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Adsorption of proteins from aqueous solutions on hydrophobic surfaces studied by neutron reflection†

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The two proteins, β-casein (β-CN) and β-lactoglobulin (β-Lg), were adsorbed on hydrophobic silicon substrates from buffer solutions at pH 8, 7, 5 and 3. The structure, in terms of thickness and composition of the adsorbed species, was determined by means of neutron reflectivity. At pH 7 β-CN forms a structure that is described by two layers, a compact layer adjacent to the solid surface and a looser layer protruding into the solution. β-Lg adsorbs as a uniform layer. At lower pH both proteins adsorb more, with thicker layers, and β-Lg also adsorbs as a non-uniform layer. The adsorption of both proteins is irreversible. The merits of contrast variation are discussed and, in particular, the importance for the systems studied of the use of water of scattering length density 4.5×10^{-6} Å^{-2} is described. Owing to the large size of the proteins, this contrast, intermediate between those of D_2O and silicon, allows details masked by the higher critical angle of D_2O to be revealed.

Introduction

Neutron reflection has been widely used for studies of adsorption at flat surfaces, both air/liquid and solid/liquid. It has proved to be a valuable technique and provides detailed information about the structure and composition of adsorbed layers.[1–3] Since many biological processes occur at interfaces, the possibility of using neutron reflection to study structural and kinetic aspects of model systems is of considerable interest. However, the number of such experiments performed so far is small. The reason for this is probably that it is well known that the most effective use of neutron reflection involves extensive deuterium substitution and this is not usually an available option in biological systems. Here, we examine ways in which neutron reflection can be made sensitive to non-deuterated materials and illustrate some of the ideas by applying them to two proteins of different shape adsorbed at the hydrophobic solid/liquid interface.

Many proteins are amphiphilic because they contain a mixture of amino acids with hydrophobic chains and with ionic or polar side-chains. Like surfactants, they aggregate in solution and may be surface active even at very low concentrations, although the mechanisms of aggregation and adsorption are more complicated than for surfactants. The adsorption of proteins at interfaces is important in areas relevant to biology,[4] medicine,[5] food processing (stabilization of foams and emulsions, and fouling of equipment)[6] and biotechnology,[7] and therefore considerable effort has been devoted to studying what are the important factors, which will include size, shape, charge, hydrophobicity and thermodynamic and thermal stability. There is no comprehensive model of protein adsorption, although a large number of studies of protein adsorption at various surfaces have been made, such as colloidal particles,[8–14] metal surfaces,[15,16] silica surfaces,[17–21] modified silicon surfaces,[22,23] polymer surfaces[24,25] and air/liquid interfaces.[26–33]

The two proteins used in this study were the milk protein β-casein (β-CN) and the whey protein β-lactoglobulin (β-Lg). They are similar in size but their shapes in solution are different and this influences their adsorption behaviour. The lack of a tertiary structure makes β-CN a flexible molecule which is able to change conformation easily. It is therefore suitable for providing steric stabilization. β-Lg is a globular protein with a more compact conformation resulting from extensive secondary and tertiary structure caused by the presence of disulfide bonds. Some basic features of the two proteins are as follows. Sedimentation field-flow fractionation and dynamic light scattering of β-CN adsorbed on polystyrene lattices[11,12] confirm that it is a flexible molecule with a hydrophobic tail, which may be the site of its adsorption on non-polar surfaces, and a hydrophilic portion that tends to extend into the aqueous environment. Such adsorption behaviour has been confirmed by neutron reflectivity in a preliminary study[23] and has been deduced from ellipsometric measurements.[34] The structure of β-Lg is sensitive to both pH and temperature and the dimer may be found in equilibrium with the octamer in the range of pH from 3.5 to the isoelectric point at pH 5.3, or with the monomer at other values of pH. At high pH (>7.5) there is a time dependent irreversible denaturation of the protein following its dissociation to a monomer.[35] As the pH varies, the protein also undergoes conformational changes which are reversible up to pH 7.5. The monomer chains in the dimer have the form of spheres of ~36 Å in diameter.

Contrast variation in neutron reflection from adsorbed proteins

In a neutron reflection experiment, the specular reflection, R, is measured as a function of the wavevector transfer, q, per-

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Table 1 Properties of the various interfacial species

Species	d^a/ g cm^{-3}	V^b/ Å^{-3}	IEP^c	b^d/ 10^{-4} Å	ρ/ 10^{-6} Å^{-2}
H_2O	0.9975	30		−0.168	−0.56
D_2O	1.105	30		1.905	6.35
Si	2.32	20		0.415	2.07
SiO_2	2.16	47		1.585	3.41
$C_{18}D_{37}$	0.78	540		36.65	6.76
β-CN	1.348	29 600	4.6	532.6	1.80
β-Lg	1.335	22 800	5.3	392.7	1.72

[a] Density, from refs. 11 and 47. [b] Volume calculated from density. Volume of β-Lg is that of the monomer. [c] Isoelectric point.[35] [d] Scattering length calculated from constituent atoms (the amino acid compositions were taken from refs. 35 and 48 and known neutron scattering lengths.[49]

pendicular to the reflecting surface where

$$q = \frac{4\pi}{\lambda} \sin \theta \tag{1}$$

θ is the glancing angle of incidence and λ the wavelength of the incident neutron beam. $R(q)$ is related to the scattering length density across the interfance, $\rho(z)$, by

$$R(q) = \frac{16\pi^2}{q^2} |\hat{\rho}(q)|^2 \tag{2}$$

where $\hat{\rho}(q)$ is the one dimensional Fourier transform of $\rho(z)$:

$$\hat{\rho}(q) = \int_{-\infty}^{+\infty} \exp(-iqz)\rho(z)\mathrm{d}z \tag{3}$$

$\rho(z)$ being a function of the distance perpendicular to the interface.

