperonin action (Hartl 1994), where proteins are prevented from (irreversible) aggregation, it could be suggested that

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the presence of a gradient in dielectric constant, as provided by an extensive available surface, might be able to shift this balance in self-association. Both in life sciences as in technological applications, as in foam (air–water) or emulsion (oil–water) formation, interfaces are largely abundant.

Chicken egg ovalbumin adsorbs spontaneously at air–water interfaces (Kudryashova et al. 2003; Wierenga et al. 2003). The kinetics of the adsorption process is relatively slow, compared to that of other proteins like, for example, β -lactoglobulin, which is related to the limited hydrophobic exposure of ovalbumin (Wierenga et al. 2003), and to the electrostatic repulsions of the net charge of -10 at neutral pH (de Jongh et al. 2004). Only minor changes at a secondary folding level are found upon adsorption of this protein to the air–water interface, and no indications for a major loss of globular packing could be derived on the basis of tryptophan fluorescence data (Kudryashova et al. 2003).

This work aims to describe both the role of self-association of ovalbumin on the formation of surface layers at the air–water interface and how a formed surface layer can influence the protein aggregation state. To this end, two forms of ovalbumin were prepared, one that was heat-treated providing (noncovalently) aggregated protein, and the other in which the net surface charge of the protein was increased by limited chemical modification of lysine residues, yielding proteins with a strongly reduced aggregation tendency. The formation of surface layers of these proteins was studied using a combination of infrared reflection adsorption spectroscopy (IRRAS) and fluorescence techniques, providing insight into local protein concentration at the interface, and the structural properties and molecular mobility of adsorbed molecules. Changes in surface pressure were monitored using both the Wilhelmy balance in a Langmuir trough and the automated drop tensiometer and are related to the self-association properties of ovalbumin.

Results

Structural characterization

Native (N-), heat-treated (T-), and succinylated (suc-) ovalbumin (OVA) were analyzed for their secondary structure content using ATR infrared spectroscopy (Fig. 1). The amide I-band shape is sensitive for the secondary structure content (Kalnin et al. 1990), and can be analyzed using a band-shape fitting procedure (Dong et al. 2000; Kudryashova et al. 2003). This yields for N-OVA $\sim 47\%$ β -strands and 35% α -helices, in close agreement with the X-ray structure as resolved previously (Stein et al. 1991). In comparison to N-OVA, in T-OVA 10% – 20% of β -structure appears to be unfolded to random coil, while the secondary structure of suc-OVA was not affected by processing (spectrum not shown). A clear shoulder around 1624 cm^{-1} is observed for T-OVA (Fig. 1B), indicative of the presence of antiparallel

Figure 1. (A) FTIR-ATR spectra of N-OVA and T-OVA. The film was dried from $100\text{ }\mu\text{L}$ of 10 mg/mL solution of the protein in 10 mM phosphate buffer (pH 6.9). (B) Enlargement of panel A showing the amide I region of N-OVA and T-OVA. The dashed lines illustrate the frequency assignment of the secondary structure types present. (β) β -strand; (α) α -helix; (r.c.) random coil; (anti- β) antiparallel β -strand.

β -sheet structure, typical for aggregated protein (Dong et al. 2000; Kudryashova et al. 2003). The secondary structure contents found by IR are confirmed for the OVA variants by analysis of the corresponding far-UV CD spectra (data not shown).

To accurately determine the amount of aggregated protein in the T-OVA sample, gel-permeation chromatography was applied. The experiment (data not shown) demonstrates that $\sim 90\%$ of the protein is aggregated in T-OVA. The mass of the aggregates was estimated to be ~ 500 – 700 kDa (12-mers to 16-mers), based on calibration of the column with standard reference globular proteins with different masses. The size of the aggregates was also confirmed more qualitatively by gel electrophoretic analysis using native gels (data not shown). Moreover, SDS-PAGE analysis under reducing and nonreducing conditions demonstrated that only 5% of the proteins residing in aggregates were involved in covalent dimers.

Since it was shown elsewhere (Hagolle et al. 1998; P.A. Wierenga and H.A. Kusters, unpubl.) that the development of surface pressure of aggregated T-OVA was faster than that of (refolded) monomeric heat-treated OVA, separation of the monomeric component from the T-OVA sample was omitted to avoid unreproducible loss of material during the

fractionation (e.g., using gel filtration columns) and to prevent rearrangements of the mostly noncovalently associated protein in the aggregate.

Suc-OVA appears to be monomeric under the conditions used in this study, just like N-OVA (Kosters et al. 2003; data not shown).

