

The Effect of Solution pH on the Structural Conformation of Lysozyme Layers Adsorbed on the Surface of Water

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Neutron reflection has been used to study the effects of solution pH and ionic strength on the surface excess and layer thickness of lysozyme layers adsorbed at the air/water interface. All the measurements were made in null reflecting water (NRW) so that all the specular signal arose from the protein layers. At the low ionic strength of 0.02 M, the adsorption was found to reach a maximum at the protein isoelectric point (IP) of pH 11, with the effect of pH on the adsorbed amount and layer thickness being more pronounced at the higher lysozyme concentration. At the low lysozyme concentration of 0.03 g dm^{-3} , the thicknesses of the adsorbed layers are $30 \pm 3 \text{ \AA}$ over almost the entire pH range, close to the short axial length of the globular dimension of lysozyme, and the area per molecule is $1700 \pm 200 \text{ \AA}^2$, suggesting the formation of a sideways-on monolayer. At the high lysozyme concentration of 1 g dm^{-3} , a number of conformational transitions occur within the adsorbed layers with respect to pH and these variations correlate well with the change in the number of net charges within lysozyme with pH, suggesting that the preferred conformation of protein molecules is dominated by the combined effect of steric and electrostatic repulsion within the adsorbed layer. Subsequent measurements at the high ionic strength of 1 M showed no obvious variation in either layer thickness or surface excess with pH or with bulk protein concentration. The thickness was found to be constant at $30 \pm 3 \text{ \AA}$ and the area per molecule to be $1500 \pm 100 \text{ \AA}^2$, corresponding to the formation of a close-packed sideways-on monolayer. These results clearly show that salt addition has screened the charges within lysozyme molecules.

Introduction

When a globular protein molecule is adsorbed at the air/water interface two essential questions will immediately arise: the possibility of surface induced denaturation and the extent of immersion of the protein molecule in water. Because the distributions of polar, nonpolar, and charged groups on the outer surface are not generally uniform, the protein will have some regions that are more hydrophobic and some that are more hydrophilic. The difference in hydrophobicity renders globular protein molecules surface activity. At the air/water interface the protein may then adsorb either unchanged with the hydrophobic portion out of the water, or denatured, where it minimizes its hydrophobic free energy by folding in such a way as to expose its apolar moieties selectively to the air. Accurate determination of the dimension of the adsorbed protein layer and its extent of immersion in the aqueous subphase may make it possible to identify which of the two has occurred and thus provide some information about the general balance of hydrophobicity and hydrophilicity in the molecule. We have recently studied the adsorption of lysozyme at the air/water interface using specular neutron reflection, a technique capable of determining the structure of a lysozyme layer along the surface normal with resolution at the level of a few \AA . In this paper we report the results on the measurement of the surface excess and layer thickness under different solution pH and salt concentrations.

Study of protein adsorption at the air/water interface is of fundamental relevance to the understanding of its effect and functionality in biological membranes and is also important to many technological applications ranging from food products to

drug delivery.¹ The in situ structural conformation of the adsorbed protein molecules in the layer is the key to the establishment of the relationship between the molecular structure of protein and its surface and interfacial properties. Many investigations have been made to probe the structural conformation of globular proteins originated from different sources.^{1–5} These include surface tension measurements,^{6,7} proteolytic enzymes to detect accessibility of hydrolysis-sensitive bonds in the adsorbed layer, and enzymatic cleavage combined with measurements of hydrodynamic thicknesses of protein layers surrounding colloidal aggregates.^{8,9} Little direct information at the molecular level can be obtained from these methods.

Neutron reflection has been used to study the adsorption of β -casein and β -lactoglobulin at the air/water interface.¹⁰ The structure of the β -casein layer was found to be largely composed of two regions: a densely packed layer on the top of water surface and a loose sublayer extending into the water substrate. Such a structural conformation is well expected for a flexible protein as in the case of synthetic polymers. In comparison with β -casein, β -lactoglobulin has a more compact and ordered conformation in solution due to the presence of disulfide bonds giving the molecule considerable secondary and tertiary structure. If β -lactoglobulin retains its globular feature upon adsorption, the density distribution profile normal to the surface should be approximately uniform or symmetrical and the layer thickness should be close to one of the axial lengths of its globular dimension. That the density profile very much resembles that of β -casein, shows that at least β -lactoglobulin has been partially denatured after adsorption. In comparison with β -lactoglobulin, lysozyme is more rigid and has a well defined globular structure. We have recently studied the adsorption of lysozyme at the air/

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water interface¹¹ and found that over a wide protein concentration range, the adsorbed layers can be described by a uniform layer distribution model and the thicknesses of the layers match one of the axial lengths of the globular structure. This observation shows that under the solution conditions studied the adsorbed lysozyme molecules retain their globular framework. Comparison of the thickness of the layer with the dimension of the globular structure thus allows a reliable determination of the in situ conformation of the protein molecules on the surface of water.

