Effect of pH on the Adsorption of Bovine Serum Albumin at the Silica/Water Interface Studied by Neutron Reflection

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In a previous study of BSA adsorption onto the hydrophilic silica/water interface using neutron reflection, we examined the concentration dependence of the surface excess of BSA at a pH close to its isoelectric point (IP). The surface excess was found to reach a plateau at a very low bulk protein concentration, suggesting a high affinity of BSA for the oxide surface. This work has now been extended to an investigation of the structure and composition of the BSA layer above and below its IP. It is found that adsorption of BSA is strongly dependent on pH, although the protein concentration has little influence on the surface excess at pH 3 and 7. Changing the pH from the IP substantially reduces the surface excess. The structure of the adsorbed layers below a bulk BSA concentration of 0.5 g dm^{-3} can be fitted to a single uniform layer distribution over all pH conditions studied, which suggests that there is no significant denaturation. Denaturation generally leads to a more fragmented peptide distribution and a nonuniform density distribution normal to the surface. The thicknesses of the layers below 0.5 g dm^{-3} were all smaller than the dimension of the short axis of the globular solution structure for BSA, indicating that the molecules are adsorbed sideways-on with their long axes parallel to the solid surface and that adsorption onto the hydrophilic surface results in some structural deformation. The reversibility of BSA adsorption at the hydrophilic silica/water interface was also examined directly. Adsorption was found to be irreversible with respect to changes in BSA concentration but reversible with respect to solution pH at low BSA concentrations only.

Introduction

Serum albumins are the most abundant protein in blood and bovine serum albumin (BSA) is one of the globular proteins whose amino acid sequence and physical properties have been well characterized.[1] BSA is approximately a prolate ellipsoid and in normal condition its major and minor axes are respectively equal to 140 and 40 Å. BSA has been widely used as a model protein for studying the physical and biological aspects of the adsorption of a protein at a solid surface and a number of such studies have been made at the hydrophilic silicon oxide/water interface.[2−4] Here we present the determination of the structural conformation of the BSA layers adsorbed at the silica/water interface using specular neutron reflection.

There are two strands of interest in studying protein adsorption.[5,6] For applications such as the development of biochemical sensors, adsorption conditions are tuned to optimize the total amount of globular proteins physically adsorbed or covalently bound onto the solid substrate. In contrast, in the use of medical devices inhibition of protein adsorption is the aim since deposition of many proteins onto medical devices is highly undesirable. Adsorption of small proteins may stimulate the subsequent attachment of fibrous proteins, leading to adverse biological consequences. For both groups of interests the basic route to achieving their objectives is to understand the factors determining adsorption. The first step must then be to characterize the structure and functionality of the adsorbed or immobilized protein layer. Studies have been made on particulate absorbents and at flat interfaces. In a particulate dispersion where the interfacial area is large the activity of the bound protein can be probed by protein assay, but it is then very difficult to identify the structural conformation. Techniques such as infrared (IR) and circular dichroism (CD) can give information about the chemical environment of some functional groups, and hence infer whether or not there is any helical structure within the globular framework, but they provide no information about the packing of protein molecules within the surface layer. Ellipsometry has been used to study protein adsorption at a flat surface[5,7] but the thickness of the protein layer is typically below 100 Å and the thickness of the layer is a parameter too strongly coupled to its refractive index profile for it to be possible to make an unambiguous separation of the two. This is exacerbated by the extensive incorporation of water into the adsorbed layer, which makes the composite refractive index of the protein layer too similar to that of pure water.

Specular neutron reflection has recently been applied to the study of proteins adsorbed at the solid/water interface. In the measurement of dimensions along the normal direction, neutron

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reflection has greater interfacial resolution than any other technique presently available, especially if isotopic substitution is used to vary the contrast between water, protein, and solid substrate. Variation between H_2O and D_2O also allows a reliable determination of the extent to which water penetrates the protein layer. This information combined with the known dimensions of globular proteins often allows us to infer the physical state of an adsorbed protein layer. Thus, neutron reflection has recently been used to examine adsorption of hen egg-white lysozyme at the bare silica/water interface and on the hydrophobic surface obtained by chemical grafting of a self-assembled monolayer of octadecyltrichlorosilane (OTS).[8,9] While lysozyme on the hydrophilic oxide surface retains its globular structure and is reversible with respect to pH variation, the protein layer adsorbed on hydrophobic OTS was seriously denatured.

In our previous study of BSA adsorption at the hydrophilic silica/water interface,[10] the concentration dependence of the surface excess at pH 5, close to the isoelectric point (IP) of BSA, was examined. Adsorption was found to reach a plateau at a very low BSA bulk concentration, suggesting that BSA has a high surface affinity. We have now extended these measurements to examine the concentration dependence of BSA adsorption at pHs above and below the IP. Adsorption is again found to reach saturation at very low protein concentration at pH 3 and 7, although the surface excesses are greatly reduced. Furthermore, while adsorption with respect to pH is found to be reversible over a wide BSA concentration range, adsorption with respect to BSA concentration is found to be completely irreversible.

Experimental Section

The BSA sample was free of fatty acid and was used as supplied (Sigma, Cat No. A0281, Lot No. 10H9304). The molecular weight of BSA is 66 267 calculated from the known amino acid sequence, and its isoelectric point is 4.7−4.8.[11] The solution pH was controlled by using phosphate buffer and the pH varied by changing the ratio of Na_2HPO_4, NaH_2PO_4, and H_3PO_4, keeping the total ionic strength fixed at 0.02 M. There were small differences in pH between H_2O and D_2O but this was controlled to within 0.2 pH units. D_2O was purchased from Fluorochem (99.9% D) and its surface tension was typically over 71 mN m^{-1} at 25 °C, indicating the absence of any surface active impurity. H_2O was processed through an Elgastat ultra pure water system (UHQ) and its surface tension at 25 °C was constant at 71.5 mN m^{-1}. The glassware and Teflon troughs for the reflection measurements were cleaned using alkaline detergent (Decon 90) followed by repeated washing in UHQ water. All the experiments were performed at 25 °C.

Neutron reflection measurements were made on the white beam reflectometer CRISP at the Rutherford-Appleton Laboratory, ISIS, Didcot, UK[12] using neutron wavelengths from 1 to 6 Å. The sample cell was almost identical to that depicted by Fragneto et al. in ref 13 with the aqueous solution contained in a Teflon trough clamped against a silicon block of dimensions $12.5 \times 5 \times 2.5$ cm^3. The collimated beam enters the end of the silicon block at a fixed angle, is reflected at a glancing angle from the solid/water interface, and exits from the opposite end of the silicon block. Each reflectivity profile was measured at three different glancing angles, 0.35°, 0.8°, and 1.8°, and the results were combined. The beam intensity was calibrated with respect to the intensity below the critical angle for total reflection at the silicon/D_2O interface. A flat background determined by extrapolation to high values of momentum transfer, κ ($\kappa = (4\pi \sin\theta)/\lambda$, where λ is the wavelength and θ is the glancing angle of incidence), was subtracted. For all the measurements the reflectivity profiles were essentially flat at $\kappa > 0.2$ $Å^{-1}$, although the limiting signal at this point was dependent on the H_2O/D_2O ratio. The typical background for D_2O runs was found to be 2×10^{-6} and that for H_2O to be 3.5×10^{-6} (measured in terms of the reflectivity).