Adsorption kinetics of N-OVA, T-OVA, and suc-OVA at the air/water interface

InfraRed reflection absorption spectroscopy (IRRAS) and automated drop tensiometry (ADT) techniques were applied to study the adsorption kinetics of the ovalbumin forms at the air/water interface. Typical examples of IRRAS spectra of N-OVA and T-OVA are shown in Figure 2. Analysis of the IRRAS spectra using a spectral simulation method (Meinders et al. 2001; Meinders and de Jongh 2002) was performed to evaluate the interfacial and subphase protein concentrations (c_1 and c_2 , correspondingly) as well as the surface layer thickness (d) as a function of time. It can be observed in Figure 2 that both the water contribution around 3300 cm^{-1} (and a smaller one around 1680 cm^{-1}) and the proteins bands ($1700\text{--}1400\text{ cm}^{-1}$) are simulated well, and that these contributions are opposite in signal. This latter is related to the fact that where now proteins reside at the interface, the corresponding volume of water must be depleted from the interface. The “derivative”-like shape of the vibrations bands is related to both contributions of the complex refractive index, and is indicative for the presence of thick layers with a relatively high protein concentration in the surface layer compared to the subphase (Meinders et al. 2001).

From the spectral simulation, the concentration in the surface layer (c_1), the apparent layer thickness (d), and the subphase concentration (c_2) are derived. These values are presented in Figure 3 as a function of time of adsorption for N-OVA and T-OVA. It can be observed that at an N-OVA bulk concentration of 10 mg/mL , the surface layer protein

Figure 2. The overlaying experimental and simulated IRRAS spectra of N-OVA and T-OVA adsorbed from a bulk protein concentration of 10 mg/mL in 10 mM phosphate buffer ($\text{pH } 6.9$). The equilibration time for adsorption was 60 min .

Figure 3. The dependencies of (A) the surface layer thickness (d), (B) protein concentration in the surface layer (c_1), and (C) the ratio of the protein concentration in the surface layer and the subphase (c_2) as a function of adsorption time. The data are derived from global analysis of IRRAS spectra for N-OVA (open circles) and T-OVA (closed circles). The protein bulk concentration was 10 mg/mL in 10 mM phosphate buffer ($\text{pH } 6.9$).

concentration (c_1) is close to an initial “burst” phase saturation already within the first minutes of the IRRAS measurements ($1\text{--}2\text{ min}$) (Fig. 3B). The local concentration is, however, doubled in the following 5 h . This condensation is accompanied by a thinning of the surface layer (Fig. 3A), while the ratio between the surface and subphase protein concentration increases (Fig. 3C).

For T-OVA the accumulation of proteins at the interface is clearly slower; the surface protein concentration, layer thickness, as well as the ratio between the surface and subphase protein concentration require more time to reach equilibrium compared to N-OVA. Since c_1/c_2 is $\sim 10\%$ higher after 6 h of equilibration for T-OVA compared to N-OVA, and the layer thickness is also larger, it is evident that the total adsorbed amount is higher for the thermo-aggregated protein.

It was already demonstrated recently that the adsorption of suc-OVA displays a strongly reduced kinetics (related to the increased electrostatic repulsion), and slightly lower adsorbed amounts compared to N-OVA (Wierenga et al. 2003).

Development of surface pressure

While recording the development of the surface pressure on-line with the kinetics presented in Figure 3, it was observed that, although the adsorption kinetics appears slower for T-OVA, the development of surface pressure was much faster (data not shown). To illustrate this, drop tensiometric analysis was performed on samples with a lower bulk concentration (0.1 mg/mL) to allow the process to be monitored with a better time resolution. Indeed, it can be observed in Figure 4 that, while N-OVA displays a lag time of ~ 600 sec, as also reported previously (Wierenga et al. 2003), the adsorption of T-OVA results in an immediate development of a surface pressure, despite the observation that the average surface layer concentration is lower than that of N-OVA (Fig. 3). Apparently, the initial surface activity ($\delta\pi/\delta c$) for T-OVA is higher than that of N-OVA. In equilibrium, however, at this bulk concentration the surface pressure of T-OVA is significantly lower compared to N-OVA (respectively, 15 and 20 mN/m). Also, for suc-OVA, a slightly lower equilibrium surface pressure was found (Wierenga et al. 2003).