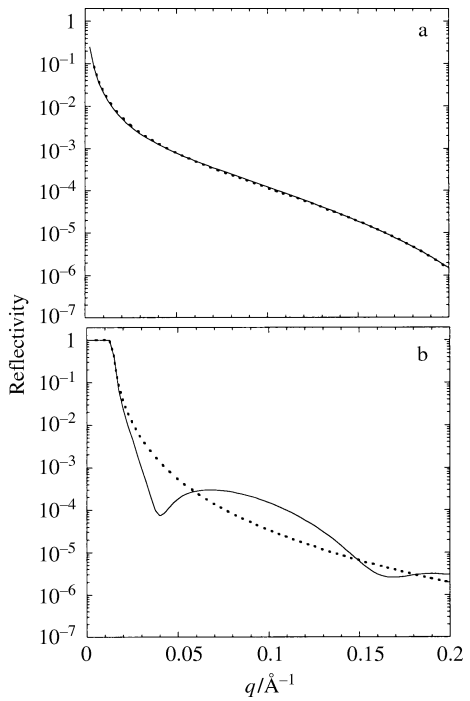

Fig. 1 Calculated reflectivity profiles for a layer of globular protein adsorbed on a hydrocarbon layer grafted on to a solid substrate using different contrast conditions. The protein layer is taken to have a thickness of 35 Å and a volume fraction of 0.6. The remaining 0.4 is water. The scattering length density of the protein is 1.8×10^{-6} Å^{-2}. The hydrocarbon layer is 27 Å thick and has a scattering length density of a typical fully deuterated solid hydrocarbon (6.5×10^{-6} Å^{-2}). (a) At silicon–hydrocarbon–water in water matched to the silicon (2.07×10^{-6} Å^{-2}) and (b) in D_2O. The dashed lines represent the calculated signal from the corresponding interfaces without protein.

The scattering length density is related to the composition of the adsorbed species by

$$\rho(z) = \sum_j n_j(z) b_j \tag{4}$$

where $n_j(z)$ is the number of nuclei per unit volume and b_j is the scattering length of nucleus j. The latter is an empirical quantity known for most nuclei. Neutron reflection often depends on the large difference between H and D to provide contrast between materials. The scattering lengths of the constituent fragments of any species adsorbed at the surfaces are the fundamental quantities from which the interfacial properties are derived and in Table 1 we list them for the materials used in this work.

It is clear from eqn. (2) that the property to which the neutrons are sensitive is the scattering length density ρ, defined by eqn. (4). To maximize the signal from the interface, the contrast in the scattering length density of that interface must be made as large as possible. One way of achieving this is to arrange for the two bulk phases on either side of the interface to have the same value of ρ but for the interface to be as different from this value as possible. At the air/liquid interface this is done by adjusting the H–D composition of the water so that it is has the same ρ as air ($\sim$0) and then preferably using deuterated adsorbed material. The two bulk phases are then said to be contrast matched and, apart from an incoherent background which may easily be subtracted, the reflected signal is due entirely to the adsorbed layer. Biological materials are often difficult or impossible to deuterate and an adsorbed protonated layer gives a small signal. It is just about possible to determine the main features of the layer from such data and this has been done by Dickinson and co-workers[26,27] in studies of the adsorption of β-CN at the air/water interface. An alternative way of studying adsorption at the air/liquid interface is to have a high contrast between the two bulk phases and then rely on the displacement of the high ρ phase at the interface by a low ρ adsorbing material. This would be the situation if proteins were adsorbed at the surface between D_2O and air. Any D_2O displaced from the interfacial region will have a moderate effect on the reflectivity and packing arguments may be used to deduce the protein adsorption indirectly. A possible difficulty with this is that any protein lying above the surface of D_2O will make a relatively small contribution to the reflectivity so that the displaced D_2O does not represent all of the protein. There may also be ambiguities in the interpretation of the displaced layer that stem from the problem of loss of phase information in eqn. (2). However, even when these difficulties cannot be resolved from this one reflectivity profile on its own, the combination of such a measurement either with the null reflecting water experiment described above, or with an X-ray reflectivity experiment,[36] would add significantly to the sensitivity. A further complication with proteins is that some of the hydrogens in the protein are exchangeable, but this effect can be often estimated sufficiently well that it should not impair the accuracy of the experiment.

At the solid/liquid interface the situation as regards choice of contrast is changed because the scattering length densities of solids are generally non-zero. The two solids most suitable for neutron reflection experiments are silicon ($\rho = 2.07 \times 10^{-6}$ Å^{-2}) and quartz ($\rho = 4.14 \times 10^{-6}$ Å^{-2} for crystalline quartz). In the situation where the signal is predominantly from the protein layer only, *i.e.*, the H/D ratio of the water has been adjusted to match the solid, the signal from an adsorbed protein layer will be weak because the layer already contains a significant fraction of water, which gives no signal.