The procedure for polishing the large face (111) of the silicon block has been previously described.[10] Before reuse the surface was cleaned with 5% Decon solution and the whole block was thoroughly rinsed with UHQ water, followed by soaking in acid peroxide solution (600 mL of 98% H_2SO_4 in 100 mL of 25% H_2O_2) for 6 min at 120 °C to optimize the surface hydrophilicity.[14] The block was then thoroughly rinsed with UHQ water to remove acid and exposed to UV/ozone for 30 min to remove any traces of organic impurities.[15] This procedure was found to produce surfaces with reproducible thickness and roughness of the oxide layer, and which were completely wetted by water. The surface hydrophilicity was further examined by measuring the reflectivity in buffered D_2O from a 0.15 g dm^{-3} BSA solution at pH 5, which gave reflectivity profiles identical to those obtained in our previous work using different blocks, confirming the reproducibility of different blocks as regards hydrophilicity.

Neutron Reflection

The results from neutron reflection measurements are usually presented in terms of neutron reflectivity $R(\kappa)$ as a function of κ. $R(\kappa)$ is the ratio of the intensity of the reflected to that of the incoming beam and its decay with increasing κ is primarily determined by the variation of scattering length density $\rho(z)$ along the surface normal direction. The scattering length density depends on the chemical composition through the equation[16,17]

$$\rho = \sum n_i b_i \tag{1}$$

where n_i is the number density of element i and b_i its scattering amplitude (scattering length). Thus, neutron reflectivity contains information about the structural distribution of the interfacial layer.

Since the scattering lengths of D and H are of opposite sign, the scattering length density of water can be varied over a wide range. This means that for a given protein layer the interfacial structure can be highlighted in different ways by using different mixtures of H_2O and D_2O. The combined analysis of more than one reflectivity profiles for the same interfacial layer greatly improves the certainty of the structural information obtained.

Information about surface excess, Γ, or area per molecule, A, can be obtained directly from the derived scattering length density ρ and thickness of the layer d using

$$A = \frac{\sum m_i b_i + n_w b_w}{\rho d} \tag{2}$$

where m_i is the number of component i with scattering length b_i, n_w is the number of water molecules associated with each protein, and b_w is the corresponding scattering length per water molecule. The values of m_i and b_i depend on the chemical composition of the protein and $\sum m_i b_i$ denotes the total scattering length for a protein molecule. The surface excess Γ is related to A by

$$\Gamma = 1/N_a A \tag{3}$$

where N_a is Avogadro's number. The volume fraction of the protein in the layer, ϕ_p, can be obtained directly using the

following equation:

$$\rho = \phi_p \rho_p + (1 - \phi_p)\rho_w \quad (4)$$

where ρ_w is the scattering length density of the bulk water. The value of n_w can be evaluated from the following equation

$$n_w = (Ad - V_p)/V_w \quad (5)$$

where V_w and V_p represent the volume of water and protein molecules, respectively.

Although rigorous relationships between neutron reflectivity and the distribution of interfacial scattering length density can be written, structural information is often obtained by fitting models to the experimental profiles. Such fitting is usually done by assuming a structural model and calculating the corresponding reflectivity using the optical matrix method.[18] The calculated reflectivity is then compared with the measured one and the structural parameters subsequently modified in a least-squares iteration to obtain a good fit. The parameters used in the calculation are the thicknesses of the layers, d_i, and the corresponding scattering length densities, ρ_i. Since the scattering length density of a given layer varies with isotopic composition, the fitting of a set of isotopic compositions to a single structural model greatly reduces the possibility of ambiguity in the interpretation, although it adds to the complexity of the fitting procedure.

Results and Discussion

1. Characterization of the Oxide Layer. The structure of the native oxide layer on the surface of the silicon block is best characterized before the adsorption measurement proceeds. This is because the oxide layer contributes to the neutron signal and any uncertainties this introduces can be minimized if its structural parameters have already been determined accurately. Although the surface on the current block had already been characterized in our previous work,[10] peroxide cleaning and subsequent UV treatment might alter its composition. The characterization was made at the solid/water interface because the presence of water and different isotopic compositions of the water are known to assist in the determination of the structure of the oxide layer. This is particularly the case if defects are present. Two water contrasts were used, pure D_2O and a mixed water contrast producing a scattering length density close to that of the bulk silicon. This was achieved using a 3:2 ratio of H_2O to D_2O. The combined fitting of the two reflectivity profiles gave a thickness of 12 ± 3 Å and $\rho = 3.41 \times 10^{-6}$ Å^{-2} for the oxide layer, in good agreement with that found using the data shown in Figure 1 in the previous paper.[10] Since the scattering length density of the layer is exactly that expected for amorphous oxide, there is no penetration of water into the layer, suggesting a surface reasonably free from defects. Also consistent with this observation, no roughness was required in the fitting, suggesting that the oxide surface is relatively smooth.

2. BSA Adsorption under Different pH. The amount of protein adsorbed on the oxide surface was determined by measuring neutron reflectivity profiles in D_2O because D_2O highlights the adsorbed protein at the solid surface. Figure 1 shows the variation of neutron reflectivity with BSA concentration for a set of BSA concentrations at pH 7. The effect of bulk BSA concentration on the adsorbed surface layer can be seen by comparing the reflectivities with that from the bare silica/D_2O interface, which is shown as a dashed line in Figure 1. BSA adsorption results in a lowering of reflectivity compared with that from the bare solid/D_2O interface. It can therefore be seen from Figure 1 that although the bulk concentration is varied over a range of 400 times, there is little change in the reflectivity profiles, suggesting weak adsorption of the BSA at this pH.

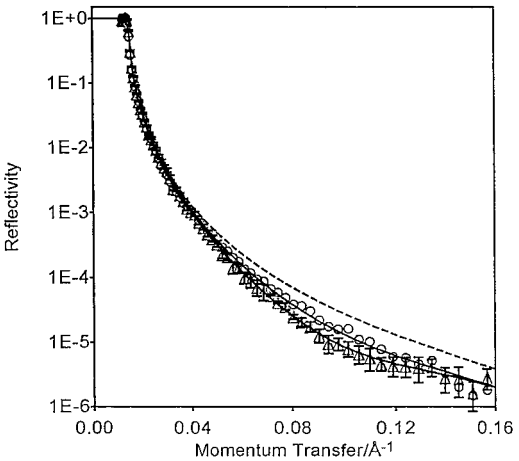

Figure 1. Plots of neutron reflectivity profiles at the silica/D_2O interface at BSA concentrations of 0.005 g dm^{-3} (○) and 2 g dm^{-3} (△). The solution pH was 7 and the ionic strength was 0.02 M. The reflectivity from the bare silicon oxide/D_2O interface is also shown as a dashed line for comparison. The continuous lines were calculated using the optical matrix method with an oxide layer thickness of 12 ± 3 Å. The thicknesses and the surface excesses for the protein layers are given in Figures 3 and 4.