Conformational properties at the air/water interface

The conformation of T-OVA and N-OVA at the air/water interface was studied using IRRAS. Analysis of the amide I-band shape of IRRAS spectra using spectral simulation provides both information on the protein secondary structure and the degree of the aggregation of the protein in the surface layer (Meinders et al. 2001; Meinders and de Jongh 2002; Kudryashova et al. 2003). The amide I regions of experimental (exp) and simulated (sim) IRRAS spectra of N-OVA and T-OVA are shown in Figure 5. The fitting of the IRRAS spectrum of N-OVA (Fig. 5A) yields a structure where the content of β -sheet is 10% lower compared to

Figure 4. The dependencies of the surface pressure on time for N-OVA and T-OVA upon adsorption at the air/water interface measured by the automated drop tensiometer. The protein bulk concentration was 0.1 mg/mL in 10 mM phosphate buffer (pH 6.9).

Figure 5. The amide I region of IRRAS spectra of N-OVA (A) and T-OVA (B) adsorbed at the air/water interface and estimation of the secondary structure from the band-shape analysis of the spectra. The protein bulk concentration was 10 mg/mL in 10 mM phosphate buffer (pH 6.9). The adsorption equilibration time was 60 min. (exp) Curves correspond to experimental IRRAS spectra; (sim) curves correspond to simulated IRRAS spectra.

ovalbumin in the bulk solution. Analysis of the IRRAS spectrum of T-OVA shows that, compared to T-OVA in the bulk solution, no further unfolding takes place upon adsorption to the interface (Fig. 5B), but the content of antiparallel β -strands was slightly reduced. Taking into account that the degree of initial unfolding of T-OVA (in solution) is 10% compared to N-OVA (see Fig. 1), the degree of unfolding for N-OVA and T-OVA at the interface (at the secondary structure level) is thus comparable.

Previously we have shown that the presence of antiparallel β -structure (at 1624 cm^{-1}) can be used to monitor the degree of aggregation of ovalbumin at the interface from IRRAS spectra (Kudryashova et al. 2003). Figure 6A shows a number of IRRAS spectra of T-OVA at different time points of adsorption. It can be clearly observed that the intensity at 1624 cm^{-1} decreases in time (especially when it is viewed relative to the 1650 cm^{-1} intensity), suggesting that the protein disaggregates. Spectral analysis yields that upon adsorption to the interface during 1 h, the degree of aggregation in T-OVA is reduced by 30% compared to T-OVA in the bulk solution (Fig. 6B). After 4 h of incubation, only 25% of the original degree of aggregation remained.

IRRAS spectra of suc-OVA (data not shown) were comparable to those of N-OVA and did not reveal any indication

Figure 6. Disaggregation of T-OVA upon adsorption at the air/water interface. (A) Amide I region of IRRAS spectra of T-OVA for various times of adsorption. (B) The degree of aggregation of T-OVA as a function of the time as derived by spectral simulation of IRRAS spectra, where the ATR spectrum of T-OVA was taken as input spectrum. The protein bulk concentration was 10 mg/mL in 10 mM phosphate buffer (pH 6.9). The initial degree of aggregation was estimated from gel-permeation chromatography.

for the presence of aggregated material at the interface. Moreover, time-resolved fluorescence anisotropy measurements showed that the rotational correlation time of suc-OVA at the interface (16 nsec) was comparable to that of N-OVA (16–18 nsec) (see Kudryashova et al. 2003). This suggests that suc-OVA does not form aggregates at the interface.

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was applied to study the molecular dynamics of the proteins at the interface. FCS allows the direct determination of the translation diffusion of the proteins by monitoring the attached fluorescent dye. In addition, the molecular concentration can be determined. Using the same confocal setup as for FCS, one can also measure the depth profile of the protein concentration by scanning the laser as a function of the distance from the bottom to the air/water interface with a submicrometer resolution and simultaneously measure the fluo-

rescence. Figure 7A gives an example of such a depth profile of N-OVA at a bulk concentration of 10^{-4} mg/mL. By testing a whole series of different bulk concentrations, the observed fluorescence intensity in the bulk can be used to calibrate the observed intensities. This calibration is shown in Figure 7B and appears linear in the region tested. Using this calibration curve, the surface layer concentrations obtained using FCS for different bulk concentrations of N-OVA are shown in Figure 7C and correspond well to the results obtained using IRRAS.