Experimental Section

The neutron reflection measurements were made on the white beam time-of-flight reflectometer SURF at the Rutherford-Appleton Laboratory, ISIS, Didcot, UK.¹² The procedure for performing the measurements was similar to that described previously in ref 13. Protein solutions were poured into a Teflon trough to give a positive liquid meniscus. The trough was mounted on an active anti-vibration table. Alignment was made using a laser beam which shared the same beam path as the neutron beam. The absolute reflectivities were calibrated with respect to D₂O. A flat background determined by extrapolation to the 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. The wavelength ranges from 0.5 to 6.5 Å. For each sample, the reflectivity was measured at three different incidence angles: 0.5°, 0.8° and 1.5° to produce a κ range up to 0.5 Å⁻¹ and the resultant reflectivity profiles were combined with appropriate scaling factors. All the experiments were performed at 25 °C.

Lysozyme (from chicken egg white) was purchased from Sigma (98%) and was used as supplied. It is a globular protein with molecular dimension of 30 × 30 × 45 Å³. D₂O (99.9% D) was from Fluorochem. Its surface tension was typically over 71 mN m⁻¹ at 298 K, indicating the absence of any surface active impurity. H₂O was processed through an Elgastat ultrapure water system (UHQ), and its surface tension at 298 K was constant at 71.5 mN m⁻¹. The solution pH was controlled by using phosphate buffer (Na₂HPO₄/NaH₂PO₄), keeping the total ionic strength fixed at 0.02 M. There were small differences in pH between H₂O and D₂O but this was controlled to within 0.2 pH units. The glassware and Teflon troughs for the reflection measurements were cleaned using alkaline detergent (Decon 90) followed by repeated washing in UHQ water.

Results and Discussion

(A) Effect of Solution pH. The isoelectric point (IP) of lysozyme is about 11 and over the normal pH range lysozyme is positively charged. The exact number of net charges on lysozyme at a given pH can be found from the work of Tanford et al.¹⁴ Alternatively, the net charges can be calculated from the dissociation constants of the amino acid groups within lysozyme because the ionizable groups within lysozyme are already known.¹⁵ Furthermore, because lysozyme is among one of those whose crystalline structure is well characterized, information about the precise location of different charged groups on the outer surface of the globular framework is also available.¹⁶ This information is useful for exploring the preferred contact region with the water surface when lysozyme is adsorbed onto the surface of water, as has been demonstrated previously.¹¹

The possible change of the adsorbed layer structure with solution pH can be obtained by measuring neutron reflectivity at a fixed lysozyme concentration. Figure 1 shows the measured reflectivity profiles plotted as a function of κ under different

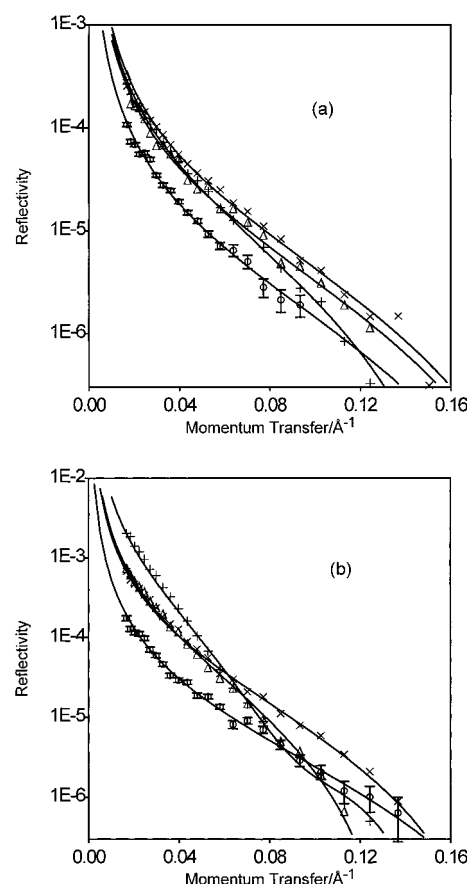


Figure 1. Neutron reflectivity profiles from lysozyme adsorbed on the surface of null reflecting water at (○) pH 2, (Δ) 7, (+) 11, and (×) 12.5. The bulk lysozyme concentration was at (a) 3×10^{-2} g dm⁻³ and (b) 1 g dm⁻³. The total ionic strength of the phosphate buffer was 0.02 M. The continuous lines were calculated using the optical matrix method, and the fitted surface excesses and thicknesses are given in Figures 2 and 3.

solution pH with lysozyme concentrations fixed at 0.03 g dm⁻³ (Figure 1a) and 1 g dm⁻³ (Figure 1b). The reflectivity, defined as the ratio of reflected beam intensity to that of the incoming one offers structural information about the adsorbed layers in terms of layer thickness τ and adsorbed amount (surface excess) Γ . Since both the adsorbed layer and the substrate contribute to the reflected signal, it is preferable to eliminate the contribution from the substrate so that direct structural information about the layer distribution is obtained. At the air/water interface this can be easily achieved by adjusting the ratio of H₂O and D₂O so that the scattering length density of water ρ is the same as that of the air; that is, $\rho = 0$. The molar ratio of H₂O to D₂O for achieving zero scattering length density is approximately 11:1. Because such water does not reflect any neutrons it is usually termed null reflecting water (NRW). All the reflectivity profiles shown in Figure 1 were thus measured under NRW. The total ionic strength was kept constant at 0.02 M.