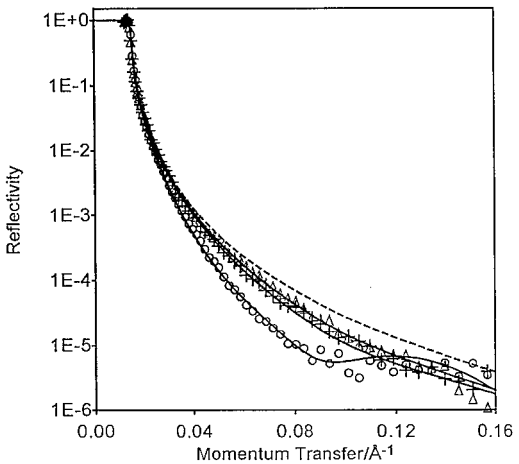

Figure 2. Plots of neutron reflectivity profiles at the silica/D_2O interface at a solution pH of 3 (△), 5 (○), and 7 (+). The BSA concentration was fixed at 0.005 g dm^{-3} and the ionic strength was 0.02 M. The reflectivity from the bare silicon oxide/D_2O interface is also shown as a dashed line for comparison. The continuous lines were calculated using the optical matrix method with an oxide layer thickness of 12 ± 3 Å. The thicknesses and the surface excesses for the protein layers are given in Figures 3 and 4.

The isoelectric point for BSA is at pH 4.8[11] and the surface excess is known to attain its maximum value at this pH.[10] Figure 2 compares a set of reflectivity profiles in D_2O at a fixed BSA concentration of 0.005 g dm^{-3} at pH 4.8 (IP), pH 3, and pH 7, with the reflectivity profile from the bare silica/D_2O interface. The profile measured at pH 5 is the one most different from that of the bare silica/D_2O interface, suggesting that surface adsorption is maximized at this pH. Furthermore, although the relative differences between the profiles at these three pH values do vary with respect to concentration, the same qualitative trend as that shown in Figure 2 is always observed. Thus, over the whole BSA concentration range studied adsorption is always greatest at pH 5.

TABLE 1: Scattering Length Densities of BSA in Different Water Contrasts[a]

	pH		
	3	5.1	7
$D_2O \times 10^6/Å^{-2}$	3.34	3.33	3.24
$CM4 \times 10^6/Å^{-2}$	2.83	2.79	2.78
$CMSi \times 10^6/Å^{-2}$	2.41	2.41	2.41
$H_2O \times 10^6/Å^{-2}$	1.85	1.90	1.90

[a] The total molecular volume was taken to be 79 111 Å^3 (see ref 33). The degree of ionization of different amino acid groups was taken from ref 34.

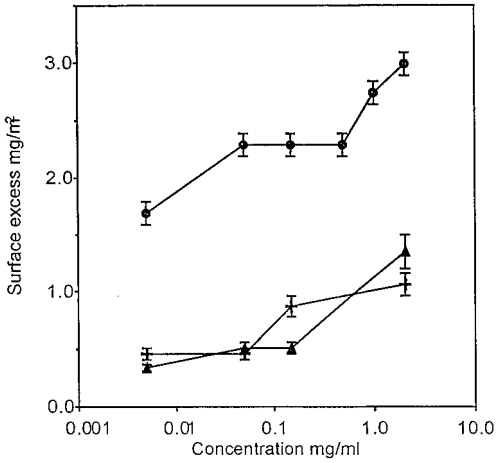

Figure 3. Variation of surface excess as a function of BSA bulk concentration at solution pHs of 3 (△), 5 (○), and 7 (+).

Quantitative information about the amount of protein adsorbed can be obtained by fitting models to the measured reflectivity profiles using the optical matrix formalism already outlined. It was found that a single uniform layer was sufficient to describe the adsorbed BSA layer over a wide concentration range. The continuous lines in Figures 1 and 2 were calculated for a uniform layer of protein adsorbed on the silicon oxide layer, assuming the structure of the oxide layer to be as already determined in pure water, and they are clearly good fits to the observed data. The volume fraction and surface excess of the adsorbed protein in the surface layer can be calculated using eqs 2−5 by taking the scattering length density of pure protein to be 3.3 x 10^{-6} Å^{-2}. As we have discussed elsewhere,[10] a good fit of the single uniform layer model to the measured reflectivity profiles indicates that the layer really is uniform along the normal direction to a very good level of approximation. The poor fit observed for some of the profiles over the higher κ range mainly results from a relatively large statistical error associated with the uncertainty of background subtraction. However, in all cases the differences between measured and calculated curves is within experimental error. Since BSA contains many labile hydrogens, the scattering length density of the protein will vary with the ratio of H_2O and D_2O because of exchange of the labile hydrogens. We have assumed here that exchange is complete and it should be noted that the reliability of this assumption directly affects the scattering length density of pure protein and hence the amount of protein adsorbed. Table 1 lists the values of ρ for pure protein under different water contrasts. Since incomplete exchange will have an effect on the results in different water contrasts, we return to discuss the validity of this assumption after all the results have been presented.

Figure 3 summarizes the variation of surface excess with BSA bulk concentration at the three different pH values. At each pH the surface excess at the lowest concentration is already some

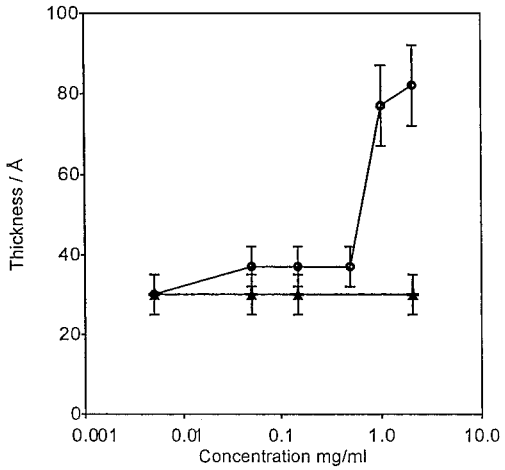

Figure 4. Variation of total BSA layer thicknesses as a function of BSA bulk concentration at solution pHs of 3 (△), 5 (○), and 7 (+).

