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Adsorption of Denaturated Lysozyme at the Air–Water Interface: Structure and Morphology.

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ABSTRACT: The application of protein deuteration and high flux neutron reflectometry has allowed a comparison of the adsorption properties of lysozyme at the air–water interface from dilute solutions in the absence and presence of high concentrations of two strong denaturants: urea and guanidine hydrochloride (GuHCl). The surface excess and adsorption layer thickness were resolved and complemented by images of the mesoscopic lateral morphology from Brewster angle microscopy. It was revealed that the thickness of the adsorption layer in the absence of added denaturants is less than the short axial length of the lysozyme molecule, which indicates deformation of the globules at the interface. Two-dimensional elongated aggregates in the surface layer merge over time to form an extensive network at the approach to steady state. Addition of denaturants in the bulk results in an acceleration of adsorption and an increase of the adsorption layer thickness. These results are attributed to incomplete collapse of the globules in the bulk from the effects of the denaturants as a result of interactions between remote amino acid residues. Both effects may be connected to an increase of the effective total volume of macromolecules due to the changes of their tertiary structure, that is, the formation of molten globules under the influence of urea and the partial unfolding of globules under the influence of GuHCl. In the former case, the increase of globule hydrophobicity leads to cooperative aggregation in the surface layer during adsorption. Unlike in the case of solutions without denaturants, the surface aggregates are short and wormlike, their size does not change with time, and they do not merge to form an extensive network at the approach to steady state. To the best of our knowledge, these are the first observations of cooperative aggregation in lysozyme adsorption layers.

Introduction

The relationship between protein structure and the interfacial properties of its solutions is an important problem for fundamental surface science. The interest in this subject has also been stimulated to a significant extent by the broad applications of protein emulsions and foams in the food, cosmetic and pharmaceutical industries. The possibility of predict the stability and properties of fluid disperse systems on the basis of the molecular structure(s) of the components is of major significance for applied science in these areas. Such predictions are, however, unattainable without detailed information on the interfacial properties of protein solutions as resolved using a variety of surface-sensitive techniques.^{1–4} Additionally, the general knowledge of protein conformations at fluid interfaces is still quite limited, and the degree of denaturation of the globular structure of proteins in the surface layer is a subject of extensive discussion. The most common technique used to study protein solutions is probably surface tensiometry, but it does not provide direct information about the protein structure.⁵ Even in the case of one of the most studied model proteins, lysozyme, the conclusions of different research groups are frequently inconsistent. The radiolabeling technique,⁶ neutron reflectometry (NR),^{7,8} ellipsometry,⁹ and surface dilatational rheology^{10–12} do not indicate significant changes of the tertiary structure at the air–water interface. On the contrary, external reflection FTIR spectroscopy shows clear changes of the lysozyme secondary structure at the air–water interface by comparison with the adsorption layers of other proteins,¹³

suggestive of possible concomitant changes of the tertiary structure. Further, a comparison of results from X-ray reflectometry (XRR) with those from modeling work led to the conclusion that lysozyme unfolds in the course of adsorption.^{14,15}

The dynamic surface properties of lysozyme solutions have some important peculiarities in comparison with those of solutions of other globular proteins. Numerous authors have discussed a long induction period of the kinetic dependencies of surface tension of lysozyme solutions.^{10,13,16–22} Stebe and co-workers, using fluorescence microscopy, have shown that this phenomenon is a consequence of two-dimensional phase transitions in the surface layer.²¹ At the same time, the strong dependence of the induction period on the solution age,²² and on the method of injection of the protein into the subphase,¹³ have not yet been explained. The adsorption kinetics of lysozyme is characterized by a strong energy barrier at the air–water interface even at pH values close to the isoelectric point.²³ Moreover, the kinetic dependencies of the adsorbed amount can be non-monotonic and go through a local maximum after a fast increase during the first adsorption step. Slow protein adsorption implies difficulties in obtaining equilibrium of the adsorbed layer. Indeed adsorption can be practically irreversible and the surface properties can depend on its method of formation.²⁴ Therefore some discrepancies between the conclusions of different authors can be connected with the non-equilibrium nature of the systems under study.

