Thermal Denaturation of Interfacial Protein Layers

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We report for the first time the structural measurements at nanometre resolution of the denaturation of β-lactoglobulin and lysozyme at an air–water interface using the technique of neutron reflectivity. The incipient denaturation shown previously[1] for myogloblin is also studied for these molecules at room temperature, and denaturation is provoked by increasing the temperature of the solutions progressively to 75°C. The change in the adsorbed protein layer thickness, its scattering length density and density distribution perpendicular to the surface as a function of increased temperature are reported and the data analysed in terms of a two-state model for the denaturation process. These measurements are relevant to an understanding of the way in which proteins at interfaces act as templates, for example, in biomineralization.

Introduction

X-Ray and neutron reflectivity measurements from free liquid surfaces and solid interfaces allow the structure of proteins at those interfaces to be probed to fractions of nanometre resolution. We have found[1] that myoglobin adsorbed at the air–water interface is distorted by the unbalanced interfacial forces and has a density profile corresponding to the thickness of an α-helix. We conclude that this molecule loses most of its tertiary structure but retains its secondary structure upon adsorption. For molecules more tightly bound by internal disulfide bridges, for example lysozyme, there is much less distortion.

Many techniques including film balance,[2,3] surface radioactivity,[3] surface potential,[3] ellipsometry[3,4] and circular dichroism[5] have been used to study the structure and behaviour of biological macromolecules such as proteins. A virtue of neutron reflectivity is the ability to allow contrast variation between the self-assembled protein monolayer and the significant amounts of water that may be incorporated into the layer. This contrast is readily achieved by deuterium labelling. The technique is therefore sensitive to the distribution of protein fragments through the adsorbed layer along the surface normal direction. It confers an important advantage over ellipsometric methods for which water/protein intermixing produces an optical refractive index close to that of water. Lu et al. have applied neutron reflectivity to study lysozyme at the air–water[6] and at the silica–water[7] interface for protein concentrations of up to 4 g dm^{-3}.

In this paper we use neutron reflectivity to investigate the changes in interfacial structure of ruminant milk β-lactoglobulin and hen egg white lysozyme as a function of temperature so as to induce thermal denaturation at the interface. Our work here offers two novel features of value in studying such systems. First, denaturation in the surface layer is compared with that in the solution. Second, we are able to use the layer thickness as a good order parameter of the denaturation process.

Apart from intrinsic interest in the structural stability of surface layer proteins, a practical interest relates to the mechanism of template-induced biomineralization as it is proteins at interfaces that do this. We have shown, by X-ray and neutron reflectivity methods,[8,9] that highly crystalline silicate films may be produced at the air–water interface with surfactant micellar structures acting as 'templates'. The methods used may allow us to understand biomineral formation, such as that of bone, shells and diatoms. These formations are natural composites of ceramic and organic phases, exhibiting high strength, and intricate architectures. The properties derive from the high level of control that biopolymers (largely proteins) exert over the nucleation and growth of crystals. In shells, the 'moving front' formation leads to dense ceramics that are formed in aqueous environments at ambient temperature.

For example, abalone shells are 3000 times stronger than the calcium carbonate component in isolation, while the proteins present in the shell comprise less than 2% of it. They nevertheless control the microarchitecture and provide the ductility and resilience of the composite; since the protein is

at an interface we are interested to find whether it remains in its globular form, is partially denatured at the interface (as for myoglobin) or it is the protein primary structure which acts as the 'template' for shell nucleation. Figure 1 shows a scanning electron micrograph of a New Zealand abalone shell (Paua shell) at four levels of magnification. The remarkable layer-like deposition, the high regularity and the thickness of the carbonate and the protein interlayer structures show how strong the control of the protein 'template' is.

Experimental

The proteins were used as received from SIGMA or Aldrich chemicals. The β-lactoglobulin from bovine milk contains genetic variants A and B and has a purity of greater than 95%. The hen egg white lysozyme also had a purity greater than 95%. Protein solutions were either 1 or 10 mg mL^{-1} in D_2O or air contrast matched water (ACMW) de-aerated with high-purity nitrogen. ACMW is a mixture of H_2O and D_2O so that the final scattering length matches that of air. This has the advantage that when molecules adsorbed at the ACMW/air interface are examined, the signal arises entirely from the adsorbed layer. The air/liquid interface reflectometry was performed in temperature controlled hermetically sealed troughs under a nitrogen atmosphere at 25°C initially, and then at a series of temperatures up to 75°C.

The reflectivity was measured at the SURF reflectometer of the ISIS spallation neutron source at Rutherford Appleton Laboratory, Oxfordshire, U.K. This instrument was ideal for these temperature-dependent studies as it is a time-of-flight instrument with a beam approximately 2 mm high incident on the sample; it is therefore relatively insensitive to the liquid surface level. Similar experiments at a single wavelength angle scanning reflectometer with a small beam size would be very difficult because of the need to keep the air–liquid interface in the same geometric position to an accuracy of about 10 microns. The data were collected with the beam incident on the sample at an angle of 1.5°; with the wavelength range available this resulted in a Q range of 0.048–0.6 Å^{-1}. As the samples contained significant amounts of hydrogenous material the Q range was effectively limited to a maximum of about 0.25 Å^{-1} before the incoherent background (ca. 4×10^{-6}) swamped the signal.