50−70% of the maximum value measured and then only increases slowly with bulk concentration. At pH 5 the surface excess tends to a limit of 3 mg m^{-2} as compared with a limit of 1 mg m^{-2} at the other two pH. In the case of the adsorption at a bulk concentration above 0.5 g dm^{-3} at pH 5 it was necessary to use a two-layer model to obtain a good fit to the data, but below this concentration all the reflectivity profiles were fitted using a single uniform layer model. The sharp upturn around 0.5 g dm^{-3} at pH 5 may result from the presence of an additional weak diffuse layer but this only introduces an extra uncertainty into the coverage of less than 0.5 mg m^{-2}. We discuss this further below.

The other structural parameter obtained from the data is the thickness of the protein layer. Figure 4 shows the variation of the thickness with bulk concentration of BSA at the three pH values. It can be seen from Figure 4 that at pH 3 and 7 the thickness of the protein layer is constant within error at 30 ± 3 Å. At pH 5, the thickness increases slightly over the low-concentration region, where the surface excess also shows a small increase, and tends to a limiting value of 37 ± 3 Å as the concentration approaches 0.5 g dm^{-3}. Over the low concentration range, the thickness of the protein layer is in all cases shorter than the length of the minor axis of 40 Å for the BSA globular structure. The dimension of the layer therefore indicates that the adsorbed BSA molecules adopt a sideways-on orientation; i.e., the molecules adsorb with their long axes parallel to the solid surface. From the dimensions of the molecule and this orientation, it is possible to make an indirect estimate of the saturated surface coverage. At pH 3 and 7, the limiting surface excess, about 1 mg m^{-2}, corresponds to an area per molecule of some 11 000 Å^2. Assuming that BSA is rigid, the limiting cross-sectional area in sideways-on adsorption is approximately 5600 Å^2 (40 × 140 Å^2), equivalent to a surface excess of about 2 mg m^{-2}. This may simply indicate that at these two pH values the surface is never fully covered with protein and that about half of the oxide surface is uncovered. It should be noted that specular neutron reflection can give no information about the in-plane structure, at least not at these dimensions. The limiting surface excess of 2.5 mg m^{-2} at pH 5 and at the concentration just below 0.5 g dm^{-3}, however, corresponds to a saturated monolayer with an area per molecule of 4400 Å^2, which is smaller than the minimum area estimated for sideways-on adsorption. This clearly indicates that BSA molecule is not rigid and some degree of structural deformation or tilting has occurred upon adsorption. Over the concentration range above 0.5 g dm^{-3} at pH 5 a two-layer model had to be used to fit the reflectivity

profiles, as has been indicated previously. The sharp rise in the total layer thickness is clearly caused by the inclusion of the second diffuse layer.

In several previous studies on protein adsorption at the solid/solution interface, time-dependent adsorption has been observed. For example, using ellipsometry, Mcguire et al.[19] found that at both hydrophilic and hydrophobic silica/water interfaces lysozyme adsorption was strongly time dependent, with the surface excess typically taking an hour to reach equilibrium. However, in the present system no variation of the reflectivity with time was observed at any of the three pH values. Similarly, we observed no time effects in the adsorption of lysozyme at the hydrophilic and hydrophobed silica/water interfaces using neutron reflection.[8,9] The time scales possible with neutron reflection are not as short as those that can be probed with ellipsometry. Thus, it typically took 5 min to load the neutron sample cell with protein solution and a further 5 min before the neutron measurement could be started. However, with the white beam neutron source it typically only takes 5 min to obtain sufficient statistical accuracy at a fixed beam incidence angle for an adequate comparison to be made of successive reflectivity profiles. Since the signal changes detected by ellipsometry are on a slower time scale than the neutron measurements, any significant change in structure or coverage should have been observed in the neutron reflection experiment. Since no such change was observed, we can only conclude that the variations observed by the optical technique result from changes in the distribution of the counterions in the layer or the extent of dehydration of the adsorbed layer, neither of which is detectable by neutron reflection.

A number of determinations of the surface excess of BSA at the silica/water interface have been made, on both planar and particulate interfaces.[2−4] Adsorption of BSA on the hydrophilic surface of an optical waveguide made of mixed titanium oxide and silicon oxide at pH 7.4 has been studied by Kurrat et al.[4] using the integrated optical signal. When saturation was reached after about half an hour, the BSA surface excess at a bulk concentration of 0.084 g dm^{-3} was found to be 1.5 mg m^{-2}, as compared with 0.5 mg m^{-2} obtained by neutron reflection at pH 7. In particulate suspensions, the surface excess can be obtained by titrating BSA solutions before and after equilibration with the silica particles. Norde et al.[2] have used this method to obtain the surface excesses up to bulk concentrations of 0.15 g dm^{-3} at pH values of 4, 4.7, and 7. As expected, their surface excess at pH 4 is systematically higher than our values at pH 3 because our measurement is further away from the IP. There is excellent agreement close to the IP, although the pH of our system is slightly higher than theirs. At pH 7, agreement is good at a bulk concentration of 0.005 g dm^{-3}, but discrepancies start to appear as the concentration increases. Thus, at 0.05 g dm^{-3}, the surface excess from the work of Norde et al. is 1.2 mg m^{-2} as compared with our value of 0.5 mg m^{-2}, a difference well outside our range of error. As the concentration is further increased adsorption tends to a saturation value of 1.4 mg m^{-2} for the particulate system, as compared with 1 mg m^{-2} for the neutron work. A similar study using silica powder has also been carried out by Kondo et al.[3] Their BSA surface excesses show a steady increase with bulk concentration but are still in acceptable agreement with the neutron results if the combined errors are taken to be 15%.

Comparison of surface excess from the hydrophilic silica/water interface with studies of BSA adsorption on other surfaces allows us to examine the effect of surface properties on BSA adsorption. Using X-ray photoelectron spectra (XPS) Fitzpatrick et al.[20] measured the amount of BSA adsorbed onto mica by preadsorbing BSA onto mica at the IP. The surface excess was found to be 5.5 ± 1 mg m^{-2} at a bulk BSA concentration of 0.005 g dm^{-3}, compared with the neutron value of 1.8 mg m^{-2} at the silica/water interface. The large difference clearly demonstrates that difference in the physical nature of the two surfaces in terms of hydrophobicity, charge type or density has a profound effect on the amount of protein adsorbed. Studies of protein adsorption on polymer surfaces also show that protein adsorption is strongly affected by surface charge and hydrophobicity.[21−25]