Another characteristic feature of lysozyme is the relative stability of its tertiary structure in the surface layer to the action of chemical denaturants.²⁵ While the denaturation of the globular structures of bovine serum albumin (BSA) and β -lactoglobulin (BLG) occurs at lower denaturant concentrations in the surface layer than in the bulk phase,^{26–28} Perriman et al. have observed the opposite behavior in lysozyme solutions.⁸ This result agrees with dilational surface rheology measurements.^{10,11} The addition of strong denaturants to dilute lysozyme solutions leads to smoother changes of the kinetic dependencies of the dynamic surface elasticity than for solutions of other globular proteins, where strong peaks of the surface elasticity are observed.^{26–28} Moreover, the addition of urea does not change the shape of the corresponding kinetic curves up to very high concentrations of the denaturant (~ 10 M).¹⁰

Globular proteins can be adsorbed in an already unfolded state from solutions containing denaturants, or the adsorption can be accompanied by unfolding if the proteins are relatively stable in the bulk phase.²⁸ The kinetic dependencies of the dynamic dilational surface elasticity indicates strong conformational transitions of protein molecules in the course of adsorption from solutions containing denaturants but do not give quantitative structural details of this process. Measurements of NR and XRR as a function of surface age can be more informative in this respect, but corresponding kinetic studies have been limited in the past due to the low scattering contrast of the protein and resulting long acquisition times. For example, XRR data for lysozyme solutions in 3.5 M guanidine hydrochloride (GuHCl) at three different surface ages (0.5, 2 and 18 h) have shown strong changes of the density profile normal to the interface, but they did not allow tracing of the main adsorption steps.⁸

Recent developments of the NR technique, and the means to deuterate protein molecules,²⁹ have resulted in the possibility of exploiting higher neutron flux in conjunction with samples that scatter more strongly than were previously available. In the present work, NR is applied to characterize the adsorption of deuterated lysozyme from solutions of two concentrations in the absence of denaturant in the bulk phase, and from solutions at the higher of the two protein concentrations in the presence of a high concentration of a strong denaturant (urea or GuHCl). Our approach is to resolve directly the surface excess during adsorption, and provide the thickness and volume fraction of the adsorption layers at steady state. Further, as many studies have been conducted using oscillating barriers over the years, the possibility of this approach introducing significant changes in the rate of formation of the adsorbed layer and its structure is investigated using two methodologies: adsorption at fixed surface area (static conditions), and adsorption during cyclic perturbations of the surface area (dynamic conditions). Another feature of our approach consists of the direct observation of strong differences between mesoscopic morphologies of the surface layer during the adsorption of lysozyme both in the absence and presence of strong denaturants using Brewster angle microscopy (BAM).

Experimental Section

Yeast expression of perdeuterated lysozyme (dLYS)

A recombinant *Pichia pastoris* expression system was designed for the production of hen lysozyme following the basic protocol described by Mine et al.³⁰ The coding sequence for hen egg lysozyme (PDB 1HEL) was synthesized by GeneArt, Regensburg, Germany and inserted into the plasmid pPICZaA

(Invitrogen) 3' to the vector-encoded *Saccharomyces cerevisiae* α -factor secretion signal. *Pichia pastoris* X33 cells were transformed with the linearized recombinant plasmid DNA. Colonies secreting lysozyme were isolated after selection on zeocin plates. Perdeuteration of the secreted lysozyme was carried out using *Pichia pastoris* high cell density fermenter culture in deuterated minimal medium.²⁹ dLYS expression was induced and maintained over 5 days by the daily addition of 1 % d₄-methanol (Euriso-top; ≥ 99.8 %).

Purification of dLYS

The culture supernatant was concentrated about tenfold using a Vivaflow 200 cross flow cassette with a MWCO of 5,000 (Sartorius) and buffer exchanged against 50 mM Tris (Sigma Aldrich; ≥ 99 %)-HCl pH 7.8. Lysozyme was isolated by cation exchange chromatography on a SP-Sepharose column (GE-Healthcare), which was eluted with an NaCl (Sigma Aldrich; ≥ 99.8 %) gradient from 0–1 M in 50 mM Tris-HCl pH 7.8. dLYS was further purified by size-exclusion chromatography on a Sephadex S75 column (GE-Healthcare) and concentrated to 1.2 mg/mL in deuterated buffer (20 mM Tris-DCl pD 7.8, 150 mM NaCl). The deuteration level of dLYS was measured by mass spectrometry. Deuterium in non-exchangeable positions replaced hydrogen at a level close to 100 %. The dLYS sample was stored at 5 °C.

Additional sample preparation details

To match the experimental conditions used in previous work, we performed the adsorption measurements at concentrations that were 3–4 orders of magnitude lower than that of the stock solution and in 40 mM standard phosphate buffer (SPB; Na₂HPO₄–NaH₂PO₄; Sigma Aldrich; both ≥ 99 %) at pH 7. The ionic strength of solutions was increased by the addition of NaCl to 150 mM, which was required to prevent aggregation of the deuterated protein. It was observed that fast dilution of the dLYS stock solution with SPB resulted in its precipitation. Therefore dilution was performed by the drop-wise addition of SPB buffer without or with dissolved denaturants into the dLYS stock solution. Urea (Roth; ≥ 99.6 %) and GuHCl (Sigma Aldrich; ≥ 99 %) were used as received and dissolved in SPB prior to their respective additions to the protein stock solution. Pure H₂O was generated by passing deionized water through a Milli-Q purification system. D₂O (Sigma Aldrich; 99.9 atom% D) was used without further purification. The purity of the buffer solution was checked using ellipsometry and the measured phase shift was equivalent to that of pure water, indicating the absence of surface-active impurities. All dLYS solutions were used fresh without storage: measurements of the surface properties were started within 1 h after sample preparation. All the measurements were carried out at 22 ± 1 °C.