Data Treatment

The collected data were scaled relative to a D_2O scan to obtain reflectivity on an absolute scale. These data were then modelled using CXMULF, a program based on the optical transfer matrix method of classical optics. The original method is due to Abelès,[10] adapted by Penfold,[11] and illustrated by Brown et al.[8] This method uses the approach that the interfacial structure can be described as one or more layers. A simulated annealing least-squares approach is then used to minimize the difference between the model and the data. The model parameters used are the film thickness (Å), scattering length density (SLD/Å^{-2}) and a Gaussian interfacial roughness (Å). For these
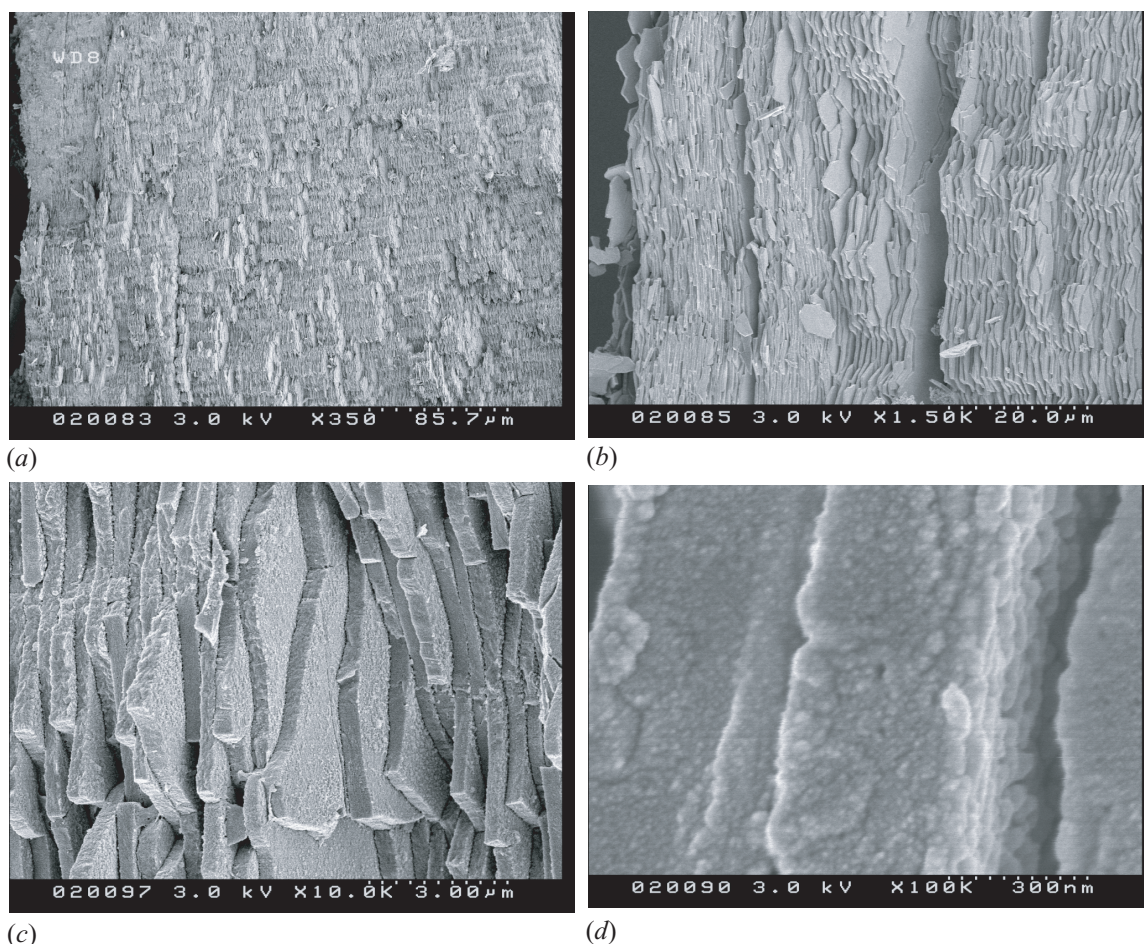

(a)

(b)

(c)

(d)

Fig. 1. Series of SEM (scanning electron microscope) images showing the nacre of a paua shell. The nacre is comprised of slabs of aragonite between a lamella protein matrix and the plates grow in stacks as can be seen in (*a*). The sheets of aragonite are approximately 400 nm thick [see (*c*,*d*)], in agreement with 'critical transitions'. The bumps seen on the surface of the aragonite in (*d*) are the same thickness as the organic matrix is reported to be (critical transition 10–50 nm). Ms Hana Robson-Marsden is gratefully acknowledged for recording the SEM images.

experiments, the sub-phase scattering length density and the interfacial roughness were constrained, leaving only the film SLD and thickness to be fitted.

Within the proteins there are many hydrogen atoms which may exchange with deuterium when the protein is in a solution containing D_2O. For each residue the number of exchangeable proteins is known;[12] therefore one can calculate an SLD which takes into account the solvent that the protein has been solubilized into. After preparation the solutions were allowed to equilibrate for excess of 24 h; we therefore assume in our calculations that all of the exchangeable protons had in fact exchanged. We also calculate the SLD for the 'pure' protein, that is, we do not include any contributions for water molecules within the structure. The protein volume and density have been calculated only from the mass and volume[13] of the individual protein residues. This approach then enables us in a first approximation to make an estimate of the protein proportion of the fitted interfacial layers from a comparison of the fitted SLD and the calculated SLD.

More detailed information regarding the protein/sub-phase contributions to the scattering can readily be obtained when data are acquired from an ACMW sub-phase, where the surface excess of the protein can be determined directly. In this case there is no contribution from any solvent associated with the protein visible to the neutron beam, and thus the total contribution to the SLD is that provided by the protein. The surface excess (Γ, g m^{-2}) can then be determined as:

$$\Gamma = \frac{10^{20} M_w \rho_b \tau}{N_A \Sigma b_{prot}} \qquad (1)$$

in which M_w is the molecular mass of the protein (g mol^{-1}); ρ_b is the measured SLD of the layer (Å^{-2}); τ is the thickness of the layer (Å); and Σb_{prot} is the sum of the scattering lengths of the elements which make up the protein, allowing for any isotopic exchange (Å).

When the experiment is carried out on a D_2O sub-phase it is impossible to determine the contribution of the material at the surface layer distinct from that of interpenetrating sub-phase molecules without information about volumes of the particles at the surface. In terms of proteins, if it can be assumed that the protein has not denatured at the surface then the scattering length density of the protein ($\rho_{b,prot}$, Å^{-2}) can be calculated from the equation:

$$\rho_{b,prot} = \frac{\Sigma b_{prot}}{V_{prot}} \qquad (2)$$

in which V_{prot} refers to the volume occupied by the protein, from its solution dimensions (Å^3). This then enables the proportion of the surface taken up by the protein (x) to be determined as the measured scattering length density of the solution is made up of the contributions from the protein and the sub-phase.

$$\rho_{b,tot} = (1-x)\rho_{b,D_2O} + x\rho_{b,prot} \qquad (3)$$

Once the fraction of the surface which consists of protein has been determined the surface excess of the protein can be calculated using also the measured thickness of the layer and the following equation:

$$\Gamma = \frac{10^{20} M_w x \tau}{N_A V_{prot}} \qquad (4)$$

From the area per molecule and the layer thickness, it is possible to gain an insight into the arrangement of the protein units.

Results

Time Dependence of the Interface Structure at 25°C

As a starting point for the experiments, the neutron reflectivity from the protein solutions at 25°C was studied soon after making up the solutions and several hours later. A lysozyme solution at 1 mg mL^{-1} in ACMW at pH 2 and a 1 mg mL^{-1} β-lactoglobulin solution at pH 2 were measured. Figure 2 shows the reflectivity from the lysozyme solution initially (lower curve) and after 5 h (upper curve). The scattering length density profiles for these two experiments are also shown. The lower (full curve) is for the data obtained immediately after the solution was placed into the reflectometer trough, and the dotted curve shows the profile after 5 h. The initial, somewhat broader, monolayer at the
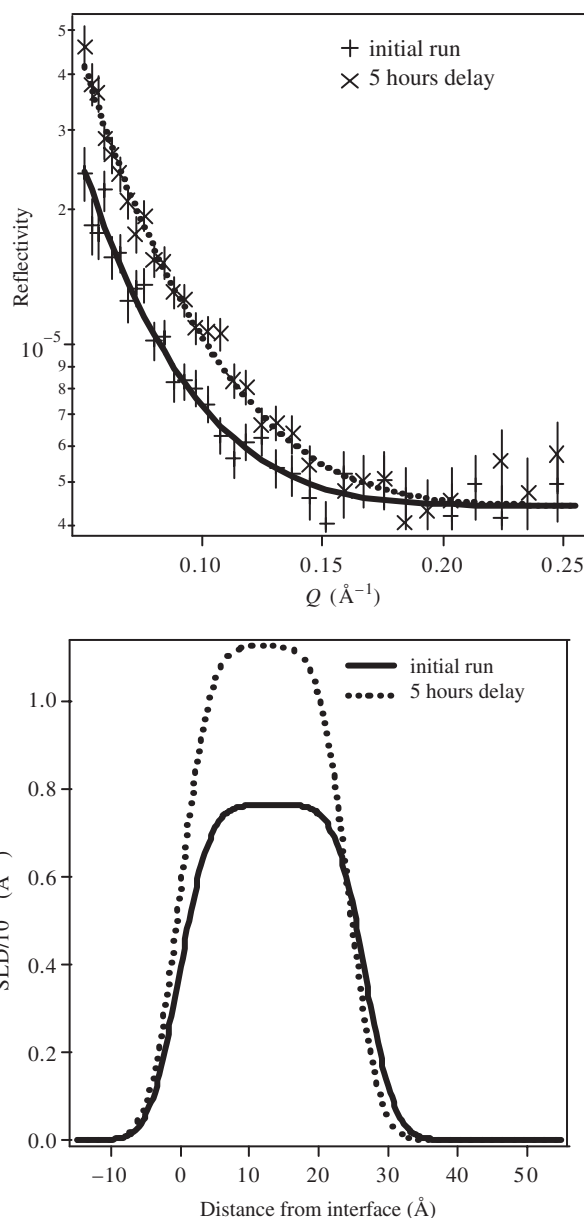