At two positions in the profile shown in Figure 7A (indicated by the arrows), the translational diffusion time of the protein was derived from the autocorrelation function. In the bulk, the diffusion time of N-OVA is 0.3 msec (Fig. 8A, curve 1), yielding a diffusion coefficient of 0.8×10^{-10} m²/sec, which corresponds well to literature data obtained from adsorption kinetics (0.5 – 1.0×10^{-10} m²/sec) (de Feijter and Benjamins 1987; Pezennec et al. 2000) or those determined using a static imaging method (0.7 – 0.8×10^{-10} m²/sec) (Gibbs et al. 1991; Culbertson et al. 2002). Adsorption of N-OVA to the interface increases the translational diffusion time of the protein from 0.3 to 9 msec (Fig. 8A, curve 2), yielding a diffusion coefficient in the surface layer of 0.03×10^{-10} m²/sec.

T-OVA contains, next to 90% aggregated material, a minor fraction monomeric protein. From the bulk FCS signal two populations with different diffusion times (0.6 and 2.5 msec) were found. A small population reflects the diffusion of monomeric T-OVA with a diffusion coefficient of 0.6×10^{-10} m²/sec. This slower diffusion of monomeric T-OVA compared to N-OVA could be explained by the irreversible denaturation of T-OVA, thereby adopting a larger volume upon refolding (de Groot and de Jongh 2003). The second and major population found in T-OVA in the bulk (Fig. 8B, curve 1) can be assigned to the aggregated T-OVA molecules with diffusion times ~10 times longer compared to N-ovalbumin ($D = 0.09 \times 10^{-10}$ m²/sec). Such a diffusion constant is in a good agreement with that expected for a particle with an estimated molecular weight of 600 kDa, as found by gel permeation chromatography as described above.

T-OVA adsorbed at the interface shows also two populations of protein with different diffusion times (of 9 and 25 msec) (Fig. 8B, curves 2 and 3). The shorter diffusion time could correspond to monomeric molecules at the interface, while the longer diffusion time suggests the existence of the large protein clusters at the interface, which on this time-scale appear “frozen.”

Influence of the aggregation state on the rheological properties of ovalbumin

To study the influence of the aggregation state on the exerted surface pressure of the protein, pressure–area iso-

Figure 7. (A) Example of a depth profile of the protein concentration obtained for N-OVA by confocal laser scanning the sample from the glass bottom (just below 5.9 mm) to the air/water interface and measuring the fluorescence intensity. The protein bulk concentration was 10^{-4} mg/mL in 10 mM phosphate buffer (pH 6.9). (B) Calibration curve of the observed fluorescence intensity in the bulk phase as a function of the protein bulk concentration. (C) The dependence of the protein surface concentration on the protein bulk concentration estimated from the depth profiles of N-OVA using the calibration curve.

therms of the ovalbumin forms were recorded after 1 h of equilibration, while studying the molecular properties during compression of the surface layer on-line using IRRAS. The pressure–area isotherm of N-OVA (Fig. 9A) is composed of two regions—from 230 to 130 cm^2 , a region where the surface pressure gradually increases from 15 to 20 mN/m, while at surface areas smaller than 130 cm^2 the surface pressure increases sharply. As was also reported in our previous work (Kudryashova et al. 2003), during compression of a surface layer of N-OVA in the first region, both the surface concentration as well as the layer thickness are unchanged. It was also observed that during this initial compression N-OVA aggregated up to a degree of 20%–25% (Kudryashova et al. 2003). Only when very limited surface area was available was an increase in protein surface concentration observed (Kudryashova et al. 2003).

Upon compression of a surface layer composed of T-OVA, the surface pressure (Fig. 9B) as well as the surface protein concentration increase steadily (Fig. 10A), while the layer thickness does not change (Fig. 10B).

Also, the pressure–area isotherm was recorded for suc-OVA (Fig. 9C), as a protein with a strongly diminished tendency to aggregate due to electrostatic repulsion of the large number of carboxylic groups introduced onto the protein surface. As can be observed, the surface pressure hardly increases upon compression of a formed surface layer. To ensure that aggregation is suppressed in the case of suc-OVA upon adsorption at interfaces and upon surface compression as well, time-resolved fluorescence anisotropy (TRFA) measurements were performed, providing informa-

Figure 8. Autocorrelation curves obtained by fluorescence correlation spectroscopy in the bulk (curves 1; see arrow in Fig. 7A) and at the interface (curves 2/3) for N-OVA (A) and T-OVA (B). In all panels the fitted (thick lines) and experimental (thin lines) curves are shown. For T-OVA two populations are found at the interface.