At the solid/liquid interface there are some attractive options for improving the sensitivity to the adsorbed protein layer. One of the interests in adsorbing proteins on surfaces is

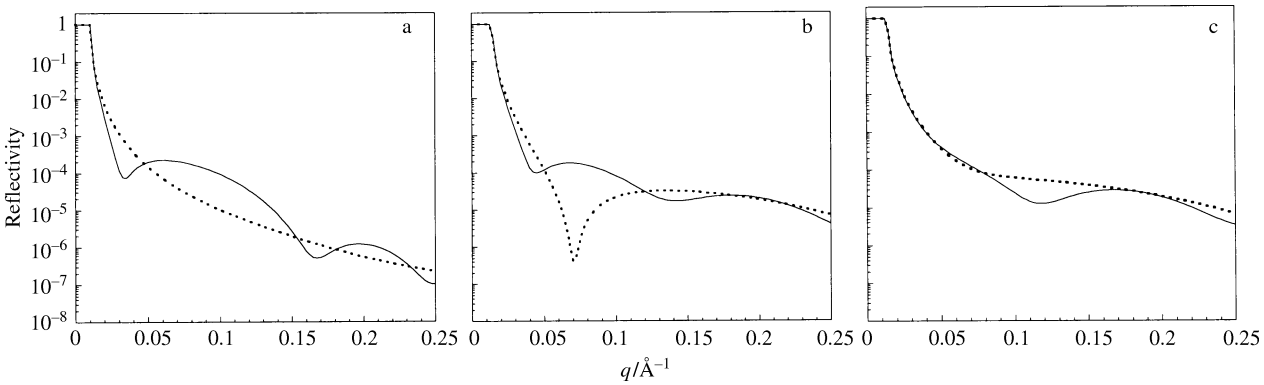

Fig. 2 Calculated reflectivity profiles calculated for a typical layer of a globular protein adsorbed on a partially deuterated hydrocarbon layer grafted on to a silicon substrate in D_2O. The protein layer is taken as in Fig. 1. The hydrocarbon layer is divided into three 9 Å thick segments, (i), (ii) and (iii), the last being next to the protein–aqueous phase. The scattering length density of a layer is taken to be -0.5×10^{-6} Å^{-2} if it is protonated and 6.5×10^{-6} Å^{-2} if it is deuterated. (a) Layers (i), (ii) and (iii) all deuterated; (b) layers (i) and (ii) deuterated and (iii) protonated; (c) layer (i) deuterated and (ii) and (iii) protonated. The dashed lines represent the calculated signal from the corresponding interfaces without protein.

to examine the way in which specific interactions with the surface modify the adsorbed layer. The easiest way of achieving this is to graft an organic layer on to either quartz or silicon and confer on the outer part of this layer an appropriate chemical functionality. While this may produce the right type of surface for studying the protein adsorption, it will be difficult to obtain an adequate signal. The effect of the protein layer is generally small and this will impair the resolution of the structural determination. Such an experiment has been done by Liebmann-Vinson *et al.* on human serum albumin[37] and their signal from the adsorbed protein was far from what one might have wished.

In the experiment of Liebmann-Vinson *et al.*,[37] protonated material was grafted on to the silica to generate the surface for protein adsorption. The contrast situation changes drastically if deuterated material is used instead. Simulations of a protein layer adsorbed to a grafted layer are shown in Fig. 1. Fig. 1(a) shows that the protein remains more or less invisible in the null situation, where the water is matched to the substrate, but in D_2O [Fig. 1(b)] there is a very large contribution to the reflectivity from the protein. Hence specific deuteration of the grafted layer may be used to optimise the signal. We have used this method previously to study the structure of β-CN adsorbed at the hydrophobic solid/water interface.[23] In general, there should be no difficulty in preparing grafted deuterated layers of a range of chemical functionality.

In many real protein layers, the structure will be more complex than that shown in Fig. 1. The question arises as to what resolution could be expected in the reflection experiment and how it could be improved. The resolution of the neutron experiment is inherently low (of the order of 10 Å) but if contrast variation techniques are used this can be improved significantly.[2] We now illustrate a strategy for improving the resolution in experiments on protein layers. It is clear from Fig. 1(b) that the presence of a fully deuterated grafted layer of hydrocarbon between the substrate and protein layer greatly enhances the signal from the protein. A further option is to deuterate only part of the grafted layer. The effect of deuterating a C_{18} layer in progressively increasing fragments of C_6, C_{12} and C_{18} with the hydrogenated part always on the protein side of interface is shown in Fig. 2, for the same single layer protein. In each case large differences are observed in the signal but if the protein forms a single layer the extra profiles would add no new information to that which could have been obtained from Fig. 1(b). However, in the case of a two layer system, such as that typically adopted by β-CN, the effect of changing the width of the deuterated label is to probe different Fourier components of the scattering length density profile of the protein, and the extra experiments now give new information about the two layers. The model calculation is shown in Fig. 3. Hence, by using more subtle, but still chemically accessible, labelling it becomes possible to enhance the ability of the experiment to resolve the different protein layers considerably. Given the range of grafting chemistry that is already well established,[38,39] the number of possibilities of design of reflection experiments to address specific problems about protein