Fig. 2. Reflectivity and fit from lysozyme solutions at 1 mg mL^{-1}, pH 2.0, when first added to the trough and 5 h later.

air–water interface 'heals' and becomes sharper and more reflective with time.

The analogous data for 1 mg mL^{-1} β-lactoglobulin solutions at pH 2, again in ACMW, are shown in Figure 3. There is a small change in reflectivity, possibly in the opposite sense to that for lysozyme. The reflectivity for the surface which was exposed for 6.5 h may be slightly lower than that of the freshly prepared solution. The corresponding scattering length density profile also shown in Figure 3 for the aged solution (dotted line) is somewhat broader and of slightly lower intensity than that for the fresh material.

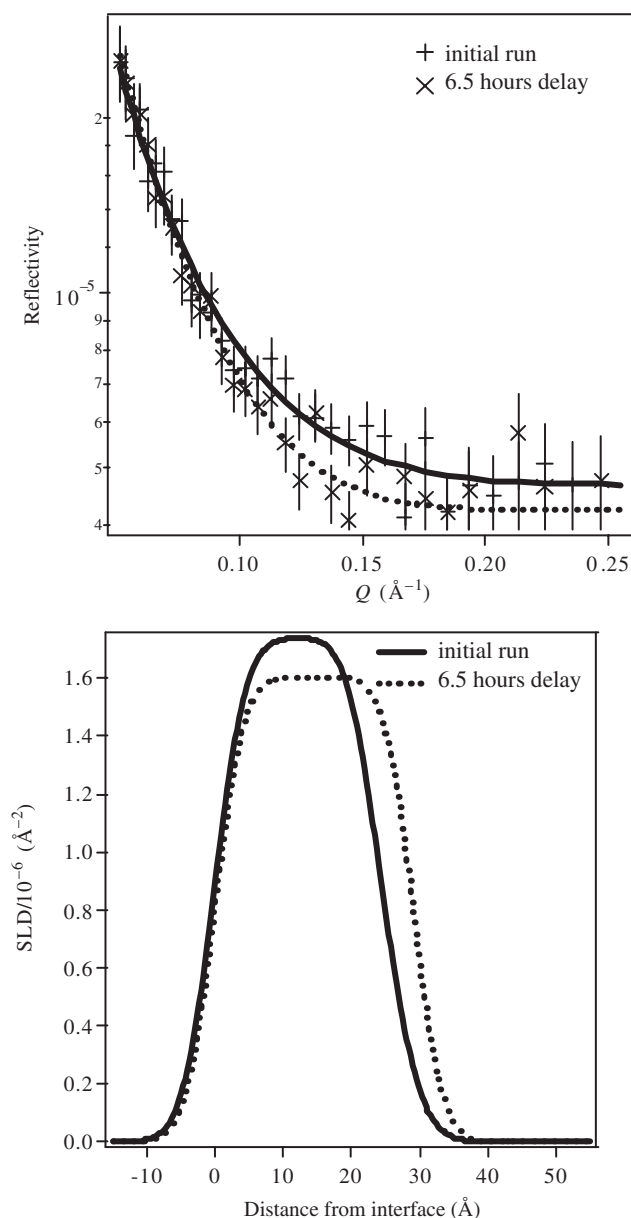To a first approximation, 'healing' of the lysozyme film is greater than that for the β-lactoglobulin.

Fig. 3. Reflectivity and fit from β-lactoglobulin solutions at 1 mg mL^{-1}, pH 2.0, in ACMW, when first added to the trough and 6.5 h later.

Temperature Dependence of the Reflectivity

Lysozyme

The most important qualitative result is that measurements were possible at all with the reflectometer up to 75°C. Good precision in fitting the reflectivity curves enabled the calculation of a scattering length density profile for each protein adsorbed at the air interface ACMW system at pH 2. The data are summarized in Table 1 for a model that comprises of a single layer of the protein at the air–water interface. Multiple-layer fits were not needed for lysozyme concentrations of 1 mg mL^{-1} or less.

Table 1 shows that the fitted scattering length density for lysozyme in ACMW is about 1.1×10^{-6} Å^{-2}, compared with a calculated value of 2.15×10^{-6} in ACMW, putting an upper limit of about 50% on the protein fraction of the monolayer. Su et al.[14] obtain a volume fraction of 0.45 for a single layer of lysozyme from a pH 4.0 solution at a solid/liquid interface. If we calculate an SLD for lysozyme based upon the crystal structure volume, we attain an SLD of 0.91×10^{-6} Å^{-2} (ACMW exchange). The inclusion of water within the lysozyme, allowing for exchange, further increases the SLD to 0.92×10^{-6} and 1.10×10^{-6} Å^{-2} for 100 and 2000 water molecules respectively. We consider it unlikely that the adsorbed layer would correspond to a densely packed monolayer of lysozyme, with no associated sub-phase. The SLD calculations undertaken using the protein residue volumes are therefore valid and are used exclusively throughout the paper.

The SLD of the lysozyme layer then increases slightly to 1.3×10^{-6} Å^{-2} (protein volume fraction ca. 70%). Beyond 55°C, the SLD decreases below the initial value indicating that there is a gradual increase in the proportion of sub-phase 'within' the protein at the interface. The model parameters are displayed in Figure 4. Focusing on the layer thickness, it appears that this might be a good order parameter for the denaturation process and we use it below for analysis of the denaturation. At low temperatures, the layer thickness is as expected for lysozyme at the concentration used. As the temperature rises, however, the layer thickness increases, gradually following a sigmoidal behaviour, to a thickness of about 150% greater than the starting value. There is some

