Unfolding and Intermolecular Association in Globular Proteins Adsorbed at Interfaces

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The conformational transitions that occur on heating solutions of globular proteins, unfolding and aggregation, were compared with the analogous transitions undergone by proteins adsorbed at interfaces. Fourier transform infrared spectrometry in solution and in the attenuated total reflection geometry revealed, for the globular proteins hen egg lysozyme and bovine serum albumen, both qualitative and quantitative differences between the transitions as they occur in bulk and adsorbed at an interface. In the bulk, unfolding is a sharp transition, followed sequentially on further heating by the relatively sharp onset of the intermolecular association associated with heat set gelation. In contrast, for adsorbed proteins, we found that both processes occur simultaneously over a wide range of temperatures. Proteins were more structurally stable adsorbed at a relatively hydrophilic, solid surface than at a liquid, hydrophobic surface; in the latter case, onset temperatures for both unfolding and intermolecular association were substantially lower than for bulk solutions.

Introduction

When a protein solution is heated, there are usually dramatic property changes. Frequently these culminate in the very visible process of coagulation; however, it is now well-known that this is presaged by pronounced changes in molecular conformation. These conformational transitions, unfolding followed by intermolecular association and aggregation, have now been extensively studied in bulk systems. What has received less attention is the nature of any conformational transitions undergone by proteins which are not in free solution, but which are associated with an interface. This question of what conformation is adopted by a protein at an interface has widespread relevance; for example, knowledge of the composition and conformation of adsorbed protein layers is necessary for the design of biomedical devices, since it is this initial adsorbed layer that determines the long-term fate of the biomaterial in the body.[1,2] Also, within the food industry, interfacial interactions become significant when considering the fouling of food processing equipment,[3] or during the production of foams and emulsions, where proteins are used as stabilizers.[4] In the latter case, the stabilizing action of proteins adsorbed at an air/liquid interface may be enhanced if the molecules interact to form a strongly interacting two-dimensional network. In contrast, if any significant conformational change takes place when an enzyme is immobilized on a solid surface this will clearly compromise its catalytic activity. In any situation in which a protein is localized at an interface, its immediate environment will differ markedly from its environment in bulk solution, according to the specific properties of the interface of hydrophobicity and charge. Thus, in general, the conformational state of an adsorbed protein may differ from that of a bulk protein, a phenomenon sometimes called surface denaturation.[5,6]

The purpose of this study was to explore the conformational changes that occur in an adsorbed protein's structure as a response to specific environmental changes. To do this, we studied the process of thermal denaturation for adsorbed globular proteins and made a direct comparison with thermal denaturation in the bulk. The conformational changes of two widely studied globular proteins, hen egg white lysozyme and bovine serum albumin (BSA), were considered.

The physical changes that occur when a solution of globular proteins is heated have been investigated extensively, and two distinct transitions can be identified.[3] Initially, the protein molecules undergo a change in conformation as a cooperative transition, the unfolding transition, to a more open, less structured state. This transition is marked by an increase in the viscosity of the solution as a result of the increased molecular dimensions of the partially unfolded protein molecules (though the unfolded molecules are still very much more compact than an ideal random walk) and the absorption of a latent heat of unfolding, measurable by calorimetry. If the protein solution is made up in deuterated water, the unfolding transition may also be sensitively observed by the rapid onset of exchange of previously sterically protected labile hydrogen atoms on the interior amide groups. The unfolding transition is followed by the onset of intermolecular association or aggregation of the individual unfolded protein molecules. This process is accompanied by the divergence in viscosity and the development of a finite shear modulus associated with gelation.[7,8] For lysozyme, the initial heat-induced unfolding occurs as a sharp transition at around 50 °C (the precise temperature depending on solution conditions such as pH), followed by gelation at approximately 70 °C. Clark et al.[9] have shown

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that, in contrast to most other globular proteins, the gel formed by lysozyme is thermoplastic (work carried out at pD 2 with 100 mMol NaCl added); a clear, rubbery gel develops at 70 °C, which melts upon further heating, and reforms during cooling. On the other hand, bovine serum albumin forms a thermoset gel, which sets irreversibly during heating, more typical of other globular proteins. The conformational changes that accompany protein gelation have also been widely investigated by the spectroscopic techniques of circular dichroism (CD), Raman, and infrared spectroscopy.[7,9−12] These studies have shown that the major change in structure observed upon heating a protein solution is antiparallel β-sheet formation, and this transition has been associated with the intermolecular association or gelation of the unfolded protein molecules. A mechanism put forward for the heat-induced gelation of ovalbumin suggested that the aggregation was formed from partially unfolded protein molecules, through the cross-linking of intermolecular β-sheet structures, as a result of the exposure of hydrophobic residues.[10]