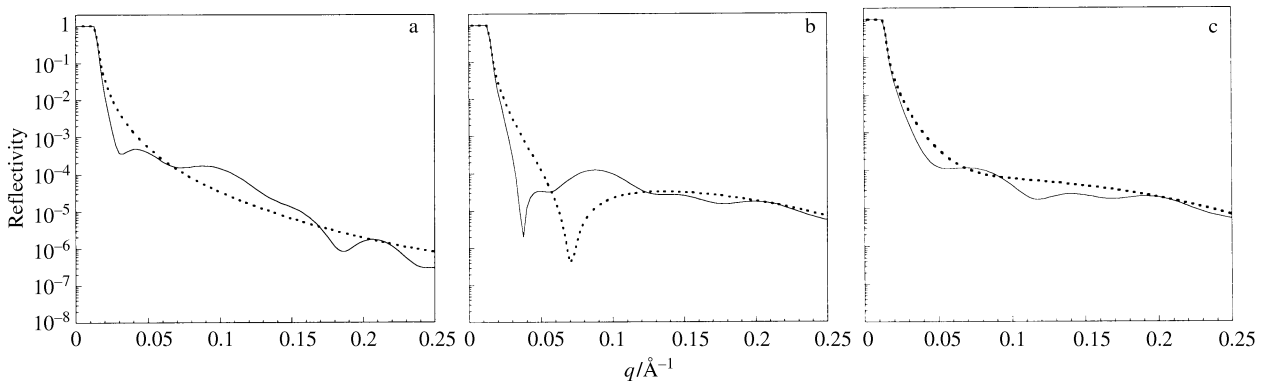

Fig. 3 Calculated reflectivity profiles calculated for an adsorbed protein forming two layers, an inner layer of thickness 30 Å and volume fraction 0.6, and an outer layer of thickness 65 Å and volume fraction 0.2, on a partially deuterated hydrocarbon layer grafted on to a silicon substrate in D_2O. The hydrocarbon layer is divided into three 9 Å thick segments, (i), (ii) and (iii), the last being next to the protein–aqueous phase. The scattering length density of a layer is taken to be -0.5×10^{-6} Å^{-2} if it is protonated and 6.5×10^{-6} Å^{-2} if it is deuterated. (a) Layers (i), (ii) and (iii) all deuterated. (b) layers (i) and (ii) deuterated and (iii) protonated, (c) layer (i) deuterated and (ii) and (iii) protonated. The dashed lines are calculated in the absence of protein. Interference features from the two component protein layers affect the different contrasts in different ways.

layers is greatly increased. We have given one option here but, of course, the labelling could be reversed to have the deuterated fragment on the outside of the grafted layer.

In a typical analysis of a neutron reflectivity profile, the measured data are compared with a profile calculated using the optical matrix method applied to different model density profiles.[40] The model will consist of a series of layers, each with a scattering length density ρ and thickness t.

An additional parameter, σ, which is the interfacial roughness between any two consecutive layers, can be incorporated if necessary. The calculated profile is compared with the measured profile and ρ and t for each layer are varied until the optimum fit to the data is found. Although any one profile is not necessarily a unique solution, as shown in the above discussion, the different isotopic contrasts can usually be used to be confident that the interpretation is correct. Once each layer has been characterised by a thickness and scattering length density, then the area per molecule or the coverage is easily determined as described in a previous paper.[41]

Experimental

Neutron reflectivity measurements were made on the small angle scattering spectrometer D17, before its recent reconstruction, at the high flux nuclear reactor of the Institut Laue Langevin (ILL), Grenoble, France. Details of the instrument are given elsewhere.[42] The reflectivity was measured with a monochromatic collimated beam of 12 Å neutrons and the momentum transfer q [see eqn. (1)] varied by varying the incident angle θ. The q range investigated was 0.008–0.145 $Å^{-1}$. D17 has a multidetector and the background was determined by interpolation of the signal from either side of the specularly reflected beam. The relative efficiency of the detector elements was calibrated with the uniform incoherent scattering from H_2O. The attenuated direct beam was measured to determine the absolute value of the reflectivity. Because the presence of the multidetector restricts the possible secondary collimation, the background is larger than on a single detector reflectometer and becomes dominant at values of q above 0.15 $Å^{-1}$. A schematic representation of the cell used is shown in a previous paper.[43] The cell was thermalised at 20 °C.

The substrate used in this study was a silicon block of dimensions 12.7 × 5.08 × 2.54 cm^3 polished by Engis (UK) cut with the large faces as (111). The block was cleaned by first sonicating in chloroform in order to eliminate any organic material on the surface, and then in RCA1 solution [aqueous NH_3–H_2O_2–H_2O (1 : 1 : 5 v/v)] at 70 °C for 5 min)[44] to produce a very hydrophilic surface. It was hydrophobed from a 1.5×10^{-3} M solution of deuterated octadecyltrimethylammonium bromide (OTS) in CH_2Cl_2. The synthesis of the deuterated material and the hydrophobing procedure were as described previously.[41] Measurements were made in 99% D_2O (obtained from the ILL), ultrapure H_2O and water$_{4.5}$ [D_2O–H_2O (0.752 : 0.248 w/w)]. Measurements on the bare silicon substrate had been taken in a previous experiment on the neutron reflectometer CRISP at the Rutherford Appleton Laboratory (Didcot, UK). Details of the experiment and results have been described.[3] The properties of the oxide in the present case were taken to be unchanged and this assumption was confirmed by measuring a reflectivity profile in D_2O, which was found to coincide with that measured for the previous experiment. The oxide was found to consist of SiO_2 ($\rho = 3.41 \times 10^{-6}$ $Å^{-2}$) with a thickness of 10 ± 2 Å and a roughness of $\sim 6 \pm 1$ Å. After the hydrophobing procedure, reflectivity profiles were measured in D_2O, H_2O and water$_{4.5}$. It was found difficult to obtain a satisfactory fit to all three profiles, which can be attributed to the hydrophobic layer not being as good as in the previous experiment on β-CN.[23] Thus, instead of a packing fraction of 0.85–0.90 in the hydrophobic layer as obtained previously, the present layer was only about 0.75, although its thickness (22 ± 2 Å) was similar to that obtained previously.[23]