Trough measurements

The surface tension was measured by the Wilhelmy plate method using a roughened glass plate attached to an electronic balance. The dynamic dilatational surface elasticity was measured by the oscillating ring method. The corresponding experimental equipment and procedures have been described in detail elsewhere.^{10,31} The surface of the solution under investigation was periodically expanded and compressed as a result of oscillations of a glass ring along its axis. The ring was partly immersed into the liquid with its axis perpendicular to the liquid surface and its internal surface was grounded to improve wetting. The ring oscillations led to regular oscillations of the liquid surface area and surface tension of the solu-

tion as a result of periodical changes of the meniscus shape at the internal surface of the ring. The surface tension of the investigated liquid was measured inside the ring using a Wilhelmy plate. The relative amplitude and frequency of the surface area oscillations were 7 % and 0.1 Hz, respectively.

The real ε_r and imaginary ε_i components of the dilational dynamic surface elasticity ε were calculated from the amplitudes of oscillations of the surface tension $\delta\gamma$ and surface area δS , and the phase shift between the oscillations of these two quantities. The imaginary part of the complex dynamic surface elasticity of the solutions under investigation proved to be much less than the real part. Only the results for the real part of the dynamic surface elasticity are presented.

NR measurements

NR is a technique used to resolve the adsorbed amount and structure of molecules adsorbed at fluid interfaces.⁴ Measurements were performed on the time-of-flight reflectometer FIGARO at the Institut Laue-Langevin (Grenoble, France).³² The neutron reflectivity R is defined as the number ratio of neutrons in the specular reflection to those in the incident beam. Profiles of $\log_{10}(R)$ were generated as a function of the momentum transfer, $q = 4\pi \sin(\theta)/\lambda$, where $\theta = 3.8^\circ$ is the incident angle and $\lambda = 2\text{--}30 \text{ \AA}$ is the wavelength range. The resolution in wavelength used was 7 %. The background was subtracted from the data through use of the area detector.

The principles and latest capabilities of the NR technique have been described elsewhere.^{4,33,34} In short, the scattering length density of a substance, ρ , is defined as its coherent scattering length, b , divided by the molecular volume, M_v , where b is equal to the sum of the values of the coherent scattering cross section, Σb_i , over all nuclei i . dLYS solutions without denaturants were prepared in ACMW (air contrast matched water; 8.1 % v/v D₂O in H₂O to give a scattering length density of zero). This approach emphasized the sensitivity of the measurement to the surface excess and thickness of an adsorption layer of deuterated protein at the air–water interface; deuteration of the protein was essential in order to resolve the thickness information so that the signal was above the background to high enough values of q . dLYS solutions with denaturants, as a result of their high concentrations, were prepared in H₂O in order to minimize the scattering length density of the subphase, $\rho = 0.15 \times 10^{-6} \text{ \AA}^{-2}$ for urea and $\rho = 0.45 \times 10^{-6} \text{ \AA}^{-2}$ for GuHCl. Data acquisitions of 5 min were made consecutively on 6 samples in parallel over several hours to optimize the use of neutron beam time, which resulted in one measurement per sample in every 0.5 h.

The analysis of the NR data was performed using the Moto-fit software³⁵ based on Abeles matrix method applied to stratified layers.³⁶ The values of the thickness, τ , and volume fraction, v_f , of a single adsorbed layer of dLYS at the air–water interface were fitted simultaneously in order to calculate the surface excess using $\Gamma = (\rho \times \tau \times v_f \times M_w) / (N_A \times b)$, where M_w is the molecular weight of the protein, and N_A is Avogadro's number. The value of ρ for dLYS was calculated as follows. The value of M_w for hydrogenous hen egg lysozyme is 14313 g/mol, which was calculated from the amino acid sequence generated using the ProtParam software (<https://web.expasy.org/protparam>). The corresponding value of dLYS in deuterated buffer is 15272 g/mol (assuming 100 % deuteration). An actual value of M_w of 14997 g/mol for dLYS in hydrogenous buffer was measured using mass spectrometry, from which 29 % of the protons were assumed to be labile. As

such, the value of b for dLYS was calculated as 10746 fm in ACMW (with no added denaturants) and 10545 fm in H₂O (with added denaturants). A molecular volume of 16960 $\text{\AA}^3$ for dLYS was calculated from the mass density of the hydrogenous protein of 1.4 g/cm³.^{37,38} As such, the values of ρ used in the model were $6.34 \times 10^{-6} \text{ \AA}^{-2}$ in ACMW and $6.21 \times 10^{-6} \text{ \AA}^{-2}$ in H₂O. The value of the residual background of the measurement used was 1.5×10^{-6} and the layer roughness values were fixed at 0.3 nm in line with capillary wave theory.^{39,40}