Adsorption of lysozyme at the air/water interface has been studied by different techniques including surface tension measurement, ellipsometry, and radiolabeling.^{6,7,17} Most of these studies have focused on monitoring time dependent adsorption. Although these studies have produced a great deal of data on the dynamic process of protein adsorption, the results can only be compared on a qualitative basis. Because the time dependent process is difficult to reproduce quantitatively, this work has been focused on studying the equilibrium situation that can be easily monitored by the variation of the reflectivity profiles with

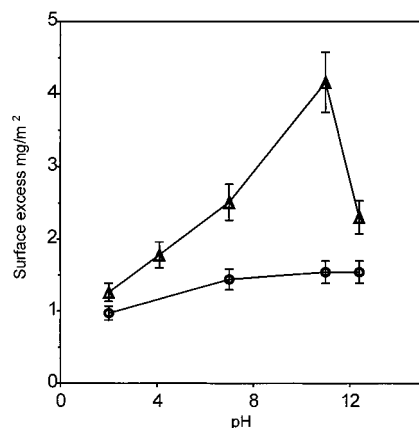


Figure 2. The variation of lysozyme surface excess with solution pH. The bulk lysozyme concentration was fixed at (○) $3 \times 10^{-2} \text{ g dm}^{-3}$ and (Δ) 1 g dm^{-3} , respectively. The total ionic strength was 0.02 M.

time. The time required for reaching equilibrium is mainly affected by lysozyme concentration. It was found that at the low lysozyme concentration of 0.03 g dm^{-3} it took some 8 h for the reflectivity profile to reach its limit, while at 1 g dm^{-3} some 2–4 h is required before the reflectivity profile was constant. All the measurements reported in this paper were made after sufficient time was given for the equilibrium to be reached.

Some basic features about the adsorbed layers can be found from the reflectivity profiles shown in Figure 1 before attempting any quantitative analysis. In general, the magnitude of reflectivity profiles determines the surface coverage and thus the relative difference between them offers information about the variation of Γ with pH.¹⁸ In comparison, the shape of neutron reflectivity profiles offers information about the thickness of the layers. The faster the reflectivity profile decays the thicker the layer. It can thus be seen from Figure 1a that there is relatively little variation of the reflectivity profile with pH at the low protein concentration of 0.03 g dm^{-3} . However, at the high protein concentration of 1 g dm^{-3} variations occur both in the magnitude and in the shape of reflectivity profiles, suggesting that both layer thickness and the adsorbed amount respond to the pH variation. At pH 11 the reflectivity profile is the steepest, indicating that the adsorbed layer is the thickest.

Quantitative information about the layer structure is usually obtained through model fitting. The continuous lines through the measured reflectivity profiles in Figure 1 were calculated using layer models based on the optical matrix formulas.¹⁹ All the fitted curves were obtained using the single uniform layer model except the one at pH 11 and at 1 g dm^{-3} . Each fit was optimized by comparing the calculated reflectivity with the measured one until the best fit was obtained. The quality of the fitting was mainly judged by visual comparison of the measured reflectivity profile with the calculated one over the κ range between 0.01 and 0.15 \AA^{-1} . This produces consistent results as determined from the values of χ^2 in the least-squares routine. The variation of surface excess with respect to pH obtained from the fitting is shown in Figure 2 and that of the thickness with pH in Figure 3.

For a uniform layer of lysozyme adsorbed on the surface of null reflecting water, Γ is calculated using the following equation

$$\Gamma = \frac{\rho \tau}{N_A \sum b_i} \quad (1)$$

where N_A is Avogadro's constant and $\sum b_i$ is the scattering length

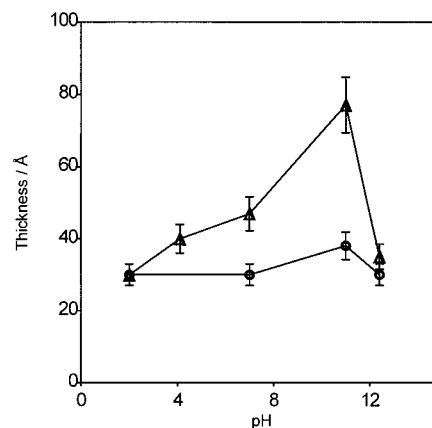


Figure 3. The variation of lysozyme layer thickness with solution pH. The bulk lysozyme concentration was fixed at (○) $3 \times 10^{-2} \text{ g dm}^{-3}$ and (Δ) 1 g dm^{-3} , respectively. The ionic strength was 0.02 M.