Of particular relevance to this study is the extensive use of FTIR spectroscopy to look at the conformation of proteins.[13,14] The IR frequencies of interest are the amide I, II, and III regions at around 1650, 1550, and 1350−1200 cm^{-1}, respectively. The amide I peak in particular is used to determine secondary structural information, such as the extent of α-helix or β-sheet character within the protein's conformation. This is a composite peak due primarily to C═O bond stretching coupled with N−H bending and C−H stretching modes.[15] Clark et al. used such changes in the structure of the amide I peak to investigate the heat set gelation of a number of globular proteins in bulk solution and correlated their FTIR data with data from small angle-X-ray scattering and electron microscopy studies.[9,16,17] They found that the appearance of shoulders at 1621 and 1684 cm^{-1} coincided with the onset of protein gelation and could be attributed to antiparallel β-sheet formation. Such changes have been shown not to occur in proteins that do not form gels on heating.[12]

The behavior of a protein at an interface is likely to differ considerably from its behavior in the bulk. Because of the different local environment at the interface, the protein may have the opportunity of adopting a more disordered state without incurring the energy penalty entailed by, for example, exposing its hydrophobic core to water.[18] The conformation of an adsorbed protein may well, therefore, differ from its native state, and the degree of change in conformation will depend on the protein and its local environment. Therefore, one would expect that the type of interface would have a considerable effect on the thermal denaturation of an adsorbed protein layer. Protein adsorption and its adsorbed conformation can be investigated by the surface sensitive technique of ATR−FTIR (attenuated total reflection FTIR) spectroscopy.[7,19,20]

In previous studies, ATR−FTIR spectroscopy has been used to compare the conformational changes of lysozyme upon heating in bulk solution and at an interface.[7] This work showed that the temperature at which protein molecules begin to form the intermolecular associations associated in the bulk with aggregation and gelation is considerably lower when the protein is adsorbed at an interface compared with the bulk. In this paper we report a substantial extension of this previous study, in which we investigate the temperature dependence and the kinetics of the unfolding and aggregation transitions of two proteins during heat denaturation, both in solution and adsorbed at an interface. We also consider the effect different interfacial properties have on the structural behavior of a globular protein by studying the adsorption and thermal denaturation of hen egg white lysozyme to three different polymeric interfaces.

Experimental Section

Sample Preparation. Hen egg white lysozyme and bovine serum albumin were received as lyophilized powders (Sigma, Dorset, U.K.), and the protein solutions were made up in a deuterated phosphate buffer (pH 7, 0.1 M) solution. D_2O (99.9% deuterium, Aldrich) was chosen rather than H_2O because water absorbs strongly in the amide region of the spectra. Another advantage of using a deuterated solvent is that it allows the unfolding of the protein to be monitored. H−D exchange occurs rapidly with hydrogens near the surface of the protein and accessible to the solvent, but very slowly for hydrogens within the core of the protein and inaccessible to the solvent. This exchange process affects the absorption frequencies of the amide II peak by shifting it to 1450 cm^{-1}, but has very little effect on the position of the amide I peak. The protein was made up in deuterated buffer the day before analysis to allow time for the exchange of all accessible hydrogens at the surface of the protein in its native conformation. Upon adsorption and during heating any unfolding of the protein brings hydrogens originally inaccessible into contact with the solvent, and this manifests itself in the spectra as a disappearance of the residual amide II peak (due mostly to N−H bending) at around 1540 cm^{-1}.[7,9,28]

For bulk solution FTIR experiments a Specac liquid transmission cell that had been modified to allow the cell to be heated was used. The cell was fitted with 4-mm CaF_2 windows and a tin spacer, giving a path length of 6 μm. A protein solution of 0.1 g/mL was used, a concentration that exceeds the bulk critical gelation concentration of both proteins. Protein adsorption experiments were performed using a 10 internal reflection, 45° fixed angle of incidence ZnSe crystal. The crystal was sealed in a squarecol ATR liquid cell (Graseby Specac Ltd., Kent, U.K.) which had been modified to allow liquid handling from outside of the IR chamber. The ATR liquid cell and the ZnSe ATR crystal were cleaned using a neutral surfactant and rinsed extensively in water before use. Polymer surfaces were created by spin coating from a 5 mg/mL toluene solution onto the smooth faces of the ZnSe crystal. This procedure produces a smooth polymer film with a thickness substantially less than the penetration depth of the infrared evanescent wave. Polystyrene (96k) and poly(methyl methacrylate) (PMMA) (95k) were both

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supplied from Polymer Laboratories Ltd, U.K. The ethylene/propylene/diene terpolymer (EPDM rubber) (Scientific Polymer Products, Inc., Ontario, NY) is a random terpolymer containing 60% ethylene and 4% diene content and with a T_g of −50 °C.