There is also some value in comparing the BSA adsorption with that from HSA (human serum albumin) because HSA has almost the same molecular weight, IP, globular dimensions, and other physical properties as BSA.[26] HSA also has some 80% of its amino acid sequence identical to BSA. A recent study of HSA adsorption on mica by Blomberg et al.,[27,28] using XPS and an HSA concentration range from 10^{-3} to 1 g dm^{-3} at pH 5.5, found that adsorption reaches a saturation limit of 2.5 mg m^{-2} at a bulk concentration of 0.005 g dm^{-3}. The surface excess then remains constant until an HSA concentration of about 0.01 g dm^{-3} after which there is a further increase. Koutsoukos et al.[29] studied the adsorption of human plasma albumin (HPA) on different solid particles at pH 4.7 and found a maximum surface excess for the adsorption on positively charged haematite (α-Fe_2O_3) at 2.7 mg m^{-2} for protein concentrations above 0.3 g dm^{-3}, close to our value for the saturation limit for BSA on the negatively charged silicon oxide surface. However, their values for the adsorption of the same protein on charged polystyrene particles suggest that adsorption reaches saturation at higher bulk protein concentrations and for a lower maximum surface excess than in the haematite system. Thus, the maximum surface excess on a positively charged polystyrene surface is 2 mg m^{-2} as compared with 2.3 mg m^{-2} on a negatively charged polystyrene surface. Finally, we note that, although HSA and BSA are similar in many of their physical properties, their surface activities appear to be different. Kurrat et al.[4] recently compared the adsorption of BSA and HSA onto the hydrophilic Si (Ti)O_2 surface from aqueous solution and found that at a protein concentration of 0.084 g dm^{-3} and pH 7.4 the saturated surface excess for HSA was 2.4 mg m^{-2} as compared with 1.3 mg m^{-2} for BSA, a difference of almost a factor of 2.

The interactions between a protein and a solid substrate have been extensively discussed in the literature. On a hydrophilic solid substrate such as silicon oxide, the electrostatic attraction between a charged surface and an oppositely charged protein molecule is often the driving force for adsorption from solution on to the solid surface. The oxide surface is negatively charged above pH 2 with a low charge density that is almost constant between pH 3 and 7.[30] At pH 3 the net charge on BSA is positive and there is an electrostatic attraction between the protein and the solid surface but electrostatic repulsion between the protein molecules within the adsorbed layer. The amount of BSA adsorbed is determined by the balance of the two forces. At pH 5, the net charge on BSA is virtually zero. At this pH it would then appear to have no attraction to the surface. However, the retention of the globular structure means that there are still positively charged portions on the outer surface, which can provide a driving force sufficient for surface adsorption. However, the lateral repulsion within the adsorbed layer is reduced relative to the lower pH and more BSA is accommodated in the layer. It is interesting to note that the driving force for adsorption at pH 3 is greater than pH 5, but the amount adsorbed is now much less. This indicates that the amount of

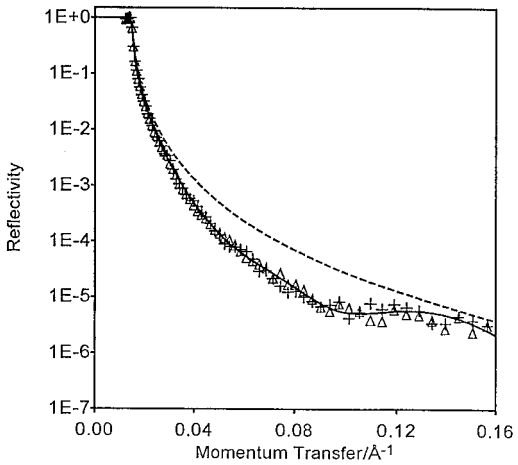

Figure 5. Comparison of neutron reflectivity at a bulk BSA concentration of 2 g dm^{-3} (△) with the reflectivity profile after the bulk BSA solution was replaced by buffered D_2O (+). The two reflectivity profiles are identical, suggesting that BSA adsorption is irreversible with respect to change in the BSA concentration. Reflectivity from the bare oxide/D_2O interface is also shown as a dashed line to indicate the change in the level of reflectivity caused by BSA adsorption. The continuous line was calculated using the parameters given in Figures 3 and 4.

protein adsorbed is dominated by the lateral repulsion rather than the attraction between BSA and the surface. A similar argument also explains the reduction of surface adsorption at pH 7 where the protein molecules now carry a net negative charge and there is strong lateral repulsion. Although there is also net repulsion between BSA and surface at pH 7, the weak attraction between the negatively charged surface and any positively charged residues remaining on the BSA will provide a driving force sufficient for weak surface adsorption. Finally, it should be noted that entropy changes resulting from changes of structural configuration of the protein globular structure and from dehydration of the protein and solid surface during adsorption may also play a role.

3. Irreversible Adsorption with Respect to Concentration. Like the adsorption of many synthetic polymers onto solid substrates, protein adsorption is often irreversible with respect to changes in the bulk concentration. Norde and Haynes[5] have recently analyzed the irreversibility of protein adsorption from a more theoretical standpoint and have identified three key factors: structural rearrangement in the protein molecule, dehydration of both surface and protein, and redistribution of charged groups in the interfacial layer. Although much effort has been made to determine the exact level of the contributions from each factor, the fact that the three processes occur simultaneously, and that there may be other smaller contributions apart from both hydrophobic effect and electrostatic interactions, makes the measurements very difficult.[5]

BSA desorption on reduction of the bulk concentration of BSA can be monitored by means of the appropriate reflectivity profiles. Figure 5 compares the reflectivity profile measured at pH 5 and at a BSA concentration of 2 g dm^{-3} in D_2O with that obtained when the bulk solution is replaced by buffered D_2O. If adsorption is reversible, BSA should be completely desorbed from the interface and the reflectivity profile in the presence of the pure buffer should be identical to the dashed line obtained from the bare silica/D_2O interface. However, the two BSA profiles before and after concentration reduction are indistinguishable; i.e. there is no BSA desorption at all. A similar result was obtained at the lower BSA concentration of 0.15 g dm^{-3}. In both desorption experiments the neutron reflectivity profiles were recorded immediately after the bulk solutions were replaced by buffered D_2O and each measurement took about 40 min. It is possible that desorption may be slow and may take longer time before any observable difference in reflectivity profiles can be detected. We have repeated both desorption measurements about 3 h after the buffered D_2O was introduced and again no measurable difference in reflectivity was found. In previous studies of lysozyme adsorption onto silica we tested the irreversibility by gradual reduction of the lysozyme concentration starting at 4 g dm^{-3}, then 0.03 g dm^{-3}, followed by the replacement of the solution by the buffered D_2O. Analysis of the reflectivity profiles suggested that there was some 10% lysozyme desorbed from the interfacial layer after the first concentration reduction and a further 20% desorption when the buffered D_2O was introduced. These results show that BSA adsorption is completely irreversible but lysozyme adsorption is only partially irreversible. The degree of irreversibility of the adsorption can be correlated with the size and rigidity of the protein. Large globular proteins tend to be more deformable and on adsorption onto a solid substrate they are more likely to be flattened, resulting in more contacts between a given protein molecule and the surface. The greater the number of contacts, the more difficult it is to detach all these contacts simultaneously. The nature of these contacts is not determined by the experiment but one possibility is the formation of hydrogen bonds between the hydroxy groups on the solid surface and those on the outer surface of the protein. A further obstruction to desorption is the positive contribution to the entropy of adsorption resulting from structural deformation of the protein. Additional free energy will then be required to be overcome before desorption occurs. Structural changes in the adsorbed BSA have been found by Kondo et al.[3] who used transmission circular dichroism to show that adsorption onto silica particles results in a substantial loss of α-helix content compared with the native structure. Subsequent desorption of the BSA by displacement with morpholine led to the recovery of almost all of the α-helix structure, suggesting that the desorbed molecules recover their native globular structure. These results were supported by the work of Norde et al.[2] using the same technique, although Norde et al. showed that some fraction of the α-helix content from the desorbed BSA was permanently lost. It is possible that morpholine may interact with BSA in a similar manner to surfactants, causing some loss in tertiary structure.