Table 1.   One-layer fits for the 1 mg mL^{-1} lysozyme, pH 2.0, in ACMW

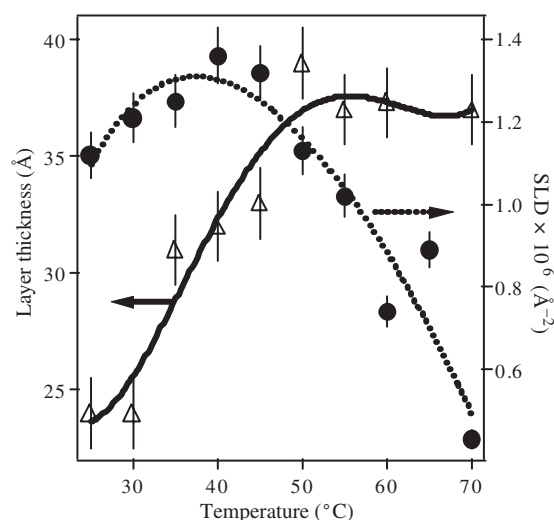| Temp. (°C)	d_1 (Å)	SLD/10^{-6} (Å^{-2})	Background/10^{-6}	χ^2
25	24(1)	1.12(4)	4.4(1)	0.8070
30	24(2)	1.21(6)	4.6(2)	0.7885
35	31(1)	1.25(3)	6.0(2)	1.34
40	32(1)	1.36(2)	5.8(2)	1.093
45	33(1)	1.32(2)	6.2(2)	1.227
50	39(1)	1.13(2)	6.6(2)	0.9515
55	37(1)	1.02(2)	5.5(2)	1.144
60	37.3(3)	0.74(2)	4.0(1)	1.139
65	37.3(3)	0.89(1)	4.7(1)	0.5667
70	37(4)	0.43(2)	4.4(1)	1.120

Fig. 4. Model parameters from lysozyme at pH 2.0, 1 mg mL^{-1}, in ACMW as a function of temperature. (Line is a guide to the eye.)

evidence of an intermediate process at temperatures near 40°C.

A good indication of the density of material at the air–water interface is given by the surface excess. Figure 5 shows this for the 1.0 mg mL^{-1} lysozyme system at pH 2 in ACMW. The maximum surface excess occurs at about 45°C. This is a lower temperature than the bulk denaturation temperature found for the wild type of lysozyme,[15] and is somewhat higher than the denaturation temperature for the Ral56Tmr variant lysozyme studied by ultraviolet circular dichroism at 205 nm wavelength. The data suggest that it has been possible by the technique of neutron reflectivity to measure the denaturation process in a 50 Å surface layer of lysozyme.

Measurements were also made in heavy water on lysozyme solutions at 10 mg mL^{-1} and pH 7 to detect qualitatively any effects of protein concentration on the
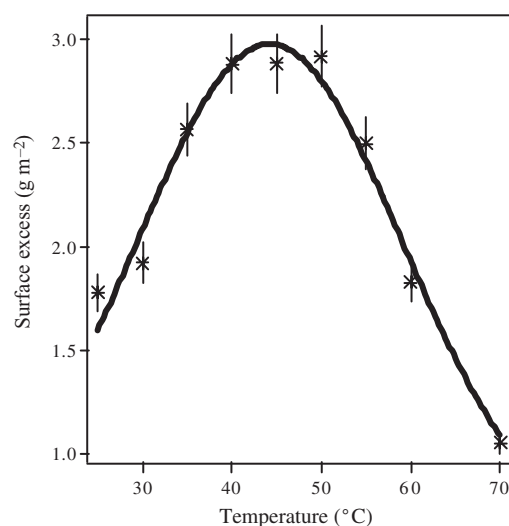
degree of deuteration of the substrate and pH. Again, good one-layer fits were obtained up to 55°C, beyond which single-layer fits were no longer acceptable (Table 2). The data show that the initial surface film is already a layer of about 37 Å, approximately equal to the thickness of the film formed after denaturation at high temperature with the 1 mg mL^{-1} solution at pH 2. This thick layer also has a large scattering length density. If all of the exchangeable hydrogen atoms on the lysozyme were exchanged, the scattering density for the protein would be about 3.5×10^{-6} Å^{-2}. The fitted profile therefore indicates that the surface layer is approximately 75% heavy water and 25% protein. This leads to a calculation of about 1000 D$_2$O molecules per lysozyme molecule. It should be noted that the quality of fit of this model to the experimental data at all of the temperatures up to 55°C is quite acceptable. The data are displayed graphically in Figure 6.

The protein layer thickness remains approximately constant at about 37 Å up to a temperature of the order of 50°C, whereupon a sharp increase in thickness occurs. Taking the layer thickness as the order parameter for the denaturation process, the midpoint of its change is at about

Table 2. One-layer fits (where appropriate) for the 10 mg mL^{-1} lysozyme, pH 7.0, in D$_2$O

Temp. (°C)	d_1 (Å)	SLD/10^{-6}(Å^{-2})	Background/10^{-6}	χ^2
25	37.2(7)	5.65(1)	2.7(1)	1.653
30	36.6(7)	5.65(1)	2.7(1)	1.370
35	34.4(8)	5.72(2)	2.7(1)	0.9297
40	34.9(8)	5.73(1)	3.6(1)	0.9100
45	37.3(8)	5.78(1)	3.6(1)	1.023
50	38.5(9)	5.80(1)	3.6(1)	1.334
55[A]	51(2)	5.74(3)	3.0(2)	2.640
60[A]	49(1)	5.57(3)	3.0(2)	5.637

[A] One-layer fit no longer acceptable.

Fig. 5. Surface excess from lysozyme at pH 2.0, 1 mg mL^{-1}, in ACMW. (Line is a guide to the eye.)
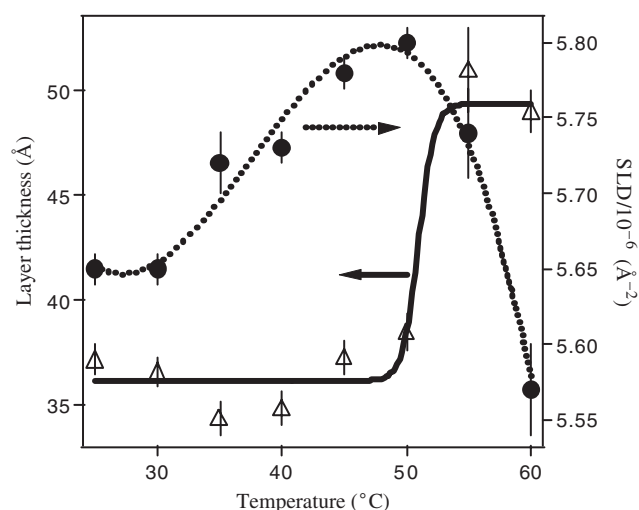

Fig. 6. Model parameters for one-layer fits to 10 mg mL^{-1} lysozyme in D$_2$O, pH 7.0. (Line is a guide to the eye.)

Table 3. Two-layer fits (where appropriate) for the 10 mg mL^{-1} lysozyme, pH 7.0, in D$_2$O
Note that sub-phase SLD = 6.35×10^{-6} (Å^{-2})

Temp. (°C)	d_1 (Å)	SLD$_1$/10^{-6} (Å^{-2})	d_2 (Å)	SLD$_2$/10^{-6} (Å^{-2})	Background/10^{-6}	χ^2
50	36(2)	5.7(2)	29(6)	6.22(7)	3.9(5)	1.020
55	36(2)	5.7(2)	27(2)	5.95(5)	4.1(5)	1.338
60	36(2)	5.38(2)	31(1)	5.85(3)	3.5(2)	1.245
65	35(1)	4.92(2)	31(1)	5.77(3)	2.4(2)	1.866
70	31(1)	5.26(3)	38(1)	5.88(3)	4.0(2)	1.010

53°C. This is considerably higher than for the monolayer system.