for lysozyme. The value of $\sum b_i$ in pure H_2O at pH 7 is $3.41 \times 10^{-2} \text{ \AA}$. In the null reflecting water there is about 8% D_2O by volume, the value of $\sum b_i$ is $3.63 \times 10^{-2} \text{ \AA}$ if the exchange between labile hydrogens and bulk water is complete. Lysozyme has some 260 labile hydrogens. Half of these labile hydrogens are associated with the polypeptide chain and the other half are on the side chains. Since most of the labile hydrogens on the side chains are associated with hydrophilic and charged groups, they are easily accessible to water and the exchange is expected to be instant. However, for the 129 labile hydrogens present on the polypeptide chain, the exchange with bulk water can be slow because of the hydrophobic encapsulation and the formation of helical structures associated with hydrogen bonding. Radford et al.²⁰ have shown that some 80% of labile hydrogens exchanges with bulk water within minutes of dissolution. If this is the case, the value of $\sum b_i$ is $3.58 \times 10^{-2} \text{ \AA}$. The difference in the value of $\sum b_i$ between 80% exchange and a complete exchange is about 1% and is therefore small as compared with the typical uncertainty of 5% in the resolution of neutron reflection. Any uncertainty in $\sum b_i$ only affects Γ , but does not affect the value of τ at all in these situations. It should also be noted that change in solution pH causes variation in the degree of ionization within lysozyme. The variation in the value of $\sum b_i$ as a result of pH shift is also negligible.

The main feature from Figures 2 and 3 is that both surface excess and layer thickness reach maxima at the IP. That the trend is less obvious for the adsorption at the lower concentration suggests that the pattern of adsorption is affected by the concentration of lysozyme. In fact, the observation shows a good correlation with the extent of the net charge within the protein. At the IP the net charge within the protein molecule is zero and the electrostatic repulsion within the adsorbed layer is minimal. As solution pH is shifted away from the IP, the net charge within the lysozyme increases and therefore the lateral electrostatic repulsion. Also, the increased charge density means that the hydrophilicity of lysozyme molecules also increases. The combined effect is such that the amount of adsorption decreases as pH is moved away from the IP. The lack of clear correlation with the IP at the low lysozyme concentration of 0.03 g dm^{-3} arises possibly from the overall lower surface concentration and hence the effect of steric and lateral electrostatic repulsion is less significant. It is important to note that although neutron measurements do not offer direct information about the extent of lateral interaction within any adsorbed layer the results to be described in the following show strong support to the description given above.

At the low lysozyme concentration of 0.03 g dm^{-3} , the thicknesses of the lysozyme layers over most part of the pH range are $30 \pm 3 \text{ \AA}$, identical to the short axial length of the globular dimension for lysozyme. This suggests that lysozyme molecules are adsorbed sideways-on over the entire pH range; that is, the molecules are adsorbed with their long axes parallel to the solution surface. This demonstrates that adsorption does not lead to the breakdown of the globular assembly. The maximal surface excess over the whole pH range at this lysozyme concentration is about 1.5 mg m^{-2} , which is equivalent to an area per molecule of 1600 \AA^2 , as compared with the minimum area per molecule of 1350 \AA^2 ($30 \times 45 \text{ \AA}^2$) required for sideways-on orientation. If proteins adsorbed in the surface layer are denatured, it would really be coincidental to find that the dimension of the layer matches one of the two axial lengths in the globular structure. Furthermore, a denatured layer distribution usually cannot be described by a uniform layer model. In a previous study of adsorption of lysozyme onto the hydrophobic solid/water interface²¹ we found that the adsorbed lysozyme layer was comprised of two regions, a dense inner layer of $10\text{--}15 \text{ \AA}$ with a protein volume fraction over 0.9, and an outer diffuse layer of some $30\text{--}80 \text{ \AA}$ with a protein volume fraction of about 0.15. Because the dense layer is shorter than the short axial length of the globular structure for lysozyme, the result clearly shows that on the hydrophobic surface lysozyme has been completely denatured. The two layer density profile on the hydrophobic solid/water interface broadly resembles those observed for β -casein and β -lactoglobulin¹⁰ and is more characteristic of the layers formed by synthetic polymers.²² At pH 11, the layer can still be represented by a single uniform layer but the thickness of $38 \pm 3 \text{ \AA}$ is greater than the short axial length and shorter than the long axial length of the globular dimension, suggesting that lysozyme molecules may be oriented with some tilting toward headways-on conformation. If it is assumed that a complete headways-on layer is 45 \AA thick, the tilting with respect to the surface normal would be about 35° . The formation of a tilted layer is supported by the closeness of the area per molecule ($1500 \pm 100 \text{ \AA}^2$) to the limiting value of 1350 \AA^2 for the formation of the sideways-on conformation, suggesting that there is sufficient steric and local electrostatic effect to force the molecules to orient vertically.