It is interesting to consider implications of the broadening of the real space density profile normal to the interface from capillary waves for an adsorbed layer of protein globules. The fitted adsorption layer thickness is the width of the density profile when the density is $0.5 \times$ its maximum value. Nevertheless, the actual thickness of an adsorbed layer of globules may be closer to the width of the distribution at a lower density due to their curvature, thus implying a larger size. As an example, the width of the density distribution is 0.5 nm greater when the density is $0.2 \times$ its maximum value. Therefore in our interpretations of the data we consider that the actual dimensions of the globules at the interface may be around half a nanometer larger than the fitted adsorption layer thickness.

BAM measurements

BAM is a technique used to image lateral inhomogeneity at fluid interfaces on the micrometer scale.^{41,42} A Nanofilm EP3 microscope was used with a 10 $\times$ objective. Due to the high mobility of the films automatic focusing was set to minimum. Both angles of polarization for the laser beam and analyser were set to zero, and in this way background subtraction was not needed.⁴³ The images were recorded at an incident angle of 53.1° (the Brewster angle of the air–water interface) for the measurement without added denaturant and 53.9° for the measurement in 5 M urea solution as a result of the refractive index difference.⁴⁴ Constant gamma correction was applied to the images to enhance uniformly the appearance of the interfacial morphologies. Note that in spite of this procedure, the optical contrast in the images presented remains rather low. This may be explained in terms of low anisotropy in the observed protein aggregates, in strong comparison with, e.g., liquid condensed domains of phospholipids.^{45,46}

Fig. 1. Kinetic dependencies of the real part of the dynamic surface elasticity (purple circles) and the surface tension (orange diamonds) of 3.5 μM lysozyme solutions.

Fig. 2. Kinetic dependencies of the surface excess of 0.35 μM (green diamonds) and 3.5 μM (black squares) dLYS solutions in ACMW without added denaturant recorded under (A) static and (B) dynamic conditions.

Fig. 3. Steady state neutron reflectivity profiles of 0.35 μM (green) and 3.5 μM (black) dLYS solutions in ACMW without added denaturant recorded under (A) static and (B) dynamic conditions; the insets are the scattering length density (SLD) profiles normal to the interface.

Results and Discussion

Lysozyme adsorption

The majority of experimental data presented in this work was obtained at a bulk lysozyme concentration of 3.5 μM . In this case the adsorption is slow enough and the main steps of this process can be followed by measuring the kinetic dependencies of surface properties.^{10,11} For reference, a description of the kinetic dependencies of the surface tension and the real part of the dynamic surface elasticity is given for solutions of hydrogenous lysozyme without addition of NaCl measured under dynamic conditions involving sinusoidal oscillations of the surface area (Fig. 1). An induction period is observed when the surface tension remains equivalent to that of pure water while the surface elasticity remains close to zero for more than 1 h after the surface formation; the values start to change slowly only after this period. Note that although it is difficult to obtain a satisfactory reproducibility of the induction period, the replotting of the kinetic data of the dynamic surface elasticity and surface pressure π as ϵ_r versus π plots, where π is defined as the surface tension of pure water minus that of the protein solution always leads to a single curve.¹⁰

NR data of pure dLYS solutions were measured both in static (constant surface area) and dynamic (sinusoidal oscillations performed in the same way as above) conditions (Fig. 2). The change in the surface excess of lysozyme with the surface age is smoother than those of the dynamic surface elasticity and surface tension, and an induction period is not observed. These kinetic dependencies of the surface excess are similar to those of the ellipsometric angle, which also does not display an induction period, unlike the changes in the dynamic surface elasticity and surface tension.¹⁰ The surface excess takes more than 6 h to reach steady state after the surface formation in the static condition, but the continuous oscillations of the surface area in the dynamic condition result in faster equilibration and at a lysozyme concentration of 3.5 μM the surface excess reaches a constant value of 1.7 mg/m^2 in about 2 h (Fig. 2).