In an attempt to make the fitting of the high-temperature and the low-temperature data consistent, two-layer fits were tried for both temperature ranges. The results from these are summarized in Table 3. Below 50°C, the two-layer fits were not very useful, the second layer having an SLD only 3% different from the sub-phase. At 55°C and higher, the quality of the fits was markedly improved by the addition of a second layer. The upper layer (closer to the air–water interface) gives a thickness of about 35 Å from 50 to 70°C, similar to the low-temperature results from the one-layer models. The second layer underneath it has a scattering density almost indistinguishable from the sub-phase at 50°C; as the temperature is increased the SLD of this layer decreases as more material is adsorbed from solution. Inspection of the main features of these variations (Figs 6 and 7) reveals that significant changes in order occur at about 55°C and possibly between 30 and 40°C. The interfacial denaturation of lysozyme is clearer for the 1 mg mL^{-1} data than for the 10 mg mL^{-1} system, where the interfacial changes are complicated by the adsorption of more material from solution which may already be partially denatured.

β-*Lactoglobulin*

The thermal denaturation of this protein at the air–water interface of ACMW at pH 2 was studied first at 25 and 65°C. The reflectivity functions at low temperature (circles) and at 65°C (triangles) are shown in Figure 8 along with the corresponding scattering length density profiles at these temperatures. The difference in the reflectivity curves is clear, that of the high temperature being more reflective, particularly at low Q values. Only a two-layer fit was appropriate to the high temperature. The parameters are summarized in Table 4. Figure 8 shows that in real space the reflectivity of the layer closest to the air–water interface (layer 1) is stronger because the scattering length density is higher and that there is a second layer beyond about 35 Å.

Experiments were also performed with β-lactoglobulin at pH 7 (the dimerized state) in ACMW over a range of temperatures. The data are displayed graphically in Figure 9 and summarized in Table 5. Quite good fits to a one-layer model were found at all temperatures up to 65°C. There was a progressive increase in layer thickness from 28 Å up to 45 Å at the highest temperature. Associated with this, the
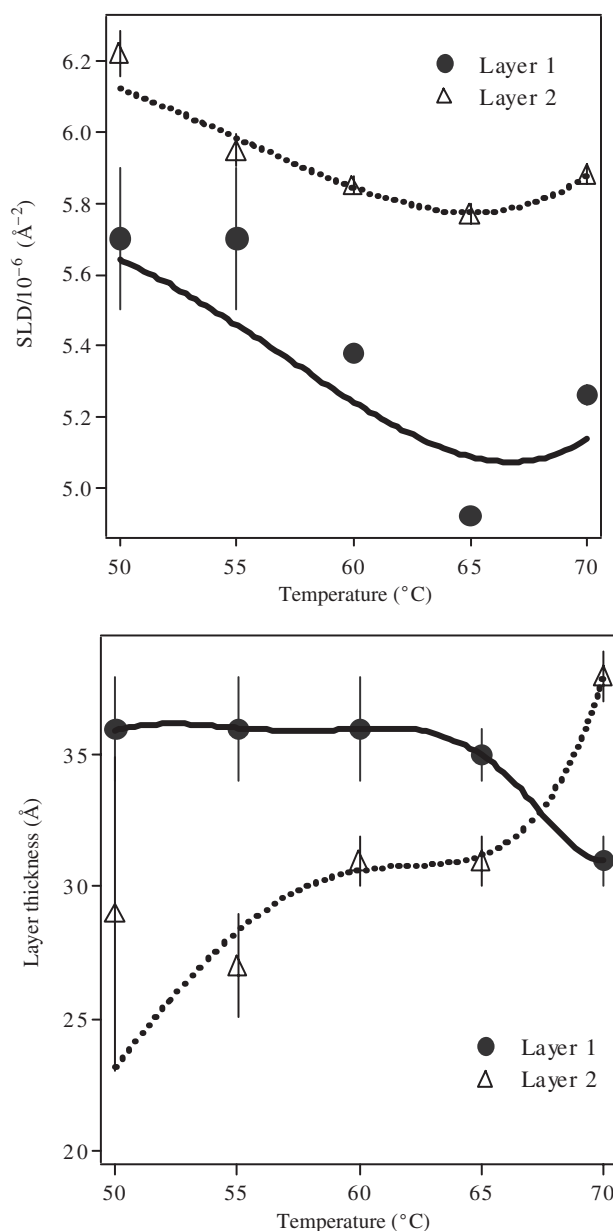

Fig. 7. Model parameters for two-layer fits to 10 mg mL^{-1} lysozyme in D$_2$O, pH 7.0. (Line is a guide to the eye.)

scattering length density up to about 45°C indicated densification of the film but, at higher temperature, entry of zero scattering length density ACMW into the film structure was observed. These data are consistent with at least partial unravelling of the protein structure above 55°C. This result is also reflected in the surface excess which shows a maximum at about 45°C—a temperature which we can take to be an indication of the surface denaturation temperature for this protein.

Discussion

In this section we first review the literature studies of the denaturation of β-lactoglobulin and lysozyme in solution and then analytically fit the profiles of the change in surface layer
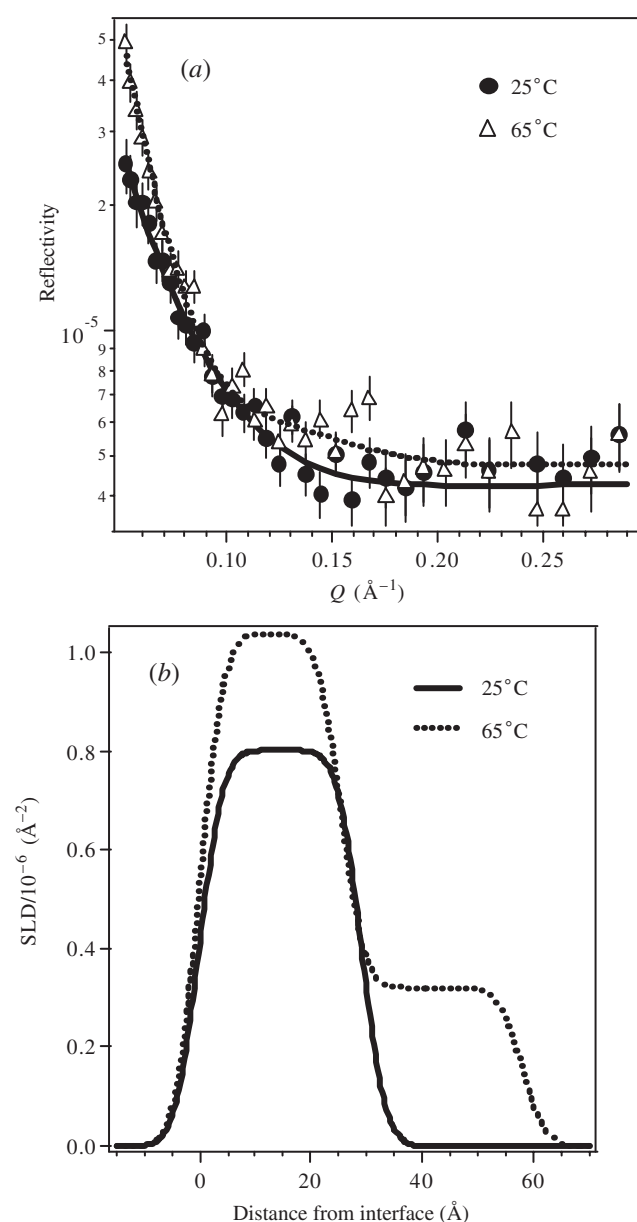
Fig. 8. β-Lactoglobulin adsorbed at the air–liquid interface: (*a*) reflectivity profiles at 25 and 65°C where the line is the fit to the data; and (*b*) SLD profiles corresponding to the fits.