The discussion given above assumes that there is no contribution to the thickness from capillary waves. The presence of thermal fluctuation on a liquid surface tends to broaden the distribution of the interfacial region. The contribution can be estimated using the following simple equation^{23,24}

$$\tau^2 = \sigma^2 + \bar{\omega}^2 \quad (2)$$

where τ is the measured thickness, $\bar{\omega}$ is the contribution from thermal roughness, and σ is the intrinsic thickness of the layer after roughness removal. Schwartz et al.²⁵ have shown that the amplitude of the thermal motion in a pure liquid is inversely proportional to the surface tension of the liquid. For pure water at 298 K, the root-mean-square amplitude is 2.8 \AA . In terms of the layer distributions, this is equivalent to 8 \AA . The level of the contribution of thermal roughness to the broadening of the layer in the presence of lysozyme can be estimated similarly. At a lysozyme concentration of 1 g dm^{-3} under pH 7 the surface tension is about $53 \times 10^{-3} \text{ Nm}^{-1}$, giving a thermal roughness of about 9.3 \AA for the lysozyme solution. If the total layer thickness is 30 \AA the use of eq 2 gives the value of σ of 28.5 \AA , a difference well within the quoted experimental error of $\pm 3 \text{ \AA}$. This result clearly shows that unlike the monolayers formed by surfactants thermal roughness has a negligible contribution

to the thickness of the protein layer. It should be noted here that in the study of adsorption of surfactant at the air/water interface roughness has been considered to be one of the major factors in causing the density profile along the surface normal to be Gaussian in shape. Although roughness is insignificant here the protein layer might still be better described by a Gaussian distribution. This is because the shape of the globular structure of lysozyme is approximately ellipsoidal and the layer density along the surface normal is more likely to be Gaussian instead of being uniform. But the lack of statistical resolution of the reflectivity data at $\kappa > 0.1 \text{ \AA}^{-1}$ means that it is difficult to distinguish between the Gaussian and uniform layer models for most of the data measured in this work.

At the higher concentration of 1 g dm^{-3} , the thicknesses of the layers vary over a wider range. Thus, at pH 2 the layer is $30 \pm 3 \text{ \AA}$ thick, equivalent to a sideways-on monolayer. The area per molecule is about $1900 \pm 200 \text{ \AA}^2$. At pH 4 the layer is $40 \pm 3 \text{ \AA}$ thick, suggesting that the layer is strongly tilted toward the headways-on conformation. The area per molecule at this pH is about $1350 \pm 100 \text{ \AA}^2$, identical to the minimum value needed for sideways-on adsorption. At pH 7, the thickness is $47 \pm 3 \text{ \AA}$ and is close to the long axial length of 45 \AA , showing that lysozyme is adsorbed with its long axis completely perpendicular to the solution surface. The corresponding area at this pH is $960 \pm 50 \text{ \AA}^2$, close to the estimated limit of 900 \AA^2 for a complete headways-on adsorption. At the isoelectric point of pH 11, the layer had to be fitted with a two layer model: an inner layer of 47 \AA with a volume fraction of 0.5 and an outer loose layer of 30 \AA with a volume fraction of 0.18. The corresponding areas per molecule were 760 ± 50 and $3500 \pm 50 \text{ \AA}^2$. This result clearly shows that as the total layer is more close packed, further reduction in the net charge within the protein leads to the adsorption of a secondary layer underneath the main layer. It is important to note that alternative structural models such as those characteristic of denatured protein density distributions did not fit the reflectivity profile. As pH was finally increased to 12.5, the total layer thickness dropped by half to the value of $35 \pm 3 \text{ \AA}$ and the good fit of the uniform layer model shows that this corresponds to a slightly tilted sideways-on monolayer. The surface excess also dropped by almost half and the corresponding area per molecule was about $1100 \pm 100 \text{ \AA}^2$.

It should be reminded that the globular structure of lysozyme is not a perfect ellipsoid and the quoted dimension of $30 \times 30 \times 45 \text{ \AA}^3$ is only a coarse approximation. The measurement of neutron reflection has no resolution to the precise shape of the molecule, and the information about the structural conformation under different pH conditions is derived from the change in the thickness of the lysozyme layer. It is useful to discuss how the charge distribution on the surface of lysozyme may affect the preferred conformation. Lysozyme is one of the several model globular proteins whose crystalline structures have been determined, and the distributions of the charged groups are hence characterized.¹⁶ Lysozyme has 20 positively charged groups, of which 1 is histidine, 8 are lysine, and 11 arginine and has 10 negatively charged groups, of which 2 are glutamic acid and 8 aspartic acid. Below the IP, the net charge is positive and above it negative. The location of the charges shows that almost all the charges are separated from each other. The dense distribution of the charges is close to the C-terminus, making this part of the molecule the most hydrophilic. The second densely charged region is around the cleft of the active site. A number of arginine groups are also scattered around the N-terminus. The only part that has almost no charged groups is the side opposite the cleft,