Figure 9. Pressure–area isotherms for ovalbumin obtained by compression of the surface layer after adsorption during 60 min. The protein bulk concentration was 0.1 mg/mL in 10 mM phosphate buffer (pH 6.9). (A) N-OVA; (B) T-OVA; and (C) suc-OVA.

tion on the rotational correlation times of naturally abundant tryptophan residues in the protein at the interface. It was shown previously using this approach that the aggregation of N-OVA at the interface upon compression is accompanied by a substantial decrease in the rotational correlation time (from 16 to 7 nsec) (Kudryashova et al. 2003). This decrease was explained by the assumption that upon aggregation only the rotational modes of protein segments can be observed; the rotation of the protein globule as a whole is “frozen.” TRFA experiments on suc-OVA demonstrated that at the air–water interface its rotational correlation time (16 nsec) is comparable to that of the protein in the bulk, even upon the maximal compression (threefold reduction) of the surface layer (data not shown).

Discussion

The self-association behavior of proteins accumulated at interfaces may have a significant influence on the rheological properties of the system. In biological systems such as cells, for example, the fluidity of a membrane can be affected and thereby a large variety of biological processes. In

technological applications, as in foams, this association behavior will greatly determine the ability of proteins to form and stabilize air–water interfaces. It is the aim of this work to investigate the relation between protein self-association and the exerted surface pressure. For that reason three forms of chicken egg ovalbumin with different self-associating properties were compared. N-OVA in solution is a monomeric cigar-shaped protein, and, as shown previously (Kudryashova et al. 2003), does not aggregate upon adsorption to air–water interfaces. Only when a formed surface layer was compressed did aggregation take place, as evidenced by the appearance of an antiparallel β -strand vibration at 1624 cm^{-1} in infrared spectra. T-OVA consists of preformed protein aggregates that are noncovalently associated. Finally, suc-OVA was used, which does not tend to aggregate due to electrostatic repulsion of charged groups.

Influence of the aggregation state on adsorption rate

The accumulation of T-OVA from the bulk solution to the air–water interface is slower compared to that of N-OVA (or suc-OVA) (Fig. 3). Despite the ~ 10 times slower diffusion constant of the aggregates, as illustrated by the FCS data, the efficiency to “load” an interface with protein is higher since every successful “hit” at the interface brings ~ 12 – 16 proteins to be at or near the interface. Also, the enhanced exposed hydrophobicity of the heat-treated proteins, as was demonstrated by ANS binding (P.A. Wierenga, unpubl.), may speed up the net adsorption process by help-

Figure 10. T-OVA concentration in the (A) surface layer (c_1) and (B) surface layer thickness (d) evaluated from IRRAS as a function of the surface area upon compression of the surface layer. The protein bulk concentration was 0.1 mg/mL in 10 mM phosphate buffer (pH 6.9).

ing to overcome the kinetic barrier for adsorption, which is present for N-OVA as reported previously in detail (Wierenga et al. 2003). That this noncovalently associated protein aggregate falls apart on the hour timescale is demonstrated in Figure 6, and coincides with the thinning of the adsorbed layer (Fig. 3A).

Influence of the aggregation state on the surface activity

Although T-OVA has slower adsorption kinetics, the initial surface activity ($\delta\pi/\delta c$) of T-OVA is higher than that of N-OVA (Fig. 4). The analysis of the secondary structure content of the adsorbed proteins demonstrated (Fig. 5) that except for the presence of antiparallel β -strands between adjacent proteins in the aggregate, there was no major conformational difference, other than the aggregation state of the protein. A possible explanation could be that N-OVA easily adopts a preferential orientation at the air/water interface (Kudryashova et al. 2003), as was demonstrated by time-resolved fluorescence anisotropy experiments, where it could be shown that the molecular mobility along the “end-over-end” direction was restricted (Kudryashova et al. 2003). The aggregated proteins in T-OVA may not be able to adopt such preferential orientation at the interface readily, thereby generating an energetically less favorable situation that causes the exerted surface pressure to be higher.

Although the adsorbed amount at the interface of T-OVA is higher compared to N-OVA (Fig. 3), in time, however, the exerted surface pressure of N-OVA exceeds that of T-OVA (>0.5 h) (Fig. 4). This could be explained by the possibility that the monomeric protein can “fill” the upper interfacial layer much more efficiently than the more bulky protein aggregates. Even the dissociation of the complexes (Fig. 6) does not result in a surface activity comparable to that of N-OVA. Apparently, the condensation process of the surface layer (Fig. 3A) in the highly concentrated surface layer ($\sim 30\%$ of the volume is occupied by protein) does not allow an optimal rearrangement of the proteins anymore.