Table 4. One- and two-layer fits for 1 mg mL^{-1} β-lactoglobulin, pH 2.0, in ACMW, as a function of temperature

Temp. (°C)	d_1 (Å)	SLD$_1$/10^{-6} (Å^{-2})	d_2 (Å)	SLD$_2$/10^{-6} (Å^{-2})	Background/10^{-6}	χ^2
25	29(3)	0.78(3)	—	—	4.2(1)	0.7037
65	26(8)	0.95(4)	32	(3)	4.8(2)	1.076

protein thickness with temperature. For this we used the approach of DeKoster et al.[16] Fitting the data in this way gives an enthalpy for the denaturation process assuming a two-state model. Finally, we consider the relationship between the change in layer thickness and the change in surface area of the low-temperature and high-temperature states of the protein at the interface.
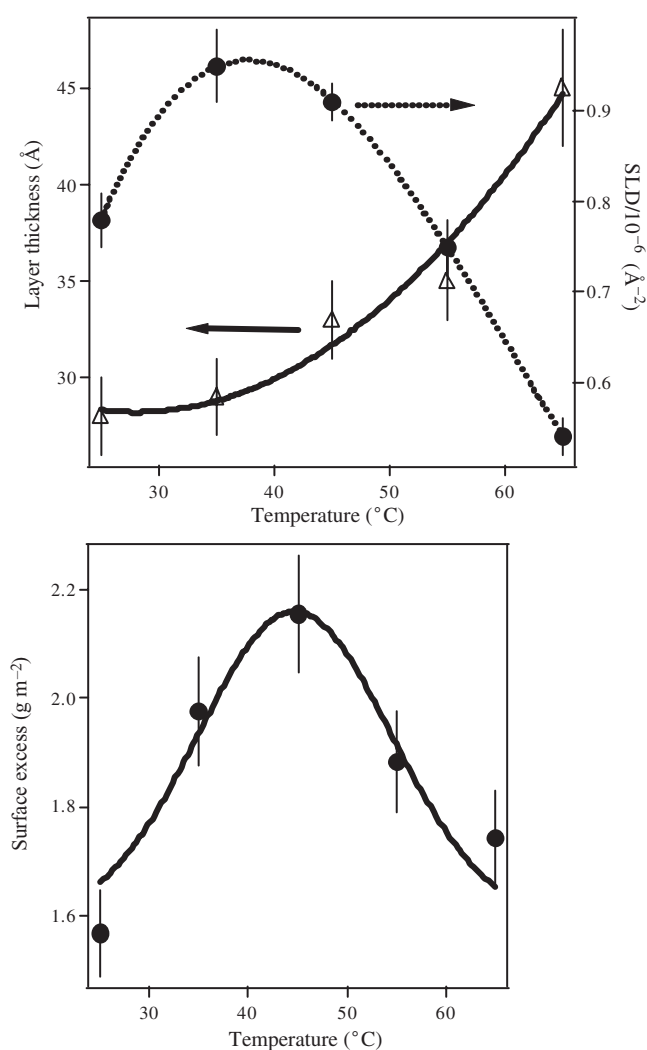

Fig. 9. Model parameters from fits to 1 mg mL^{-1} β-lactoglobulin, pH 7.0, in ACMW as a function of temperature. (Line is a guide to the eye.)

Table 5. One-layer fits for 1 mg mL^{-1} β-lactoglobulin, pH 7.0, in ACMW, as a function of temperature

Temp. (°C)	d_1 (Å)	SLD$_1$/10^{-6} (Å^{-2})	Background/10^{-6}	χ^2
25	35(2)	0.53(2)	3.2(1)	0.6724
25	28(2)	0.78(3)	4.2(1)	0.7355
35	29(2)	0.95(4)	5.5(1)	0.6433
45	33(2)	0.91(2)	6.4(2)	1.081
55	35(2)	0.75(3)	5.9(2)	0.174
65	45(3)	0.54(2)	4.9(1)	0.9438

Ruminant Milk β-Lactoglobulin

β-Lactoglobulin from ruminant milk is a globular protein with an M_w of 18.3 kD. It has 162 amino acids and exists as a dimer at physiological pH values. β-Lactoglobulin is predominantly constituted of β-sheets (50% of the secondary structures) and contains α-helix (10% of the secondary structures). Native β-lactoglobulin has two disulfide bonds and one free thiol group, which is buried within the protein structure. Four out of the five cysteine residues form two

disulfide bridges, 66–160 and 106–119, leaving Cys121 as the free thiol that appears to be responsible, through disulfide interchange, for the formation of covalent aggregates upon heating.[17]

Hen Egg White Lysozyme

Lysozyme contains 129 residues with an M_w of 14.4 kDa. The lysozyme molecule can be divided into an α-helical and a β-sheet domain with the active site cleft between them. The α-helical domain consists of residues 1–35 and 85–129 which contains four α-helices and a short 3_{10}-helix. The β-sheet domain consists of residues 36–84 that form a three-stranded antiparallel β-sheet, a long loop and a 3_{10}-helix. The conserved long loop has a disulfide at its base, disulfide 64–80, and a second disulfide pins the loop to the third α-helix, disulfide 76–94.[18]

Denaturation in the Bulk

β-Lactoglobulin is the major whey protein in bovine milk. It has 162 amino acids and molecular weight of 18298 (genetic variant A). The genetic variant B has a molecular mass of 18212 and there are two disulfide bridges. The overall structure is in the form of an eight-stranded β-barrel. Thermal denaturation experiments in bulk solution have been done on all variants, the temperature of the inflection point in the denaturation curve ranging from 72 to 76°C.

In studies of the temperature denaturation of lysozyme in the bulk, Green et al.[19] and others[20] have suggested that two distinct transitions can be identified. These proteins in solution change conformation first at a lower temperature and then subsequently denature when the temperature is raised. The first transition is characterized by an increase in viscosity of the solution, which in turn is related to the increase in molecular dimensions. For lysozyme, the first transition occurs sharply at about 50°C and the second at approximately 70°C. The work of Green et al.[19] was done at a protein concentration of 100 mg mL^{-1}, well above the concentrations used in the present experiments. Following Clarke et al.,[21] the changes observed in the infrared spectra for the bulk solution indicate formation of anti-parallel β-sheet structures as a precursors to bulk gelation. Green et al.[19] also observed that, at 50°C, complete unfolding occurred if lysozyme was incubated at 52°C for 5 h.