4. Reversible Adsorption with Respect to pH. The surface excess of BSA is strongly affected by solution pH, and the variation in surface excess must be caused by the presence of ionisable groups whose degree of ionization changes with pH, resulting in different affinity of BSA for the solid substrate and a different level of repulsion within the adsorbed layer. Since changes in pH cause a large variation in the adsorbed amount, it is of interest to examine if adsorption is reversible with respect to pH variation.

The effect of pH on the amount of BSA adsorbed at the silicon oxide/water interface was examined by varying the pH in D_2O. Figure 6a shows the reflectivity profiles measured at different solution pH at a bulk BSA concentration of 2 g dm^{-3}. Unlike the results shown in Figure 2 where each profile was recorded on first contact of the solution with the bare oxide surface, the pH in this system was changed sequentially by replacing the already measured solution with a new one at different pH but otherwise under the same composition. The measurement was started at pH 5, raised to 7, and then returned to 5, followed by a further reduction to 3 and finally back to 5. The three reflectivity profiles at pH 5 are identical, which tends to suggest that the adsorption of BSA is reversible with respect

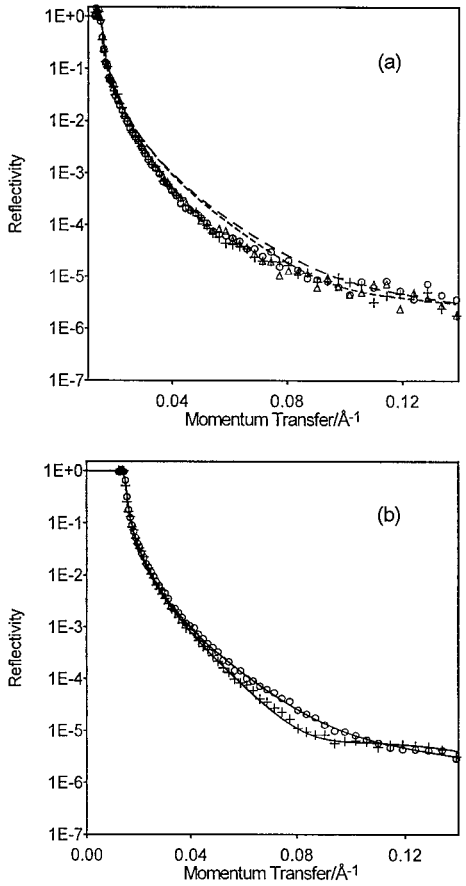

Figure 6. Effect of pH on BSA adsorption measured as the variation of reflectivity in D_2O at 2 g dm^{-3}. The adsorption was started at pH 5 (○), then raised to 7, followed by 5 (+), then 3, before returning to 5 (△). (a) The comparison of the reflectivity profiles measured at pH 5. The best fits to the measured reflectivity profiles at pH 7 (long dashed line) and pH 3 (short dashed line) are also shown to demonstrate the variation of reflectivity profiles with solution pH. (b) The comparison of the reflectivity profile measured on the fresh oxide surface at pH 7 (○) with that measured when the solution pH was shifted from 5 to 7 during the pH cycle (+). The continuous lines were calculated using $\Gamma = 1.0$ mg m^{-2} for the fresh pH 7 and $\Gamma = 1.8$ mg m^{-2} for pH 7 in the cycle.

to pH. However, a more crucial test for reversibility is to examine the desorption process. This can be done by comparing the adsorption at pH 7 (after being raised from 5) with the one obtained on direct exposure of the bare oxide to solution at pH 7. This comparison is made in Figure 6b. That the reflectivity profile at pH 7 (after being raised from 5) is lower than that corresponding to the fresh pH 7 suggests that a significant amount of BSA does not desorb when the pH is raised and that the process is therefore irreversible. The possible effect of time was also examined in this case over a period of 3 h by repeating reflectivity measurements. The reflectivity profiles at pH 7 (raised from pH 5) were identical, showing that the extent of desorption did not improve with time. Quantitative analysis shows that while adsorption onto the bare oxide/water interface gives a surface excess of 1 ± 0.3 mg m^{-2} the profile resulting from the shift of pH from 5 to 7 produces a surface excess of 1.8 mg m^{-2}. In previous work on BSA adsorption,[10] a similar pH cycle was followed with the bulk BSA concentration fixed at 0.15 g dm^{-3}. In that case adsorption was found to be completely reversible with respect to ascending or descending pH. The results are consistent because at the low BSA concentration pH variation creates sufficient driving force to overcome the energy barrier for desorption while at the high BSA concentration the barrier is too high for complete desorption.

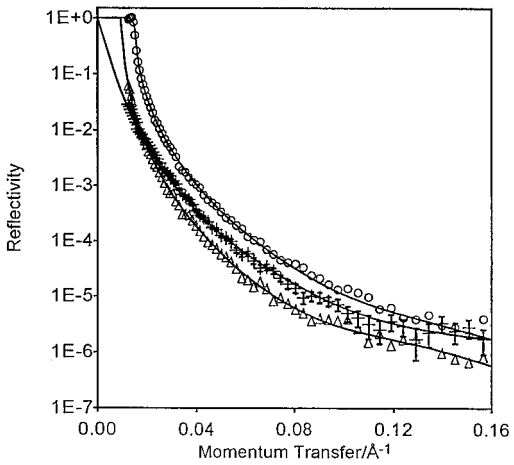

Figure 7. Single uniform layer fits to the reflectivity profiles in D_2O (○), CM4 (△), and H_2O (+) at pH 7 in the presence of 2 g dm^{-3} BSA. The continuous lines were calculated using $\tau = 30$ Å and $\Gamma = 0.8$ mg m^{-2}.