The continuous growth of the adsorbed amount at the start of adsorption (Fig. 2), when the surface tension is constant and close to the value of pure water (Fig. 1), can be described in terms of the heterogeneity of the adsorption layer.²¹ The first adsorption step consists of the gradual growth of the size and number of non-interacting protein islands (two-dimensional

aggregates) at the interface, and thereby of the total adsorbed amount. The surface tension starts to decrease only when the aggregates start to interact at the interface. In the dynamic condition, it is important to bear in mind that the oscillations of the barriers not only cycle the surface area but also apply convection in the bulk close to the sub-surface. The faster increase of the surface excess may therefore be attributed to enhanced diffusion in the liquid close to the sub-surface and/or induced changes in the heterogeneity of the interface. Indeed the influence of these mechanical perturbations on the adsorption kinetics indicates that it is mainly diffusion controlled.

The decrease of lysozyme concentration by an order of magnitude, where an induction period in the changes of the real part of the dynamic surface elasticity and the surface tension has also been observed,¹⁰ does not change noticeably the adsorption kinetics in the static condition (Fig. 2A). The difference between the steady state surface excess for the two concentrations appears only in the dynamic condition (Fig. 2B). The difference reaches $\sim 0.4 \text{ mg/m}^2$ and is a consequence of the slower equilibration at the lower concentration probably due to the lower concentration gradient in the bulk phase and thereby to the slighter influence of the induced convection.

The NR data were also modeled to give τ and v_f at the end of the experiment when the surface age was 6 h (Fig. 3 and Table 1); real space scattering length density profiles normal to the interface are shown as insets in the figure. Effectively the layer thickness determines the gradient of the reflectivity profiles with steeper curves resulting from thicker layers.

Table 1. Fitted thickness and volume fraction of the adsorbed layer of dLYS following 6 hours of equilibration in the 4 conducted neutron reflectometry experiments without added denaturant in the bulk.

Expt.	Concentration (μM)	Thickness (nm)	Volume Fraction	Surface Excess (mg/m^2)
Static	0.35	1.5 ± 0.1	0.61 ± 0.02	1.4 ± 0.1
Static	3.5	1.7 ± 0.1	0.57 ± 0.01	1.4 ± 0.1
Dynamic	0.35	1.5 ± 0.1	0.62 ± 0.03	1.3 ± 0.1
Dynamic	3.5	1.8 ± 0.1	0.67 ± 0.02	1.7 ± 0.1

The fitted adsorption layer thickness is 1.5 nm in the static and dynamic conditions for the lower of the two measured protein concentrations. These numbers correspond to steady state values at the end of the experiments. The fitted adsorption layer thickness is slightly higher at 1.7 nm (static) and 1.8 nm (dynamic) for the experiments conducted at the higher of the two bulk concentrations. Even if we take into account the point that the actual thickness of the protein molecules at the interface may be higher than the fitted adsorption layer thickness by around half a nanometer due to the broadening of the density profile normal to the interface from capillary waves in combination with the globular shape of the molecules (see Experimental Section), the obtained values are all still lower than even the short axial length of the globular dimension of lysozyme ($4.5 \text{ nm} \times 3.0 \text{ nm} \times 3.0 \text{ nm}$).⁴⁷ In comparison with these results, Lu et al. have obtained a thickness of the lysozyme adsorption layer of 3.0 nm at a higher concentration and concluded the sideways-on orientation of the globules in the surface layer.⁴⁸ Furthermore, Perriman et al. have reported a thickness of 4.7 nm at the high lysozyme concentration of 70 μM , which was associated with the headways-on orientation or the formation of a bilayer.⁸ As such, the thinner interfacial layers observed from dilute solutions in the present work

indicate a degree of deformation of the protein globules upon adsorption at the air–water interface that has not been observed in structural studies on more concentrated samples. Another explanation could be the globule unfolding at the interface; however, a strong difference in the adsorption properties of native and denaturated lysozyme (see below) as well as the numerous results of other authors^{6–9} make this possibility less likely.

BAM allows visualization of heterogeneity in the interfacial layer on the micrometer scale, and the technique was applied to a pure 3.5 μM lysozyme solution during the course of adsorption (Fig. 4). Separate two-dimensional elongated aggregates with rounded boundaries that have a mean length of several 100s of μm and a mean width of about 10–20 μm can be observed initially. The interface is probably not fully covered with protein as islands with pronounced circular holes appear to be separated by channels. Over the following hour, these patches form much larger agglomerations, although individual entities can still be distinguished. According to Stebe *et al.* the initial step of lysozyme adsorption corresponds to the coexistence of gaseous and liquid-expanded surface phases.²¹ The shapes of the aggregates in ref. 21 differ noticeably from those in Fig. 4, probably because of different protein concentrations and procedures of the sample preparation.