The solutions at different pH were made using buffers of disodium phosphate (Na_2HPO_4) and citric acid ($H_3C_6H_5O_7$).[45] The ionic strength was not constant but varied from 0.12 M at pH 3 through 0.30 M at pH 5, 0.49 M at pH 7 and 0.57 M at pH 8. The pH of all solutions was checked with a pH meter and found to be $\sim$0.05 units higher than calculated from the composition for solutions of the proteins in H_2O, between 0.1 and 0.2 units higher for solutions in D_2O and between 0.1 and 0.15 units higher in water$_{4.5}$. The protein concentration was in all cases 0.05 g dm^{-3}. The addition of salt or buffer is known to increase the solubility of these proteins in water.[35] The protein solutions were prepared a few hours before the experiments were performed and they were kept at a low temperature in order to speed up the solubilisation of the proteins in water. Over the whole range of pH studied the proteins were soluble except for β-CN in D_2O at pH 5, in which case the solution looked cloudy. This did not appear to have any effect on the reflectivity profile.

The order of the measurements of the adsorption of proteins at different pH was solutions of β-CN at pH 7, 5, 3 and 8, followed by removal of β-CN from the surface and measurements from solutions of β-Lg in the same order. β-CN was removed by using a solution of the non-ionic surfactant $C_{12}E_8$ at 10 × c.m.c. (c.m.c. = critical micelle concentration). At each value of pH measurements were made in D_2O, H_2O and water$_{4.5}$. At each pH the measurement in the first contrast was repeated two or three times until the profiles were found to coincide. It could therefore be assumed that the protein had reached its final conformation at that pH (kinetic effects are discussed further below). After each measurement the block was rinsed with the solution to be used next. It was not thought necessary to remove the protein after each measurement except when the two proteins were exchanged.

Results and discussion

Structure of adsorbed proteins at neutral pH

The reflectivity profiles of adsorbed layers of β-CN and β-Lg at pH 7 on the deuterated hydrophobic surface are shown for three different water contrasts, D_2O, H_2O and water$_{4.5}$, in Figs. 4, 5 and 6. In the absence of protein all three water contrasts give smoothly decaying reflectivity profiles with no sign of any structure. As would be expected from the earlier discussion, the greatest effect of the protein is seen for D_2O, there being the sharp negative part of an interference fringe close to a momentum transfer value of about 0.04 $Å^{-1}$, both proteins showing this effect (Fig. 4). Also, as would be expected from the earlier discussion about the effects of contrast, there is very little change in the profiles with H_2O on adsorption of protein (Fig. 6). The structure in the reflectivity profiles of Fig. 5 shows a useful feature not anticipated by the earlier discussion about effects of contrast. First, both proteins give some interference in the profile with water$_{4.5}$, although the effect is much weaker than in D_2O. However, the angle of total reflection (critical angle) is lower for water$_{4.5}$ than for D_2O and this, in principle, means that any useful shape in the reflectivity profile can be seen down to a lower value of q. The shape of the profiles in the region of 0.04 $Å^{-1}$ is different for the two proteins and detailed fitting of models shows that the difference can be attributed to longer range structure in the β-CN which we had not been able to identify in our earlier measurements. This once again emphasises the need to explore many contrasts in order to probe the Fourier components of the interfacial structure over as wide a range as possible. The properties of the protein layers deduced from the fitted parameters are given in Table 2 for β-CN and Table 3 for β-Lg. These parameters that represent a physical model

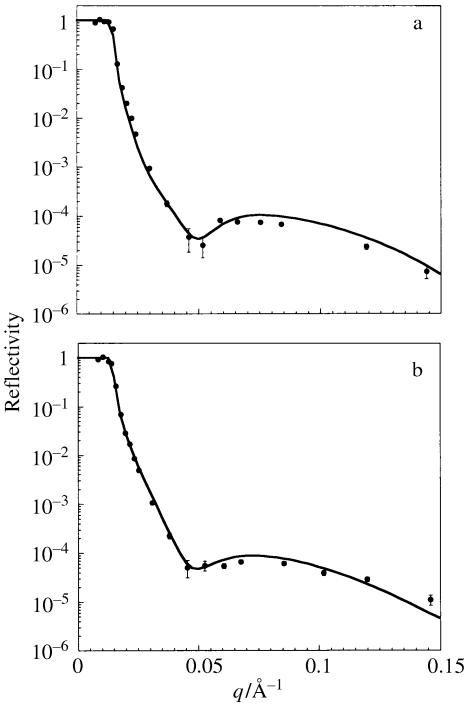

Fig. 4 Reflectivity profiles and fitted curves for the deuterated hydrophobic interface between Si–SiO_2 and D_2O with absorbed protein, (a) β-CN and (b) β-Lg, at pH 7. The continuous lines are the best fits using the parameters in Tables 2 and 3.