5. Structure of BSA Layers. A feature of the adsorption of globular proteins is that the thickness and composition of the adsorbed layer together with the known dimensions of the protein can be used to infer the orientation of the protein molecules on the surface and possibly also the extent of structural deformation caused by contact with the solid surface. Neutron reflection has a depth resolution of the order of angstroms for the thicknesses typically observed for adsorbed layers of globular proteins. Furthermore, unlike optical techniques such as ellipsometry, the change in water contrast with H_2O/D_2O ratio helps to highlight the interfacial region in different ways, which results in a much better definition of the protein distribution within the layer.

We previously found that BSA adsorbs in a sideways-on orientation at the hydrophilic silica/water interface at pH 5 at BSA concentrations up to 0.5 g dm^{-3}. Similarly, we can obtain the structure of the BSA layer at the other two values of the pH. Figure 7 shows the reflectivity from 2 g dm^{-3} BSA solution at pH 7 in D_2O, CM4 (D_2O:$H_2O \simeq 3$:1), and H_2O. We have attempted to fit the set of reflectivity profiles from the different isotopic compositions using the same single uniform layer model for the protein layer as that used previously for extracting the surface excess and the continuous lines in Figure 7 are the best fits using such a model with a thickness of 30 ± 3 Å for the protein layer. The scattering length densities were calculated using the fixed protein volume fraction of 0.18 obtained earlier and the residual space was filled with water of the appropriate contrast. The oxide layer was taken to be the same as that of the bare surface before protein adsorption and no roughness was included in the fitting. In the calculation of the scattering length of the protein, complete exchange of the labile hydrogens in BSA with the bulk water was assumed. That a single structural model fits the three isotopically different profiles with this assumption in itself suggests that the assumption is correct, since the profiles are quite sensitive to incomplete exchange. However, it could be argued that the effects of incomplete exchange were exactly cancelled by isotopically induced changes in structure. This would be a surprising coincidence but, since uncertainty in the assumption would affect our conclusions, we repeat the outline of the discussion of H/D exchange given in our previous work.[10]

The exchange of the labile hydrogens in globular proteins with D_2O has been extensively investigated by Hvidt and Nielsen[31] and more recently by Radford et al.[32] The accessibility of labile hydrogens within globular proteins to the surrounding water has been used as a measure of the masking of portions of the polypeptide in the folding and unfolding process. There are about 1015 potentially labile hydrogen atoms within each BSA molecule and some 750 of these will exchange almost instantly at pH 7 and 0 °C.[30] A further 250 exchange over a period of 2 h. The remaining 30−50 appear not to exchange within a further 24 h. Thus, over 90% of the labile hydrogens are relatively rapidly exchanged. The rate of exchange is also dependent on pH, with all of the labile hydrogens exchanging below pH 3.5 and above pH 9. The exchange rate also rises rapidly with temperature. The hydrogen atoms that are most difficult to exchange are those on the amide groups on the peptide chain because their exchange may be prevented by the formation of the structured hydrophobic environment, the so-called hydrophobic encapsulation. However, most of the labile hydrogens on the backbone are on the outer surface of the globular structure and are therefore readily exchanged, only a relatively small fraction being expected to be hydrophobically encapsulated. Structural deformation accompanying adsorption may cause sufficient disturbance to the encapsulated peptide chain fragments to allow exchange of all backbone hydrogens to proceed to completion. The response of the calculated surface excess to changes in the neutron scattering length of the layer is approximately linear and any uncertainty in the extent of exchange will convert linearly into an error in the surface excess, although the magnitude of the error will be different at each water contrast, as can be shown from eqs 4−7. Since the difference in scattering length for the labile hydrogens is largest in D_2O, the effect caused by incomplete exchange will be largest for the D_2O measurements but much less for the other contrasts. Thus, on the basis of the work of others it is unlikely that the extent of exchange is less than about 90% leading to a maximum error in the surface coverage of 10%. However, the excellent fits of the single layer model to all the data suggests that exchange is, in fact, more complete than 90% and the error is then correspondingly comparable to the experimental uncertainty of a few percentage. Note that incomplete exchange has no significant effect on the determination of the layer thickness because to a good approximation ρ and τ affect the reflectivity differently.

Similar uniform layer models have been found to be appropriate for fitting similar sets of reflectivity profiles at pH 3 and 7 over the whole concentration range. It was found that the differences in these reflectivity profiles result from variations of scattering length densities of the protein layer rather than the thicknesses, which are all found to be 30 ± 3 Å. This suggests that under these conditions weak adsorption occurs and the molecules adsorb sideways-on. Since surface excesses are low and large gaps exist between the adsorbed BSA molecules the lateral repulsion within the layer is also weak. This explanation is consistent with the thickness of the layer being significantly less that the length of short axis of the globular structure because there is space for adsorption to induce some degree of flattening of the ellipsoidal protein molecule. The single uniform layer model also fitted the reflectivity profiles below 0.5 g dm^{-3} at pH 5. As the bulk BSA concentration increases from 0.005 to 0.5 g dm^{-3} the corresponding layer thickness increases from 30 to about 37 Å, an increase of some 20%. The gradual thickening of the layer indicates that as the coverage increases the level of lateral repulsion increases and the extent of flatterning as a result of direct contact with the oxide surface is reduced.[10] Increase in concentration may also result in the increased tilting of the BSA molecule, which also contributes to the thickening of the layer. Obviously, the neutron measurement cannot distinguish the two contributions. In practice, however, both processes may occur simultaneously because BSA molecule is very flexible.

The easy deformability of the BSA molecule must arise mainly from the presence of three principal domains that are loosely joined together.[11] The native structure within each subdomain will be kept in a reasonably ordered state in aqueous solution by the large number of disulfide bridges. This is unlikely to be the case for the three main domains because the interactions between them are not covalent. The structure of the BSA molecule is commonly described as a string of three spherical domains and the question arises as to whether it is appropriate to compare the observed thickness of a uniform layer directly with the diameter of the BSA ellipsoid. Because of the effect of averaging over the distribution of matter in a sphere the assumption of a uniform layer would be expected to have the effect of giving a value of the thickness slightly smaller than the maximum dimension of 40 Å. However, in separate experiments on the more robust globular lysozyme,[8] where no change in geometry is expected on adsorption, the uniform layer thickness was found to be exactly the same as the appropriate globular dimension, indicating that the averaging effect is small and may be compensated in the fitting procedure by a small roughness of the underlying surface. The decrease from 40 to 30 Å is therefore large enough to suggest that there is some structural deformation of the BSA on adsorption.