Influence of aggregation on surface layer compression

The pressure–area isotherm of an N-OVA surface layer suggests the existence of two molecular states of the protein in the film (see Fig. 9A). During the initial stage of compression of the surface layer, it was shown previously that the protein concentration in the layer is practically not changed (Kudryashova et al. 2003), while the degree of aggregation increased steadily with increasing surface pressure. Also, upon compression the molecular mobility showed more and more preferential rotation along the long axis of the cigar-shaped molecule (Kudryashova et al. 2003). Upon reducing the available area by more than a factor of 2, the surface pressure increases sharply (Fig. 9A), suggesting a different

conformational state, most probably of “gelled” proteins. In this situation the approximate majority of the proteins have developed antiparallel β -strands, and this aggregation might prohibit proteins to desorb from the interface, triggering the strong networking.

The preaggregated proteins behave differently upon compression: both the surface pressure and surface layer concentration increase instantly and monotonically, while the layer thickness remains constant (Figs. 9B, 10A,B). The small deviation from the linear increase in the surface layer concentration upon compression (Fig. 10) could be explained by the fact that part of the T-OVA material ($\sim 30\%$ as demonstrated in Fig. 6B) was disassociated during the 60-min precompression equilibration of the surface layer. Desorption of this small part of dissociated material could occur at the initial stage of compression. At the second stage of compression, the surface layer concentration shows a larger increase. This could then correspond to the situation in which all protein at the interface is aggregated protein. Most probably, these proteins form a “gelled” state, comparable as mentioned above for N-OVA, prohibiting desorption from the interface.

When N-OVA and T-OVA are now compared with the form of OVA that does not have any tendency to evolve aggregates, namely, suc-OVA, it is evident that the inability to self-associate results in a rather constant surface pressure upon compression (Fig. 9C). This highly net-charged protein may desorb easily, thereby maintaining an energetically optimal configuration of proteins accumulated at the interface.

Summarizing, using a combination of state-of-the-art biophysical and physicochemical methods, we have shown that protein self-association is an important factor defining the adsorption rate, surface activity, and the rheological properties of the system. Especially the inability to desorb from an interface once the protein is aggregated plays a crucial role.

Materials and methods

Ovalbumin purification

A batch of ovalbumin was purified from fresh hen eggs using the following semi-large-scale procedure, based on published purification protocols (Takahashi et al. 1991). From 9-d fresh hen eggs, the egg white was separated from yolk by hand. To the total egg white fraction (~ 300 mL), 600 mL of a 50 mM Tris-HCl (pH 7.5) buffer, containing 10 mM β -mercaptoethanol, was added. This solution was stirred for 24 h at 4°C . Subsequently, the solution was centrifuged for 30 min at $14,000g$ and 4°C . The pellet was discarded, while 1800 mL of 50 mM Tris-HCl (pH 7.5) was added to the supernatant. After a 30-min period of gentle stirring, the solution was filtered over a paper filter (Schleicher & Schuell). To the filtrate, 500 g of DEAE Sepharose Cl-6B (Pharmacia) was added, followed by overnight incubation at 4°C under gentle stirring. Next, the solution was filtered over a glass filter (G2) followed by

extensive washing with 10 L of demineralized water and 5 L of 0.1 M NaCl, successively. The protein was eluted stepwise with subsequent steps of 1 L of 0.1, 0.15, 0.2, 0.25, and 0.35 M NaCl. The latter two eluents did contain some ovalbumin but appear slightly yellowish and were discarded. The 0.15 and 0.2 M NaCl batches were pooled and concentrated using a Millipore Ultrafiltration Unit with a 30-kDa molecular mass cutoff membrane. The concentrated solution was dialyzed extensively against demineralized water, freeze-dried, and stored at -40°C until further usage. The yield of this procedure is generally ~ 1.0 g of ovalbumin per egg, and the efficiency of isolation is $>90\%$. The purity of the protein was $>98\%$, as estimated from densitometric analysis of SDS-PAGE gels.

Preparation of thermo-denatured ovalbumin (T-OVA)

T-OVA was prepared by incubation of 10 mg/mL ovalbumin solution in 10 mM sodium phosphate buffer (pH 6.9) at 78°C for 30 min. The analysis of the degree of aggregation in T-OVA was carried out on a Superdex S200 column (Pharmacia Biotech). The column was equilibrated with 10 mM sodium phosphate buffer (pH 6.9) containing 50 mM NaCl at 20°C . Protein detection in the eluent was at 280 nm.