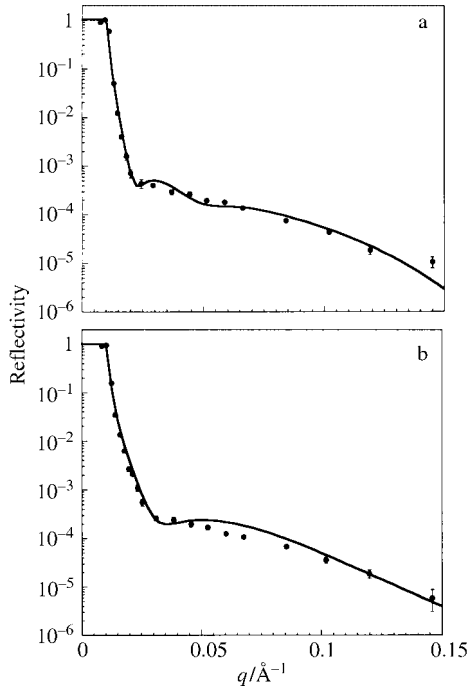

Fig. 5 Reflectivity profiles and fitted curves for the deuterated hydrophobic interface between Si–SiO_2 and water$_{4.5}$ with adsorbed protein, (a) β-CN and (b) β-Lg, at pH 7. The continuous lines are the best fits using the parameters in Table 2.

are easier to interpret than the individual results of scattering length density for the simultaneous fits to many contrasts used in the experiments. The remaining discussion is therefore centred on these parameters.

In the case of β-CN, a two layer model for the adsorbed protein was required to fit the data, a more compact layer adjacent to the surface of thickness 30 ± 3 Å and volume fraction 0.62 and a looser and thicker layer protruding into the solution of thickness 65 ± 5 Å and volume fraction 0.20. The thickness of both layers is higher than in the previous experiment (23 Å and volume fraction 61% and 35 Å and volume fraction 12%[23]) and closer to some of the values found in the literature.[20,21] The introduction of some roughness between the outer layer and the bulk water results partly from the lower momentum transfer features observed in the profile for water$_{4.5}$ and commented on above, and partly because the structure of the denatured protein may be sensitive to the structure of the hydrophobic layer, which must have a looser structure here than in our previous work. As for the deuterium substitution, it was found that while the hydrophobic part close to the substrate was not affected, some H–D exchange occurred in the hydrophilic part protruding in the solution. The effect is difficult to quantify in detail. The scattering length density of this layer changed from 1.8×10^{-6} Å^{-2} in H_2O to 2.7×10^{-6} Å^{-2} in D_2O.

For the globular β-Lg at pH 7, a single layer model was required to fit the adsorbed protein layer and this was expected because of its more compact structure in solution. The thickness, 32 ± 4 Å, is slightly lower than the diameter of the protein in solution, which is not surprising since the protein may be forced to flatten to some extent for better contact with the surface. This is confirmed by the experimental value of the area per molecule when compared with the cross-sectional area of the spherical protein in solution: if the radius of the sphere is 18 Å, the cross-sectional area of the monomer in solution would be about 1000 Å^2, which is only slightly lower than the value calculated for the molecule in solution, confirming that the molecule flattens at the interface. The volume fraction, 0.65, is similar to the β-CN case. Again, some roughness was required at the interface with the solution. Penetration of the protein into the underlying hydrophobic layer was detected in both cases. The values of the parameters required to fit the data are summarised in Table 2 and the calculated reflectivity profiles are shown as continuous lines in Figs. 4(b), 5(b) and 6(b).

Table 2 Properties of the β-CN layers derived from fits

Property	pH 7	pH 5	pH 3
Inner layer—			
t/Å	30 ± 3	30 ± 1	30 ± 1
ϕ	0.62 ± 0.02	0.71 ± 0.01	0.64 ± 0.01
A/Å^2	(1.6 ± 0.02) × 10^3	(1.39 ± 0.05) × 10^3	(1.53 ± 0.05) × 10^3
Γ/μmol m^{-2}	(10 ± 1) × 10^{-2}	(11.8 ± 0.5) × 10^{-2}	(10.2 ± 0.4) × 10^{-2}
Outer layer—			
t/Å	65 ± 5	95 ± 5	105 ± 5
σ/Å	35 ± 5	45 ± 5	50 ± 5
ϕ	0.20 ± 0.02	0.33 ± 0.01	0.30 ± 0.01
A/Å^2	(2.3 ± 0.15) × 10^3	(0.9 ± 0.1) × 10^3	(0.9 ± 0.1) × 10^3
Γ/μmol m^{-2}	(7.2 ± 0.5) × 10^{-2}	(19 ± 2) × 10^{-2}	(19 ± 2) × 10^{-2}

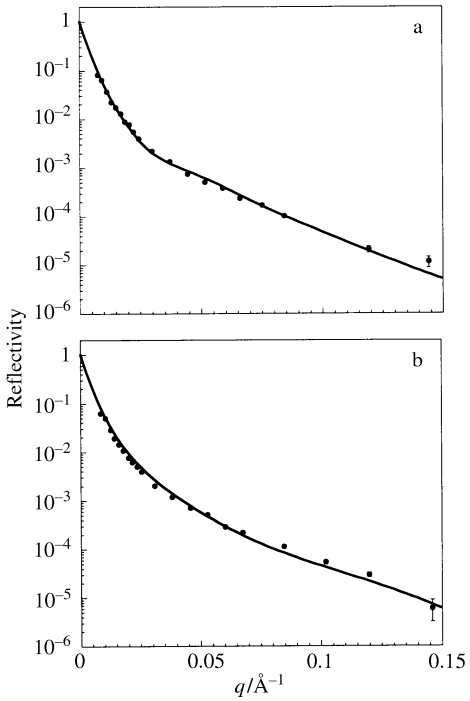

Fig. 6 Reflectivity profiles and fitted curves for the deuterated hydrophobic interface between Si–SiO_2 and H_2O with adsorbed protein, (a) β-CN and (b) β-Lg, at pH 7. The continuous lines are the best fits using the parameters in Tables 2 and 3.