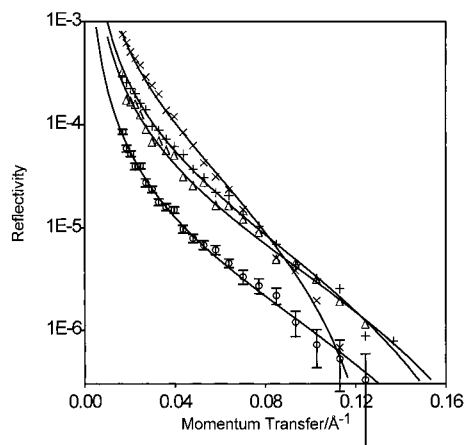


Figure 4. Neutron reflectivity profiles from lysozyme adsorbed on the surface of null reflecting water at (○) $9 \times 10^{-4} \text{ g dm}^{-3}$, 0.03 (△) g dm^{-3} , (+) 0.1 g dm^{-3} and (×) 1.0 g dm^{-3} . The solution pH was 7, and the buffer ionic strength was 0.02 M. The continuous lines were calculated using the optical matrix method, and the fitted surface excesses and thicknesses are given in Figure 5.

showing that the back of the molecule is relatively hydrophobic. Thus, when the molecule is adsorbed sideways-on, the back of the molecule is exposed to air with its cleft facing water. At the high concentration lysozyme molecule is adsorbed headways-on. The C-terminus end must be immersed in water as this part of the molecule is heavily charged. To accommodate the headways-on conformation, the N-terminus has to be tilted toward air. The current measurements in null reflecting water offer no information about the relative location of the lysozyme layer with respect to water, but in a previous study of lysozyme adsorption in D_2O at pH 7,¹¹ we found that when the molecule is adsorbed headways-on, the region out of water is about $10 \pm 3 \text{ Å}$ and the part immersed in water is about $37 \pm 3 \text{ Å}$. Since there are a few arginine groups on the N-terminus and their fully extended length is about 5 Å , it is possible that some of these charged groups are driven out of water and exist in the form of dehydrated ion pairs when the pH is below the IP. Further experiments need to be done to examine how the extent of immersion of the lysozyme layer varies with solution pH.

(B) Effect of Bulk Concentration. To support the above proposition that the adsorbed molecules retain their globular framework and the structural conformation of lysozyme molecules can switch from sideways-on to headways-on orientations, we have also examined the variation of the protein layer structure with bulk concentration. The measurements were done at pH 7 and at a total ionic strength of 0.02 M. Figure 4 shows the recorded reflectivity profiles over the concentrations ranging from 9×10^{-4} to 1 g dm^{-3} . The increased magnitude of the reflectivity profiles with respect to bulk concentration suggests that the surface excess progressively increases with bulk concentration. That the reflectivity profiles at the three lower concentrations are almost parallel suggests that the thicknesses of the layers are similar. In contrast, the sharp decaying of the reflectivity profile at 1 g dm^{-3} shows that the layer is substantially thickened. The continuous lines through the four measured reflectivity profiles were calculated using uniform layer models. The values of surface excess and layer thickness are plotted in Figure 5. To highlight the change over the low concentration range both sets of data were plotted against $\log[\text{concentration}]$. It can be seen from Figure 5 that at the bulk concentration below 0.03 g dm^{-3} the layer thickness is constant at $30 \pm 3 \text{ Å}$, indicating the formation of a sideways-on monolayer. The result also shows that up to this concentration

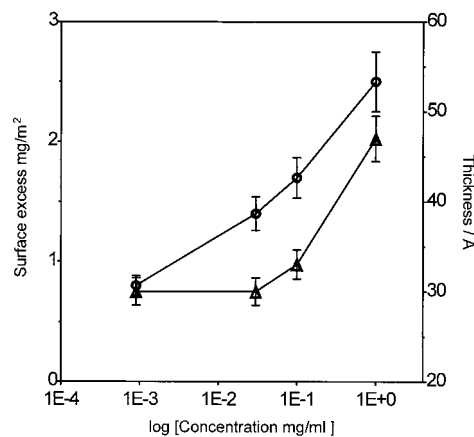


Figure 5. The variation of (○) lysozyme surface excess and (△) layer thickness with its bulk concentration. The solution pH was 7 and the buffer ionic strength was 0.02 M.

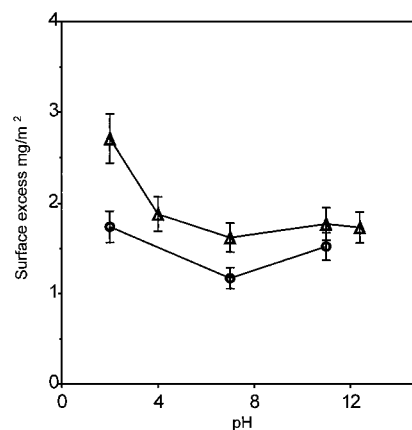


Figure 6. The variation of lysozyme surface excess with solution pH. The bulk lysozyme concentration was fixed at (○) $3 \times 10^{-2} \text{ g dm}^{-3}$ and (△) 1 g dm^{-3} , respectively. The ionic strength was adjusted to 1 M by adding NaCl.

increase in bulk concentration only leads to the increase in surface coverage but not the layer thickness. At 0.1 g dm^{-3} the protein layer is $34 \pm 3 \text{ Å}$, suggesting that lysozyme molecules adsorbed in the layer are slightly tilted. The increase in layer thickness is consistent with the area per molecule of $1400 \pm 100 \text{ Å}^2$, close to the minimum value required for sideways-on adsorption. Further increase in concentration to 1 g dm^{-3} results in the formation of a complete headways-on monolayer with the thickness of $47 \pm 3 \text{ Å}$ and the area per molecule of $950 \pm 50 \text{ Å}^2$.