Preparation of succinylated ovalbumin

Primary amino groups in ovalbumin (lysine residues) were succinylated as described in detail elsewhere (Kosters et al. 2003). A 100-mL solution of 25 mg/mL ovalbumin (0.55 mM) in demineralized water was set at pH 8.0 by the addition of 3 M NaOH using pH-stat titration equipment (at room temperature). Next, 300 mg of solid succinic anhydride was added stepwise in five portions, yielding a final concentration of 30 mM succinic anhydride. During the addition of succinic anhydride, the pH was maintained at $8.0 (\pm 0.1)$ by the addition of 1 M NaOH using pH-stat equipment. After addition, the solution was stirred for another 30 min, followed by extensive dialysis against demineralized water at 4°C and subsequent freeze-drying. The material was stored at -20°C until use. The degree of modification was 64% (13 out of 20 lysine residues were modified), as determined by both the OPA assay (detection of primary amino groups) as described by Kosters et al. (2003), and the adapted Woodward assay (detection of carboxylates), as described by Kosters and de Jongh (2003). This protein was shown to have still a globular packing with a significant amount of secondary structure, but with lower free energy of conformational stabilization (Kosters et al. 2003).

Labeling of ovalbumin with BODIPY TMR

The labeling of ovalbumin with BODIPY TMR, STP ester (B-10002) was carried out according to the “Molecular Probes” protocol (<http://www.probes.com>). Typically, 5 mg of ovalbumin was dissolved in 1 mL of 0.1 M sodium bicarbonate buffer (pH 9.0). A stock solution of BODIPY TMR STP ester (5 mg in 0.5 mL) was prepared in deionized water. While stirring the protein solution the reactive dye solution was added in several portions. The mixture was stirred for 1 h at room temperature. The reaction was stopped by the addition of 0.1 mL of 1.5 M hydroxylamine solution (pH 8.5). The conjugate was separated from nonreacted reagent on a Sephadex G-25 gel filtration column (10×300 mm), equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The degree of the modification (N) was determined using the following equation:

$$N = A_{\text{max}} \times \text{MW}/[\text{protein}] \times \varepsilon_{\text{dye}},$$

where A_{max} is the absorbance of the protein–dye conjugate at 544 nm, MW is the molecular weight of the protein, ε_{dye} is the extinction coefficient of BODIPY TMR at its absorbance maximum ($60,000 \text{ cm}^{-1} \text{ M}^{-1}$), and where the protein concentration is expressed in milligrams per milliliter. The determined average degree of modification of ovalbumin was 0.3 mol dye per mol protein.

Automated drop tensiometry (ADT) experiments

An automated drop tensiometer (ADT; ICT France) was used to measure the interfacial tension between the liquid and gas phases. The interfacial tension was determined by means of drop shape analysis of a bubble of air formed within a cuvette containing 0.1 mg/mL protein solution in 10 mM phosphate buffer at pH 6.9. The bubble was illuminated by a light source, and its profile was imaged and digitized by a CCD camera and a computer. The profile was used to calculate the interfacial tension using Laplace’s pressure equation. Temperature was kept constant at $22^{\circ} \pm 1^{\circ}\text{C}$.

Langmuir trough experiments

An automated computer-interfaced teflon Langmuir trough with two compartments for both a protein-sample and a protein-free reference solution, equipped with two moving barriers (Nima 601M; UK) was used to study the rheological properties of the air–water interface. The dimensions of the Langmuir trough were 10×26 cm (390 mL) for the sample compartment and 5×26 cm (195 mL) for the reference compartment. A pressure sensor connected to a paper Wilhelmy plate measured the surface pressure. Prior to any measurements the film balance was calibrated using standard weights. The surface tension (σ_0) of the buffer solution in the reference compartment was 72.2 mN/m .

Typically, 390 mL of a protein solution ranging from 0.1 to 50 mg/mL in 10 mM phosphate buffer (pH 6.9) (in Millipore water) was transferred to the sample compartment of the Langmuir trough. After cleaning the surface with a microtip, the surface pressure ($\pi = \sigma - \sigma_0$) of the protein solution was followed as a function of the time. The pressure–area (π – A) isotherms were recorded after 2 h of equilibration at a barrier speed of 3–6 cm/min, depending on the maximal area used, to obtain compression rates of $\sim 20\%$ of the initial area per minute (maximal area is 230 cm^2). Simultaneously, IRRAS or TRFA measurements were performed. All experiments were carried out in a thermostated room at $22^{\circ} \pm 1^{\circ}\text{C}$.

IRRAS measurements

The IRRAS spectra acquisition was described previously (Kudryashova et al. 2003). Spectra were acquired using an Equinox 50 FTIR spectrometer attached to an external reflection module (Bruker XA-500), equipped with a liquid-nitrogen-cooled MCT detector. The spectrometer was placed on an optical bench table to minimize and dampen external vibrations. The incident and reflecting angle of the infrared beam was 30° in all studies described here. The FTIR spectrometer was purged with a constant flow of dry air.