In the course of adsorption, the lysozyme layer becomes denser and the regions of the gaseous surface phase disappear gradually with the patches joined up to form a dense two-dimensional network. It is interesting that the film was rather mobile for the first 15 min of the experiment, during which time local flows can exert random influences on the interfacial morphologies. Following this time, after the surface excess

Fig. 4. BAM images of an adsorbed layer from 3.5 μM lysozyme solution after (A) 0, (B) 2, (C) 5, (D) 15, (E) 60 and (F) 120 min.

Fig. 5. Kinetic dependencies of the real part of the dynamic surface elasticity (purple circles) and the surface tension (orange diamonds) of 3.5 μM LYS solutions in (A) 6 M urea and (B) 6 M GuHCl. Some of the data are produced from ref. 10.

Fig. 6. Kinetic dependencies of the surface excess of 3.5 μM dLYS solutions in H₂O with added 6 M urea (red circles) and 6 M GuHCl (blue triangles) recorded under (A) static and (B) dynamic conditions.

exceeds ~ 1 mg/m², the film became more rigid. Finally, after 1–2 h, the BAM images become more homogeneous, indicating the approach to a continuous liquid-expanded surface phase at steady state, even though steady state in the surface excess has not been fully reached by that time (Fig. 2).

By means of comparison, the adsorption of human serum albumin (HSA) at the air–water interface also leads to the formation of a heterogeneous layer but the morphology of the solution surface is different and a sparse two-dimensional network of elongated aggregates is observed.⁴⁹ The distinctions in the shape of the aggregates may cause significant differences in the induction period. While it can exceed 1 h for lysozyme solutions, this period is only a few seconds at similar concentrations of HSA.⁵⁰ The formation of a continuous network from rounded small surface aggregates of lysozyme probably requires much higher global surface concentrations than the network of elongated HSA aggregates.

The random sequential adsorption of dimers, trimers and higher oligomers of lysozyme is not sufficient to explain the observed behavior in Fig. 4, and dynamic light scattering measurements were conducted that eliminated the possibility of formation of larger aggregates in the bulk. It follows that the patches of two-dimensional aggregates are formed directly

at the interface due to attractive forces between adsorbed molecules. The results obtained indicate that the formation of a heterogeneous adsorption layer of protein is an intrinsic surface process involving the separation of two-dimensional phases. The coexistence of surface phases has been already observed in the solutions of surfactants⁵¹ and their complexes with DNA.⁵²

Effects of added denaturants in the bulk

The addition of strong denaturants (urea and GuHCl) in the bulk of 3.5 μM lysozyme solution results in an acceleration of adsorption kinetics, and the induction period disappears (Fig. 5). The kinetic dependencies of the dynamic surface elasticity remain almost monotonic for the solutions with urea up to denaturant concentrations of ~ 10 M while the corresponding dependencies for the solutions with GuHCl have local maxima at denaturant concentrations higher than ~ 2 M, indicating changes of the protein tertiary structure.¹⁰ At the same time, stronger surface activity (i.e. lower steady state surface tension) of lysozyme in solutions with urea than with GuHCl can be explained by the increase of hydrophobicity of the protein globules. The globules become looser under the influence of urea and some relatively hydrophobic amino acid residues go

Fig. 7. Steady state neutron reflectivity profiles of 3.5 μM dLYS solutions in H_2O with added 6 M urea (red) and 6 M GuHCl (blue) recorded under (A) static and (B) dynamic conditions; the insets are the scattering length density (SLD) profiles normal to the interface.

to the surface of globules from their interiors. In the following, we discuss in turn the effects of the two denaturants present in the bulk of the samples: first urea and then GuHCl.

The kinetic dependencies of the surface excess also demonstrate acceleration of the adsorption kinetics by the addition of 6 M urea in both the static and dynamic conditions (Fig. 6). It takes less than 1 h to reach a steady state surface excess of 1.5 mg/m^2 , which is approximately the same value as in the case of solutions without the added denaturant (Fig. 2).

Although urea is a strong denaturant, it does not destroy entirely the lysozyme globular structure even at high concentrations. Instead, in solution it makes the globules looser leading to the molten globule state with the changed protein secondary structure and the higher mobility of amino acid residues inside the globule.⁵³ As a result, some hydrophobic groups can go from the globule interior to its surface leading an increase of the lysozyme surface activity with the increase of urea concentration.¹⁰ The same changes of the globular structure can result in an increase of the adsorption rate due to a decrease of the charge density and thereby of the electrostatic adsorption barrier.⁵⁴ Such acceleration can also explain the disappearance of the induction period in the changes of the surface tension and dynamic surface elasticity:¹⁰ the addition of denaturants accelerates adsorption (Fig. 6) and thereby reduce the induction period (Fig. 5). Further, it follows that the molten globules are formed in the bulk phase and the protein tertiary and secondary structures do not change noticeably after that in the course of adsorption, given the lack of subsequent changes in the surface excess. As a result the kinetic dependencies of the dynamic surface elasticity are monotonic even for urea concentrations that exceed 6 M.¹⁰