The formation of the monolayer at pH 3 and 7 and at the concentration below 0.5 g dm^{-3} at pH 5 shows no indication of serious denaturation in that there is no fragmentation in the distribution of the layer along the surface normal. At the hydrophobic solid/solution interface, denaturation of lysozyme resulted in a noticeably fragmented peptide chain distribution with the highest volume fraction next to the surface.[9] This is manifested in the reflection experiment by the need to divide the adsorbed layer into two or more layers of unequal scattering length density to obtain satisfactory fits to the data. That this is not required in the fitting of the data in this work establishes the uniform density distribution along the surface normal direction within the BSA layer under these conditions. A Further characteristic feature of the retaining of the globular framework is the reversibility of the adsorption with respect to pH. This has been found to be the case for lysozyme adsorption at the hydrophilic oxide/water interface.[8] That there is no breakdown of the BSA globular structure is also consistent with the reversibility of adsorption with respect to pH variation over this concentration and pH range.[10] In contrast, at the hydrophobic solid/water interface where serious denaturation occurs the adsorption was found to be completely irreversible with respect to the variation of solution pH.[9]

Above 0.5 g dm^{-3} the uniform layer model becomes less good for describing the adsorbed BSA layer. Figure 8 compares the measured reflectivity in D_2O with the ones calculated from the best uniform layer model with a thickness of 37 Å (dashed line) and a two-layer model consisting of a densely packed inner layer of 37 Å and a loosely packed outer layer of some 50 Å (solid line). It can be seen that there is an improvement in the two-layer model fit over the middle κ region around the dip in the interference where the reflectivity is well above its background level. We earlier commented that the inclusion of this diffuse second layer gives an additional contribution of some 0.5 mg

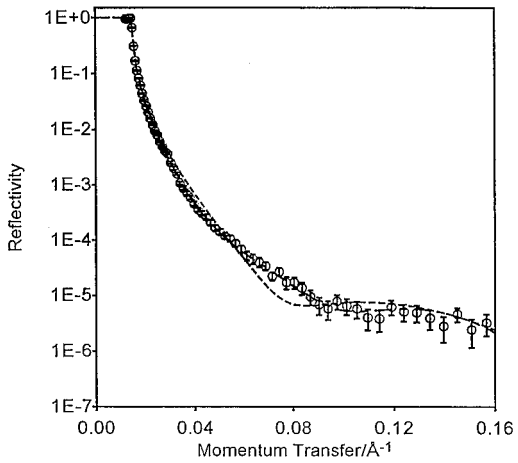

Figure 8. Comparison of the single uniform layer model and the two-layer model fits to the measured reflectivity at pH 5 in the presence of 2 g dm^{-3} BSA in D_2O. The solid line was calculated using $\tau_1 = 37$ Å for the inner layer and $\tau_2 = 50$ Å for the outer layer.

m^{-2} to the total surface excess for the high concentration profiles. The correctness of this interpretation is also supported by measurements at CM4 and H_2O at a bulk concentration of 2 g dm^{-3} and pH 5 (not shown in Figure 8 for clarity) for which the same two-layer model gave the best fit. The need to use a two-layer model at concentrations above 0.5 g dm^{-3} suggests that such a model might also be appropriate at other concentrations in this range. When this is done the apparently sharp upturn in the surface excess versus concentration plot at pH 5 shown in Figure 3 becomes smoother. However, the effect of using the two-layer model becomes less obvious as the BSA concentration decreases until it becomes difficult to justify. The uncertainty caused by the choice of model is less than 0.5 mg m^{-2} over the whole range of concentration.

The formation of a diffuse outer layer could arise either from the attachment of BSA molecules to the first adsorbed layer or from the projection of fragments from some denatured molecules. The current measurements cannot distinguish these two alternatives and there is little reliable information from any other technique that could help to resolve the situation. Blomberg et al.[27,28] using a surface force apparatus to study HSA on mica showed that the adsorbed HSA layers are more compressible than single-domain proteins like insulin and lysozyme, again suggesting that there is a lack of conformational stability in the HSA layer. The layer thickness below 0.01 g dm^{-3} at the IP was found to be between 30 and 35 Å, suggesting sideways-on adsorption, an observation in good agreement with our neutron findings. Blomberg et al. also observed that as the HSA concentration was increased to 1 g dm^{-3} the adsorbed layer thickness increased from 30 Å to some 120 Å. On the basis of the change in the layer thickness these authors suggested that the adsorbed layer switched from a sideways-on conformation to predominantly head-on adsorption. Since the surface force apparatus detects the thickness of the adsorbed layer by bringing the two mica films together, the thickness obtained must be the maximal dimension of the protein layer distribution. The neutron signal, on the other hand, is dictated by the volume fraction of protein in the layer and its sensitivity to an outer surface layer with very low protein volume fractions, when there is an inner layer of high volume fraction will be poor. Under these circumstances the thickness for the outer layer probed by neutron reflection will therefore inevitably be less than that detected by the surface force apparatus. It could therefore be said that the two techniques approximately agree on the overall dimension of the layer. However, the attribution of the change in layer

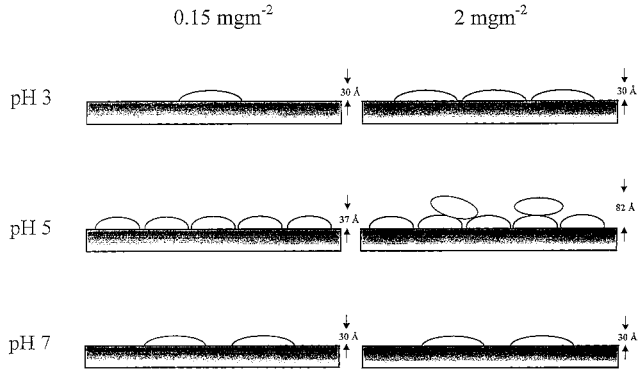

Figure 9. Schematic representation of the effect of pH and BSA concentration on BSA adsorption at the hydrophilic silicon oxide/water interface. The number of the BSA molecules is approximately proportional to the surface excess under different solution conditions, and the shapes of the molecules have been drawn to represent the structural deformation that occurs on adsorption.

structure from a sideways-on to a longways-on monolayer by Blomberg et al. is definitely not consistent with the neutron results. Neutron reflection would be very sensitive to a uniform layer of this large thickness, which is what is implied by Blomberg et al. At this point, it must also be remembered that the two surfaces involved are not the same and this may affect the layer structure.

The structural conformation of BSA at the silica/water interface is shown schematically in Figure 9. Our main finding is that BSA adsorption over a wide pH and concentration range is in the form of a uniform layer between 30 and 40 Å thick, suggesting a sideways-on conformation of the ellipsoidal protein. That the thickness of the layer is in all cases less than the length of the shorter axis of the protein, except over the high concentration at pH 5, suggests that some degree of deformation occurs on contact with the solid substrate, although there is no sign that this deformation leads to a more fragmented peptide distribution. Although at the high BSA concentration under pH 5 the measurement is sensitive enough to reveal the existence of a outer diffuse layer, the resolution is not sufficient to reveal the precise conformational arrangement of BSA molecules within this region. The represntation shown in Figure 9 is only symbolic and is primarily intended to indicate the change of surface excess with respect to pH and BSA concentration.

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