The interfacial behaviour of the two proteins can be understood in part by considering the hydropathy profiles. These are shown in Figure 10. For lysozyme, the three-point average and the nine-point average of hydropathy suggest that broad hydrophilic and hydrophobic regions exist, comprising between 20 and 50 amino acids in proximity. For β-lactoglobulin, especially in the middle of the protein's sequence, the three-point average shows extreme excursions towards both hydrophilic and hydrophobic directions. This pattern suggests that the molecule may be subject to more distortion than lysozyme by the unbalance of surface forces.

In addition to the hydropathy, the two proteins have different internal strengths due to disulfide bridging which resists the 'tearing' forces arising from the hydropathy. Unfolding is dependent primarily upon the protein structure. Table 6 shows structural information for three proteins (including myoglobin) in their crystalline and adsorbed states. The extremes define a range of disulfide involvement ranging from myoglobin, deficient in cystein residues, to lysozyme, the well-characterized 'hard' protein containing four disulfide bonds, exhibiting little conformational flexibility in the native state.

The analysis of Table 6 was based upon a comparison, for each protein, of the area/molecule derived from the molecular dimensions obtained from the literature with that determined by the 'footprint' from neutron reflectivity studies at the air–water interface. The values of the area per
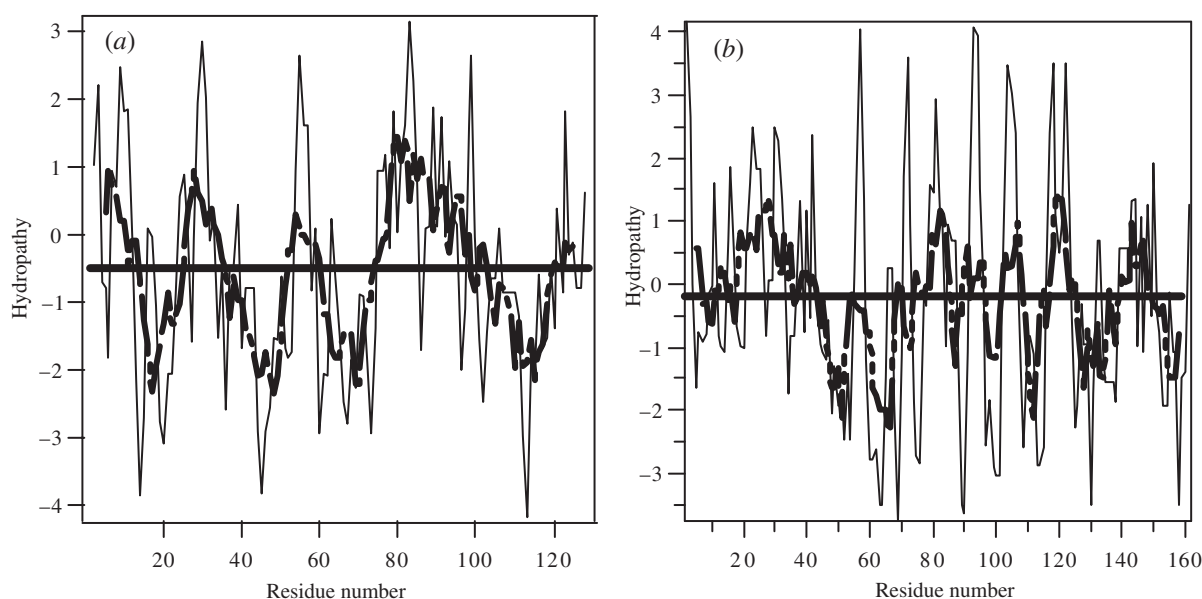

Fig. 10. Hydropathy profiles (nine-point and three-point averages) for: (*a*) lysozyme; and (*b*) β-lactoglobulin. The thin line represents the three-point average, the dashed line represents the nine-point average, and the solid line represents the overall average.

Table 6.   Properties of materials used

	Myoglobin	β-Lactoglobulin	Lysozyme
Molecular weight (Da)	16935	18265 (monomer)	14296
Residues	153	162	129
No. disulfide bonds	none	2 + 1 SH	4
Secondary structure	α (100%)	α (10–15%) + β (50%)	α (25%) + β (45%)
Molecule dimensions (Å³)	$25 \times 35 \times 45^A$	$35.8 \times 35.8 \times 35.8^B$	$45 \times 30 \times 30^C$
Area/molecule (Å²) (molecule dimensions)	1575	1282	900
Area/molecule (Å²) (neutron reflectivity)	2300	1857	1363
Layer thickness (Å)	16^A	36^D	48^D
SLD (Å⁻²)			
No exchange	1.81×10^{-6}	1.72×10^{-6}	2.00×10^{-6}
ACMW exchange	1.94×10^{-6}	1.84×10^{-6}	2.15×10^{-6}
D₂O exchange	3.13×10^{-6}	2.99×10^{-6}	3.53×10^{-6}

^{A} See Ref. [1]. ^{B} See Ref. [22]. ^{C} See Ref. [23]. ^{D} A. Perriman, unpublished data.

molecule from the reflectivity study were determined by application of the following relationship:

$$\text{area/molecule} = \frac{\sum b_{\text{protein}}}{\tau \times \rho_b} \qquad (5)$$

where $\sum b_{\text{protein}}$ is the sum of the scattering lengths of the elements which comprise the protein (Å); τ is the thickness of the layer (Å); and ρ_b is the measured scattering length density of the layer(Å⁻²). For β-lactoglobulin, $\rho_b = 0.75 \times 10^{-6}$ Å⁻²; for lysozyme, $\rho_b = 1.12 \times 10^{-6}$ Å⁻². A comparison of the area/molecule to those obtained by neutron reflectivity show that the area occupied by protein molecules at the fluid interface are ca. 1.5 times larger than in the native molecular state. These values indicate that each protein has been deformed at the air–water interface. The calculated SLD for the most rigid protein, lysozyme, in ACMW assuming substitution is 2.15×10^{-6} Å⁻². The low, measured value of 1.12×10^{-6} Å⁻² indicates that there is a significant proportion of sub-phase associated with the protein at the interface.

The layer thicknesses presented in Table 6 show that the susceptibility to deformation at the interface decreases in the order: myoglobin > β-lactoglobulin > lysozyme. The layer thickness determined for myoglobin at the air–water interface, at 16 Å, is thinner than expected from the smallest dimension of the unit cell or the molecular diameter calculated from the unit cell dimensions. This thickness corresponds to approximately the thickness of a single α-helix and suggests a substantial loss of tertiary structure. The layer thickness determined for β-lactoglobulin (36 Å) and lysozyme (48 Å) are in agreement with literature values of their molecular dimensions (35.8[22] and 45 Å[23] respectively) and also with values obtained by neutron (37 Å[24]) and X-ray (45 Å[23]) reflectivity at a solid surface.

Fitting the 'Order Parameter' for Surface Denaturation

In order to fit the change in the protein layer thickness as a function of temperature and to extract thermodynamic parameters for the denaturation process, we first of all assume that denaturation is an equilibrium between two states only whose proportions change between 25 and 70°C. These are the folded and the unfolded states of the protein. We furthermore assume that since the heating process was slow, the protein achieves an equilibrium degree of unfolding at any particular temperature where neutron reflectivity measurements were taken.