The fitted adsorption layer thickness is significantly higher in urea solution than in those without added denaturants with the value reaching 2.5 nm in the dynamic condition (Fig. 7 and Table 2). Also, the adsorption layer is much more loosely bound with a water content of 52 % compared with 33 % in the absence of denaturants. These may appear to be a perplexing results at first, but it is important to bear in mind that lysozyme has disulfide bonds between remote amino acid residues (e.g. between the 6th and 127th and also between the 30th and

115th),⁵⁵ which means that the globules cannot unfold into coils completely and thus flatten out in the interfacial layer.

The difference in the reflectivity profiles in the static and dynamic conditions also reveals a real influence of the surface oscillations on the packing of macromolecules in the surface layer and suggests irreversible adsorption. These results can be attributed to changes of the conformation of adsorbing macromolecules under the influence of denaturants and therefore of the mechanism of the adsorption layer formation.

Table 2. Fitted thickness and volume fraction of the adsorbed layer of dLYS following 6 hours of equilibration in the 4 conducted experiments at 3.5 μM with added denaturant in the bulk.

Expt.	Denaturant	Thickness (nm)	Volume Fraction	Surface Excess (mg/m^2)
Static	6 M urea	2.1 ± 0.1	0.54 ± 0.01	1.6 ± 0.1
Static	6 M GuHCl	2.0 ± 0.1	0.53 ± 0.01	1.4 ± 0.1
Dynamic	6 M urea	2.5 ± 0.1	0.48 ± 0.01	1.6 ± 0.1
Dynamic	6 M GuHCl	1.9 ± 0.1	0.50 ± 0.02	1.3 ± 0.1

BAM images were also recorded of the evolution of the interfacial layer during the course of adsorption for a sample of 3.5 μM lysozyme solution containing 5 M urea (Fig. 8); note that this slightly lower urea concentration was necessitated by the limit of the filters used. Unlike the solutions without denaturants, the images are almost homogeneous for ~ 20 min after the surface formation, during which time the surface excess almost reaches its steady state value (Fig. 6). The separation of surface phases occurs suddenly at a surface age when the adsorbed amount is close to its limit value. The new phase appears as relatively short wormlike aggregates that are rather similar in appearance to the aggregates observed in bulk solutions of whey protein isolate;⁵⁶ we note that resolution of their specific internal structure would require the application of complementary techniques. Importantly, the size of the aggregates is almost invariant with time, and only a slight increase of their density with some progression in the overall brightness occurs. The observed features indicate a cooperative and equilibrium phenomenon (like micellization in the bulk phase)

Fig. 8. BAM images of an adsorbed layer from 3.5 μ M lysozyme solution in 5 M urea after (A) 0, (B) 10, (C) 20, (D) 40, (E) 60 and (F) 120 min.

with a constant size of aggregates that are stabilized by repulsive interactions and do not coalesce. Although the overall charge of lysozyme globules is positive at pH 7, there are some negatively charged patches on the globule surfaces, which points to the cooperative aggregation of the molten globules at the interface being electrostatic in nature. This resulting morphology is in contrast to that of aggregates at the surface of lysozyme solutions without denaturants (Fig. 4), which become more homogeneous with time.

While the detailed structure of the aggregates at the interface is outside the scope of this study, as the applied techniques do not have sufficient resolution, it is interesting to note that the molten globules of lysozyme in the presence of urea have a larger number of hydrophobic groups at their surface, which can enhance their propensity for surface aggregation.¹⁰ Nevertheless, the repulsive electrostatic forces evidently do not hinder the surface aggregation until the agglomerates reach a certain size, at which point their overall charge can exceed a critical value and further growth is hindered. These results give only a rough picture of the aggregation in the lysozyme adsorption layer at high urea concentrations and further studies of the aggregation kinetics and of the critical aggregation conditions are in progress. Note that to the best of our knowledge this is the first observation of cooperative aggregation in lysozyme adsorption layers.