ATR spectra were accumulated on a BioRad FTS 6000 spectrometer equipped with an MCT detector using a germanium crystal (45° , trapezoid crystal yielding six internal total reflections).

The experimental equipment and measurement procedure were described in detail elsewhere (Meinders et al. 2000, 2001). Fourier Transform infrared (FTIR) spectra (ATR and IRRAS) were acquired from 1000 to 4000 cm^{-1} with a spectral resolution of 2 cm^{-1} . For each spectrum, 100 scans were accumulated and averaged. Reference spectra of protein free samples were recorded under identical conditions. External reflection absorption spectra of the protein are presented as $-\log(R_{\text{prot}}/R_{\text{buf}})$, where R_{prot} is the reflection spectrum of protein solution and R_{buf} is the reflection spectrum of the buffer solution. The result spectra were smoothed with a nine-point Savitsky-Golay smooth function to $\sim 8 \text{ cm}^{-1}$ resolution.

Analysis of the secondary structure of the proteins using ATR FT-IR spectra

Self-deconvolution of the ATR spectra in the amide I region to estimate the secondary structure percentage was made with GRAMS/IR software (Buck Scientific). The assignment of peaks to secondary structure elements was made according to Goormaghtigh et al. (1994) and Dong et al. (2000).

Spectral simulation

To extract information from IRRAS spectra on the local concentration in the surface layer (c_1), the layer thickness (d), and the protein concentration underneath the surface layer (c_2), the experimental spectra are simulated using the absorption spectra obtained by ATR-FT IR of the different constituents as input. The details of the theory and application of the procedure are described elsewhere (Meinders et al. 2001; Kudryashova et al. 2003). In short, the simulation is based on the stratified layer model, considering a system consisting of a top layer with a particular thickness and a subphase over the solution. Introduction of additional layers did not provide a better description of the system, while description of the system with only a single layer was clearly not sufficient. The top layer thickness, interface, and subphase protein concentrations are fit parameters.

Changes in the secondary structure of the protein in the surface layer were monitored using spectral simulation by adjusting the ATR spectrum, with reference spectra representing the secondary structure contributions (e.g., -10% β -strand and $+10\%$ random coil). The new composed absorption spectrum was then used as input spectrum in the spectral simulation. The details of the application of the simulation procedure have been described before (Meinders et al. 2001). In a similar way, the degree of aggregation of the protein adsorbed at the interface was estimated, taking into account the antiparallel β -sheet structure as an additional component of the secondary structure of the protein. The presence of the antiparallel β -structure absorbing at 1624 and 1693 cm^{-1} , well distinct from the parallel β -structures (1638 and 1686 cm^{-1}), was used as a criterion for evaluation of the degree of aggregation of ovalbumin at the interface as described before (Kudryashova et al. 2003).

Fluorescence correlation spectroscopy (FCS) experiments

The FCS setup has basically been described in detail elsewhere (Hink et al. 1999, 2003). For excitation of the Bodipy TMR-containing proteins, a green helium neon laser (543 nm) was used. The alignment and focusing of the setup was frequently checked

by measuring the autocorrelation function of 0.2 nM 5-carboxy-tetramethylrhodamine (5-TAMRA; Molecular Probes Inc.) in 10 mM buffer (pH 7.0). A description of the theoretical background and the evaluation of the autocorrelation functions have been reviewed (Hess et al. 2002). Fitting the experimental data to a 3D-diffusion model was performed as described by Hink et al. (2003). Two parameters were recovered: the diffusion time and the number of labeled protein. Since the confocal detection volume is obtained from experiments on a calibration dye, both the translational diffusion constant can be calculated from the diffusion time as well as the used concentration. A depth profile of a 10- μL protein solution (0.1 mg/mL) was made to assess local concentration by confocal scanning through the droplet from the glass plate until the air phase with a step resolution of 0.5 μm (Gluck et al. 1996).

TRFA measurements

Time-resolved fluorescence measurements were carried out using mode-locked continuous wave lasers for excitation and time-correlated single photon counting as the detection technique. The measurements were carried out using the procedure described before (Kudryashova et al. 2001, 2002, 2003). The temperature of all experiments was $22^\circ \pm 1^\circ\text{C}$.

Data analysis was performed using a home-developed computer program (Digris et al. 1999; Novikov et al. 1999). The analysis of the data was carried out as described before (Kudryashova et al. 2001, 2002, 2003).

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