We furthermore assume that the reflectivity measurement is an average value of the thickness associated with the superposition of the different states of the protein at a particular temperature. The fitting process therefore assumes that the thickness of a folded (native) protein is the minimum measured thickness, d_n, and that the thickness of an unfolded protein is the maximum measured thickness, d_u. If d_1 is the measured layer thickness from neutron reflectivity, F_u is the fraction of unfolded proteins, Equation (6).

$$F_u = \frac{d_n - d_1}{d_n - d_u} \qquad (6)$$

The equilibrium constant between the folded and the unfolded states is then given by:

$$K_{eq} = \frac{F_u}{(1 - F_u)} \qquad (7)$$

Following the approach based on that of DeKoster et al.,[16] we show that the fraction of unfolded protein, F_u, at a particular temperature, T, is:

$$F_u = \frac{i_{bt} + i_{at}.\exp\left(\left(\frac{\Delta H}{R}\right)\left(\frac{1}{T_m} - \frac{1}{T}\right)\right)}{1 + \exp\left(\left(\frac{\Delta H}{R}\right)\left(\frac{1}{T_m} - \frac{1}{T}\right)\right)} \qquad (8)$$

where i_{bt} is the intercept before the transition; i_{at} the intercept after transition; T_m the midpoint of denaturation, ΔH the enthalpy of unfolding and R the gas constant (1.987 cal mol^{-1} K^{-1}). This approach was used to fit d_l versus T data for lysozyme (Fig. 4) and for β-lactoglobulin (Fig. 9). The F_u versus T profiles and the fitted curves for lysozyme and β-lactoglobulin are displayed in Figures 11 and 12 respectively. Though a limited data set in each case caused some uncertainty in the fit to the data, the simulated profiles provide a reasonable guide to the energetics of denaturation and their relationship to the structural properties of the two globular proteins. The values derived from the fit to the data with the model described in Equation (8) are ΔH 70 kcal mol^{-1} and T_m 40°C for lysozyme. For β-lactoglobulin the values are 50 kcal mol^{-1} and 52°C respectively.

The values of ΔH for lysozyme and β-lactoglobulin determined in this study fall within the range defined by the
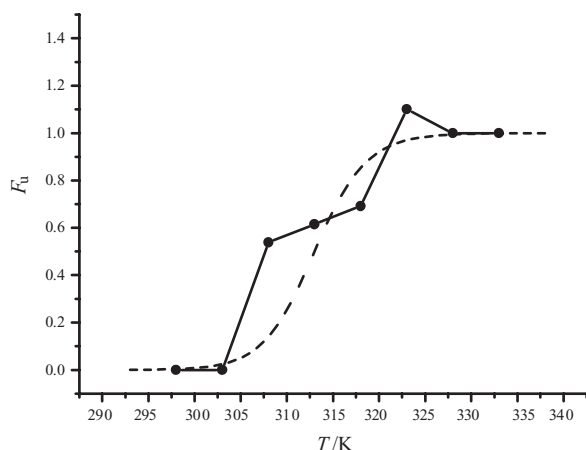

Fig. 11. Fraction of unfolded (F_u) lysozyme versus temperature (T) in 1 mg mL^{-1} lysozyme solution, pH 2.0 (data from Fig. 4). The broken line represents the simulated curve in accordance with a denaturation process that follows a two-state model.

Fig. 12. Fraction of unfolded (F_u) β-lactoglobulin versus temperature (T) in 1 mg mL^{-1} β-lactoglobulin solution, pH 7.0 (data from Fig. 9). The broken line represents the simulated curve in accordance with a denaturation process that follows a two-state model.
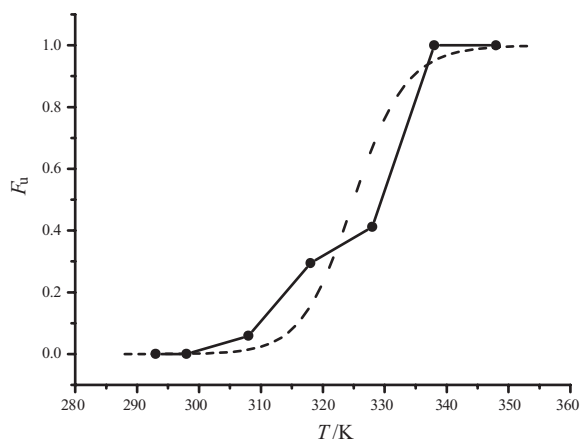
denaturation of ribonuclease A, 96 kcal mol^{-1}[16] and turkey ovomucoid third domain, 40.8 kcal mol^{-1}.[25] These values were determined by a similar analytical protocol for data acquired from spectroscopic responses to thermal and chemical denaturation of these proteins. We note that Ribonuclease A[26] (124 amino acid residues) and hen egg white lysozyme[26] (129 amino acid residues) both contain four disulfide linkages. In addition, their enthalpies of unfolding are greater than those of turkey ovomucoid (56 amino acid residues), which contains three disulfide bridges, and β-lactoglobulin (162 amino acid residues), which contains two disulfide bridges and one free thiol group. The enthalpy change between folded and unfolded states, ΔH, consists of dispersion forces, electrostatic interactions, van der Waals potentials and hydrogen bonding within the protein. Of these interactions, the major contribution to the enthalpy of unfolding comes from the disruption of van der Waals interactions between amino acid groups packed in the native state.[27] If we assume that β-lactoglobulin and lysozyme are fully unfolded under the conditions of our experiment, the higher ΔH value determined for lysozyme may be reconciled in structural terms where a greater number of disulfide linkages results in a more tightly bound protein.

Conclusions

In this study the structural distortions accompanying denaturation of the globular proteins hen egg white lysozyme and β-lactoglobulin at the air–water interface are shown. An analytical protocol for extracting thermodynamic information, based upon film thickness measurements on denaturation process, is also developed.

(i)   For relatively low (1 mg mL^{-1}) and high (10 mg mL^{-1}) concentrations of lysozyme solutions at pH 2, film thickness and surface excess measurements increase with increasing temperature. At low lysozyme solution loading, where monolayer behaviour dominates, the temperature of denaturation was 45°C, lower than that reported for the unfolding process in bulk solution. At high solution loading, the temperature of denaturation was 53°C, considerably higher than for the monolayer system.

(ii) With the exception of the two-layer structure at pH 2 and 65°C, low pH and neutral pH solutions of 1 mg mL^{-1} β-lactoglobulin solutions displayed monolayer behaviour for the temperature range studied (25–65°C) for solutions of this protein. At pH 7, where β-lactoglobulin dimers predominate, film densification and ingress of the sub-phase with increasing temperature indicated a partial unravelling of the protein structure above 55°C.

(iii) An analytical protocol involving the fraction of unfolded protein (F_u) as a function of temperature (T), determined from the layer thickness measurements for both lysozyme and β-lactoglobulin, is described. For each case, a plot of F_u versus T shows sigmoidal behaviour. The profiles can be simulated by a model in which denaturation is considered to be an

equilibrium between two states only, whose proportions change between the extremes of the temperatures studied. The enthalpy of unfolding, ΔH, and the midpoint of unfolding, T_m, can be derived from the simulation. For lysozyme, ΔH was 70 kcal mol^{-1} and T_m 40°C. For β-lactoglobulin, the values were 50 kcal mol^{-1} and 52°C. These values are significantly lower for proteins in bulk solution that have a similar number of disulfide bridges and may indicate, by the methodology described in this paper, that the enthalpy contribution of the surface toward denaturation can be determined.

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