An investigation of the interfacial properties of 3.5 μ M lysozyme solutions with GuHCl present in the bulk phase was also carried out using NR. In this case, in common with the measurements involving urea, adsorption is accelerated in both the static and dynamic conditions (Fig. 6). These results corre-

late again with the disappearance of the induction period of the evolution of the surface tension and dynamic surface elasticity (Fig. 5). At the same time, there is a peculiarity in the surface excess evolution over several hours. Although the surface excess reaches a high value within 0.5 h (i.e., by the time of the first measurement), the values gradually decrease subsequently: from 1.5 to 1.4 mg/m^2 in the static condition and from 1.6 to 1.3 mg/m^2 in the dynamic condition. These observations imply that the surface excess goes through a maximum in the course of adsorption. The non-monotonic changes of the surface excess with the surface age indicate that the protein adsorption may be accompanied by another process occurring in the sample. To exclude the influence of real-time aggregation in the bulk phase, measurements on fresh and aged samples using dynamic light scattering were conducted, and no aggregation was observed. As a result, the slowly diminishing surface excess during the experiment may be attributed to a process occurring at the interface, e.g., a relaxation of the surface structure at the expense of expulsion of a small amount of material from the adsorption layer. Further work, however, is required to elucidate the reasons for this behavior.

It is known that lysozyme does not preserve its tertiary structure in 6 M solutions of GuHCl.⁵³ The adsorption of denaturated lysozyme molecules from solutions with 6 M GuHCl leads to a noticeable maximum of the kinetic dependency of the dynamic surface elasticity, unlike the adsorption from urea solutions.¹⁰ Therefore, slow conformational changes of relatively flexible molecules of denaturated lysozyme in the adsorption layer may lead to the redistribution of segments between the distal and proximal regions of the surface layer and perhaps the compaction of the latter. This may explain the slight desorption of protein from the interface. The more pronounced effect in the dynamic condition may be understood in terms of the additional reconfiguration in the adsorption layer under the influence of surface oscillations, and desorption of some flexible macromolecules that are only loosely attached to the interface. Note that Perriman and White also observed the non-monotonic kinetic dependence of the surface excess of lysozyme with a local minimum that lasted several hours for more concentrated solutions close to the isoelectric point.²³ In this case the effect probably has another cause; i.e., the non-equilibrium aggregation of strongly interacting, neutral globules at the beginning of adsorption and slow destruction of the aggregates during the course of equilibration.

The fitted adsorption layer thickness for solutions with GuHCl also increases slightly in comparison with that for solutions without denaturants (Fig. 7 and Table 2 cf. Fig. 3 and Table 1). The effect is less pronounced than for solutions with urea. These observations can be connected with different mechanisms of the modification of the protein tertiary structure in the bulk by the two denaturants,^{10,51} where remote amino acid residues prevent complete collapse of the globules to differing extents,⁵⁵ and thereby with the different resulting protein conformations in the surface layer.

Conclusions

The results from neutron reflectometry and Brewster angle microscopy of dilute lysozyme solutions at the air–water interface, describing the kinetics of formation, structure and morphology of the adsorption layer, indicate underlying changes of the protein adsorption mechanisms when the solution contains high concentrations of denaturant (urea or GuHCl). The outcome of this study was made possible by a combination of

recent advances in instrumentation (use of a high flux neutron reflectometer) and the availability of deuterated protein (increased scattering to access the interfacial thickness).

Two different experimental approaches were compared: static samples with a fixed surface area and dynamic samples with a surface area subjected to continual periodic oscillations of small amplitude. The results show that the external perturbations can result in a higher steady state surface excess in the absence of added denaturants, and a thicker and more loosely bound adsorption layer in the case of added urea. These differences have been rationalized in terms of surface-induced effects and/or bulk convection induced near the sub-surface.

The adsorption from solutions without denaturants leads to the gradual growth of the number and size of patches (surface aggregates) of a liquid-expanded surface phase leading to the approach of a continuous adsorption layer over several hours. Adsorption from the dilute solutions studied show that the surface excess also increases over a time scale of several hours, during which time there is an induction period in the surface tension and surface elasticity. The adsorption layer thickness is less than the short axial length of the lysozyme globule, thus indicating its deformation at the interface.

The adsorption of lysozyme molecules with modified secondary and tertiary structures from 6 M solutions of urea and GuHCl is much faster, which can be attributed to the reduced charge density of the adsorbing macromolecules and the diminished electrostatic adsorption barrier. The resulting adsorbed layer is thicker, which can be explained in terms of a more loosely packed layer with a higher solvent content. In the case of urea solutions the thick layer may be connected with the adsorption of molten globules while in the case of GuHCl solution the observed effects may be explained by the adsorption of partially unfolded globules and the subsequent redistribution of the segments between proximal and distal regions of the surface layer. Lysozyme adsorption from solutions with urea also results in aggregate formation in the surface layer, but in this case the observed surface aggregation is a cooperative process and interestingly is shown to be intrinsic to the steady state adsorption layer.

We conclude with a schematic illustration of structural differences in the adsorption layers at the air–water interface that have been resolved in the present work for lysozyme globules both in the absence and presence of denaturants in the bulk (fig. 9).

Fig. 9. Schematic illustration of the key differences between the structures observed at the air–water interface in the present work.

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