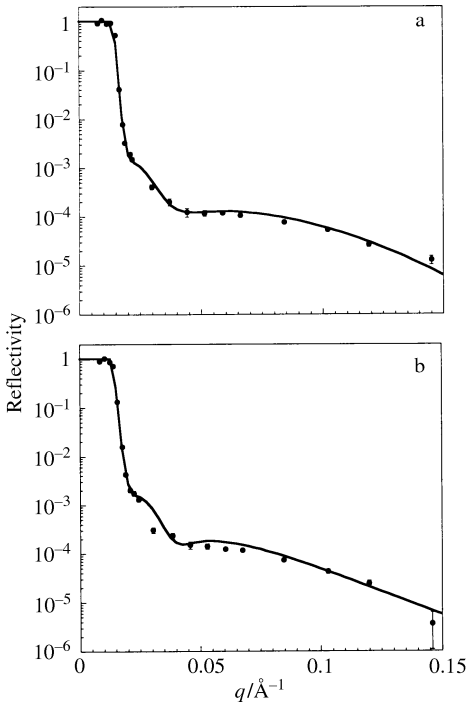

Fig. 7 Reflectivity profiles and fitted curves for the deuterated hydrophobic interface between Si–SiO_2 and D_2O with adsorbed protein, (a) β-CN and (b) β-Lg, at pH 3. The continuous lines are the best fits using the parameters in Tables 2 and 3.

Structure of proteins adsorbed from acidic or basic solution

Change in the pH of a protein solution alters dissociation in the charge on the protein molecule. This will result in a modification of the conformation of the protein and of its surface activity. In acidic solutions both the proteins become less charged and the lower electrostatic repulsion between the molecules then leads either to partial collapse of the layer or, possibly, to more adsorption. At alkaline pH there is an irreversible denaturation of both proteins. In this experiment the adsorbed layer was deposited at neutral pH. Since the proteins are strongly bound to the surface and are displaced only by concentrated surfactant solution, the main change in the protein layer on changing the pH will be the effect of the pH on the layer itself rather than from any change in the adsorbed amount or the nature of the species adsorbed.

For each pH the reflectivities in the three water contrasts D_2O, H_2O and $water_{4.5}$ were measured. As expected, the greatest sensitivity to the adsorbed layer is in D_2O. For both β-CN and β-Lg there is a marked increase in the amount adsorbed on lowering the pH to 5 (see Figs. 7–9) but little change when the pH is lowered further to 3. That adsorption is irreversible is indicated by the fact that when the pH is raised from 3 to 8 no significant change in the layer occurs. The increased adsorption at the first decrease of pH from 7 to 5 is correlated with the decreased charge within the protein molecules and thus decreased hydrophilicity. The charge decrease also means that the electrostatic repulsion within the adsorbed layer is to be decreased. The combination of these two effects may be responsible for the increased adsorption as pH is lowered. When the pH is decreased from 5 to 3, positive charges within protein molecules start to increase and so does the lateral repulsion. This would mean that the amount of protein adsorbed should decrease when pH is moved away from the isoelectric points for the two proteins. However, this is not what we have observed. That the level of adsorption does not vary with pH after the first pH shift suggests that the adsorbed protein molecules cannot be desorbed. The denatured protein fragments are well intermixed and they also have many contacts with the solid substrate. To desorb a single protein molecule, many contacts have to be detached simultaneously. This is energetically unfavourable. A polypeptide chain such as β-CN has several contacts with the solid

Table 3 Properties of the β-Lg layers derived from fits

Property	pH 7	pH 5	pH 3
Inner layer—			
t/Å	32 ± 4	32 ± 3	33 ± 1
ϕ	0.65 ± 0.01	0.66 ± 0.01	0.65 ± 0.01
A/Å^2	1.30 ± 0.1	(1.09 ± 0.1) × 10^3	(1.14 ± 0.03) × 10^3
Γ/μmol m^{-2}	12.8 ± 0.8	(15.5 ± 1.5) × 10^{-2}	(14.5 ± 0.5) × 10^{-2}
Outer layer			
t/Å		95 ± 10	120 ± 10
σ/Å	13 ± 3	40 ± 5	60 ± 5
ϕ		0.28 ± 0.01	0.27 ± 0.01
A/Å^2		(0.88 ± 0.1) × 10^3	(0.71 ± 0.06) × 10^3
Γ/μmol m^{-2}		(19 ± 2) × 10^{-2}	(23.6 ± 2) × 10^{-2}

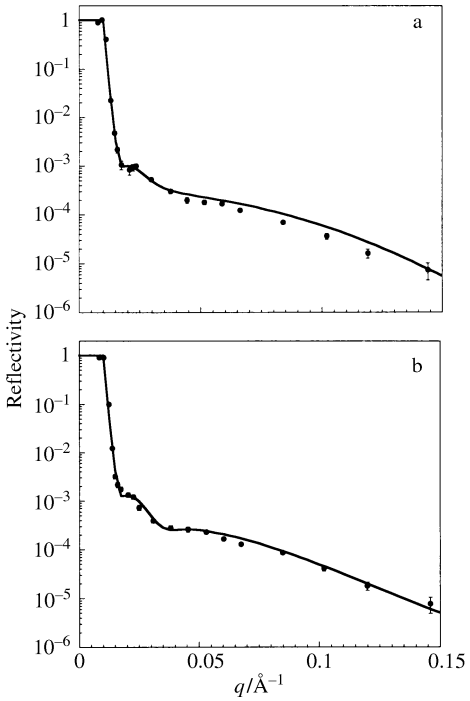

Fig. 8 Reflectivity profiles and fitted curves for the deuterated hydrophobic interface between Si–SiO_2 and $water_{4.5}$ with adsorbed protein, (a) β-CN and (b) β-Lg, at pH 3. The continuous lines are the best fits using the parameters in Tables 2 and 3.

surface and the pH change alone does not provide sufficient energy to desorb it.