(C) Effect of Salt Concentration. All the results so far presented were measured at a total ionic strength of 0.02 M. We have also examined the effect of ionic strength by adding sodium chloride to bring the total ionic strength to 1 M. The resultant surface excess plotted as a function of pH is shown in Figure 6 and the thickness variation in Figure 7. The main change is that the pattern of maximum adsorption at the IP disappears. There is a significant reduction in surface excess at pH 11 and at 1 g dm^{-3} . The maximum in the thickness at the same solution conditions also disappears. Addition of salt has resulted in an almost constant surface excess with respect to pH, except at pH 2 where surface excess has jumped from 1.2 ± 0.3 to $2.7 \pm 0.3 \text{ mg m}^{-2}$ at 1 M, an increase by a factor of 2. This surface excess increase can be correlated with the decreased solubility of lysozyme under the low pH environment. The surface excess increase is consistent with the sudden rise in the total layer thickness, indicating that, under pH 2 and at

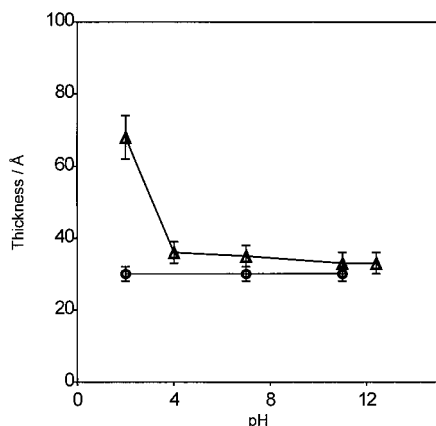


Figure 7. The variation of lysozyme layer thickness with solution pH at the total ionic strength of 1 M. The bulk lysozyme concentration was fixed at (○) 3×10^{-2} g dm⁻³ and (Δ) 1 g dm⁻³, respectively.

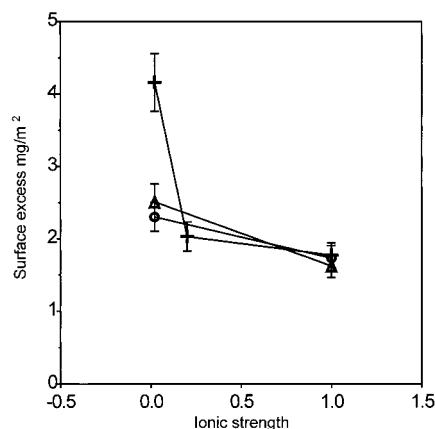


Figure 8. The variation of lysozyme surface excess with solution ionic strength at the bulk lysozyme concentration of 1 g dm⁻³ and the solution pH of (Δ) 7, (+) 11, and (○) 12.5.

1 M NaCl, water is apparently becoming a poor solvent for the protein. Given that at low pH and high ionic strength more charges and ions are associated with the molecule the solubility is expected to increase, a trend opposite to what was observed. The decreased solubility is most likely to be caused by the change in the globular conformation, leading to the exposure of some hydrophobic fragments. In comparison, at the low lysozyme concentration of 0.03 g dm⁻³ salt addition has had little noticeable effect on surface excess or layer thickness, except at pH 2 where a slight increase in surface excess is also observed. This small increase is broadly consistent with what was observed at the high protein concentration of 1 g dm⁻² at the same solution pH. Salt addition also systematically reduces the gap in surface excess between the two lysozyme concentrations, over almost all the pH range. The less dramatic variation with respect to pH at the high salt concentration is probably caused by the screening of the electrostatic charges on the protein. The reduced adsorption may be caused by association of ions with the protein molecules, lowering its tendency to adsorb.