In Tables 2 and 3, the results of the fits of the reflectivity profiles at pH 3 and 5 are summarised. The analysis assumed that the scattering length densities of the proteins do not change significantly with pH. The results show that at lower pH the thickness of the inner layer remains constant (~30 Å) although the composition varies as more protein seems to adsorb when compared with the results at neutral pH. β-Lg at low pH also exhibits a second layer and for both proteins the thickness of the outer layer and the volume fraction of the protein increase.

The measurements in these experiments were mostly repeated three times in a period of 2–4 h to ensure that equilibrium had been reached and reproducible reflectivity curves obtained. At this point it was assumed that the protein had reached its final conformation on the surface. In an earlier experiment we had found that β-CN needs time to reach its final conformation on the surface and data were collected every 90 min until successive reflectivity profiles were found to be identical. The surface used in the earlier experiment was different to that used here, although the substrate was also hydrophobed with OTS. It took about 8 h for the protein to assume its final conformation. It is interesting that one can follow conformational changes at an interface as a function of time. Clearly, the adsorption of the protein may be very sensitive to the final quality of the hydrophobed surface.

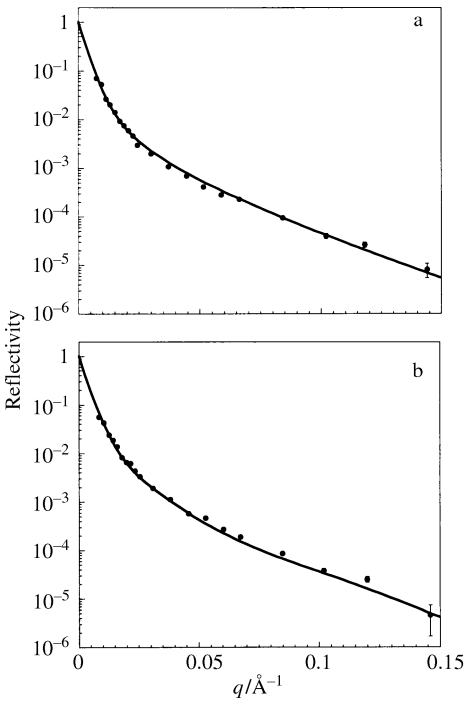

Fig. 9 Reflectivity profiles and fitted curves for the deuterated hydrophobic interface between Si–SiO_2 and H_2O with adsorbed protein, (a) β-CN and (b) β-Lg, at pH 3. The continuous lines are the best fits using the parameters in Tables 2 and 3.

Conclusion

The results described above may be explained in terms of the different conformations adopted by the two species of molecules in solution. In the case of β-CN an explanation of the structure of the adsorbed layer in terms of the overall charge of the protein has already been provided.[23] Compared with those results, a better agreement with the literature has been found here[20,21] in terms of both thickness and area per molecule. The different structures of β-CN at the two interfaces may be due either to small differences in the hydrophobic surfaces or to the different ionic strengths of the solutions. The time behaviour illustrates how sensitive the adsorbed β-CN layer is to variations in the hydrophobic layer. For β-Lg at pH 7 the one layer model for the adsorbed protein compares well with the compact structure in solution. The thickness of the layer and the area per molecule imply a slight flattening of the molecules on the surface. Again there is good agreement with literature data.[20]

At values of pH other than 7, both proteins show more adsorption and thicker layers. β-Lg now adsorbs in two layers and this may be explained by assuming either that the protein unfolds, and this has been suggested by other workers who have investigated its secondary structure,[35] or that it aggregates. It is believed that a compact globular molecule does not generally unfold to such an extent that it forms an adsorbed layer with loose loops and tails,[46] so that the formation of aggregates (additional layers on top of the first) seems more likely. Unfortunately, there are not many results in the literature of studies of β-CN and β-Lg adsorption at interfaces in the range of pH investigated here.

This study illustrates a valuable feature of investigating such systems on a flat surface rather than on a colloidal dispersion. For the latter it is essential that the adsorbed layer maintains the stability of the colloid. The stability range is usually very narrow in terms of applied conditions. On the flat surface this is irrelevant as long as there is not bulk deposition on the surface. It is therefore possible to study the adsorbed layer even at pH values where the protein in solution is denatured as long as further adsorption of the denatured product does not interfere with the measurements.

Finally, it is interesting that the contrast $water_{4.5}$ is more sensitive to the large thickness of the adsorbed layers than D_2O (which gives a greater contrast to the proteins). It is this extra measurement that is largely responsible for the differences in interpretation and increased precision of this study.

The largest scale dimensions in the protein have their main effect at low angles where they are masked by the higher critical angle of D_2O and this detail is only revealed in water$_{4.5}$.

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