The results described above have clearly shown that the effects of salt concentration and pH are interrelated. We summarize these results in Figure 8 for the variation of surface excess with respect to the total ionic strength under different pH and in Figure 9 for the thickness variation. It is clear that at the low ionic strength there is a significant effect of pH on the surface excess and layer thickness. This is especially so at the higher lysozyme concentration. As the ionic strength is increased

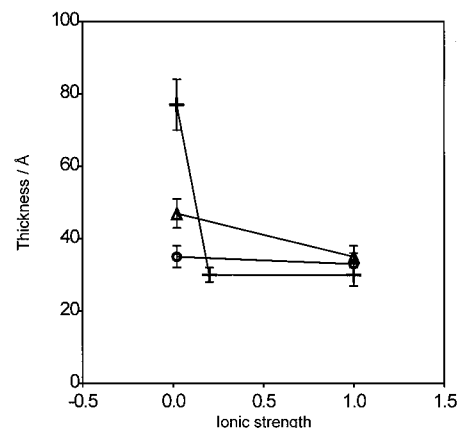


Figure 9. The variation of the total lysozyme layer thickness with solution ionic strength at the bulk lysozyme concentration of 1 g dm⁻³ and the solution pH of (Δ) 7, (+) 11, and (○) 12.5.

the gaps between different pH values become small. At 1 M NaCl all the surface excesses converge to a constant value of 1.6 ± 0.2 mg m⁻². This value corresponds to the area per molecule of 1500 ± 100 Å², close to the minimum value of 1350 Å² required for sideways-on adsorption. This observation is supported by the convergence of the thickness with the increase of salt concentration. It can be seen clearly from Figure 9 that salt addition brings the thickness to a limit of 30 ± 3 Å, again suggesting the formation of sideways-on orientation. It is thus clear that the conformational transitions of the preferred molecular orientations under different pH diminish with the increase of solution ionic strength.

In our previous study of adsorption of bovine serum albumin (BSA) at the air/water interface we have also examined both pH and salt effect in a similar manner.²⁶ While pH had a small effect on the adsorption at a low BSA concentration of 5×10^{-3} g dm⁻³ and at a low ionic strength of 0.02 M, both surface excess and layer thickness showed pronounced peaks at pH 5 at the higher concentration of 1 g dm⁻³. The increased adsorption at pH 5 was also attributed to the reduced lateral electrostatic repulsion around the IP. This adsorption pattern became less pronounced when the total ionic strength was increased from 0.02 to 1 M, indicating that electrolyte screens the electrostatic repulsions within the adsorbed layer.

We have also studied the adsorption of lysozyme and BSA at the hydrophilic silicon oxide/water interface²⁷⁻³⁰ and found that although the detailed conformational structures are affected by the presence of the solid surface, both proteins showed an increase in surface excess and layer thickness when their isoelectric points were approached. As previously discussed, the major difference between the two surfaces is that at the air/water interface the surface itself does not have an intrinsic charge which would also vary with pH. Hence, there is no electrostatic interaction comparable with that between the protein and the solid substrate, although electrostatic forces still matter within the adsorbed layer. These results taken together tend to suggest that despite the difference in surface properties, the pattern of adsorption at the low ionic strength is broadly the same and is dominated by the electrostatic repulsion within the adsorbed layer.

Conclusion

We have shown that the effects of pH and ionic strength are interrelated, both affecting the adsorption through their influence on the charged groups inside protein molecules. At low protein concentration, the effect of pH or ionic strength is small because

proteins are well separated within the adsorbed layer and the repulsive force caused by the like charges is relatively weak. At the high protein concentration the surface excess is high. Steric effects and electrostatic repulsion become significant. This has been demonstrated by the attainment of the pronounced peaks at the protein IP in both surface excess and layer thickness. Salt addition clearly reduces the drastic effect of pH, as a result of the screening of the charge repulsion within the adsorbed layer by the added electrolyte ions. The conformational structure transitions between different preferred orientations under different pH and bulk protein concentrations strongly suggest that the adsorbed proteins retain their globular framework.

The distributions of the layers over the entire solution conditions except at 1 g dm⁻³ of lysozyme and pH 11 under the ionic strength of 0.02 M can be represented by uniform layer models, suggesting that the adsorbed molecules retain their globular framework. In comparison, the distribution of the surface layer of β -lactoglobulin is similar to that formed by β -casein and has to be represented by a two layer model consisting of dense outer layer and a loose inner layer, indicating the breakdown of its globular framework after adsorption.¹⁰

It has been difficult to determine the structural conformation of an adsorbed protein layer directly. In a number of our recent studies^{11,27-30} we have demonstrated that the in situ structural conformation of an adsorbed protein layer at a planar interface can be determined by neutron reflection. The use of water contrast variation has been shown to be very effective at enhancing the resolution of the experiment. In this work we have shown that by using the null reflecting water, specular neutron reflectivity only arises from the adsorbed protein layers. Accurate determination of the layer structure allows us to infer not only the structural conformation of protein molecules under different solution conditions but also the major forces involved in the interfacial adsorption. Conventional statements about the interaction have typically been that the conformation of protein molecules adsorbed at interfaces is determined by a balance of various forces, including van der Waals attraction, electrostatic repulsion, hydrogen bonding, and the more complex interactions resulting from the hydrophobic effect and changes in conformational entropy associated with adsorption. Such vague statements do little to help to reveal the true nature of the interfacial phenomena involving protein molecules. Our neutron reflection studies of the adsorption of model globular proteins at the air/water and hydrophilic solid/water interfaces have shown that the dominating factor in these cases is the lateral electrostatic repulsion.

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