Infrared Spectroscopy and Experimental Procedure. A Mattson Galaxy 4020 FTIR spectrometer with a deuterated triglycerine sulfate (DTGS) detector was used to collect the infrared spectra. The spectrometer was continually purged with dry air during each experiment to remove water vapor from the chamber. Data were collected as interferograms at 2 cm^{-1} resolution, of which typically 120 interferograms were collected and coadded, except during the initial stages of adsorption where 28 interferograms were coadded. Heating was carried out using a Eurotherm temperature controller producing stable temperatures to within ±0.5 °C and calibrated using a thermocouple.

IR spectra of the adsorbed protein layers were collected as ATR spectra using the ZnSe ATR crystals. The crystal was sealed within the ATR liquid cell inside the IR chamber for at least 30 min before the experiment was started to allow the system to purge under a flow of dry air, removing atmospheric H_2O. The stability of the level of trace water in the purge gas was carefully controlled, but even so slight variations in the level of trace water between background runs and experiment probably account for the background in the amide I and II region of the spectrum. Deuterated phosphate buffer was added to the ATR cell and left to equilibrate until no difference was observed in the appearance of consecutive spectra. This was of particular importance when the ZnSe crystal was coated with a polymer, since polymers such as PMMA may hydrate and reorientate upon contact with water. The reference spectrum of the substrate and buffer was thereafter used as the background against which all subsequent protein spectra were ratioed. The buffer solution was then replaced with lysozyme solution and the adsorption observed for 2 or 4 h, during which IR spectra were acquired continuously. Finally, the lysozyme solution was replaced by a buffer-only solution and the heating experiment initiated. Heating was performed in 2 °C increments at a rate of 1 °C/min, and the cell was allowed to equilibrate for 5 min before acquiring an IR spectrum. The system was heated from 28 to 90 °C and then cooled to 30 °C with spectra taken every 2 °C on heating and every 5 °C on cooling. Additional control experiments were also performed at each surface before beginning lysozyme adsorption. In these, the substrate was immersed in buffer-only solution and heated to 90 °C at a rate identical to the heating process described above. This control was necessary because during the heating experiment small changes in the optical properties of the ATR crystal and its coating, if any, can lead to slight changes in the ATR spectra on heating. In all cases the effects were minor and reversible. Spectra of the bulk protein solution were carried out using the Specac liquid transmission cell. Heating was performed in the same manner as for the ATR experiments, and all spectra were ratioed against a blank buffer solution.

The protein spectra, following ratioing against the background, were analyzed by peak integration within a fixed range to determine the area under the amide I and II peaks. Any contribution to the integral from the small temperature-dependent changes in background revealed by the control heating experiments were subtracted from the peak integrals. Integrals were calculated for the amide I peak (C═O stretch) between 1600 and 1700 cm^{-1}, for the shoulders at approximately 1620 and 1680 cm^{-1}, the amide II peak at 1540 cm^{-1} which was of weak intensity (containing mostly N−H bending), and the more intense peak at 1450 cm^{-1}, the amide II′ peak, a composite peak that includes the N−D bend frequency.

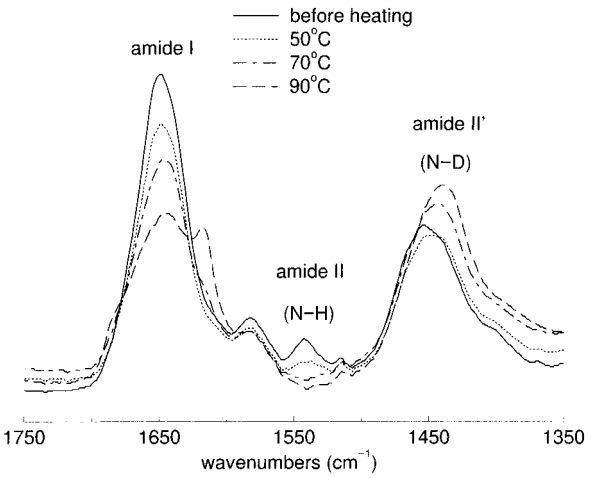

Figure 1. The amide region of the IR transmission spectra during the thermal denaturation of lysozyme in bulk solution.

Results and Discussion

Thermal Denaturation of Lysozyme and BSA in Bulk Solution. Lysozyme (14.3 kDa) contains a single chain of 129 amino acids with four disulfide bridges and has dimensions of approximately 4.6 × 3 × 3 nm^3.[21−23] Albumin (65 kDa) consists of approximately 580 amino acids and has three distinct domains containing disulfide-bonded helical subdomains.[24] Both lysozyme and albumin have a highly helical native structure containing approximately 50% α-helix. Lysozyme, however, is more rigid because of its extensive disulfide cross-linking which limits its flexibility. As we shall see, this degree of structural stability means that it only partially unfolds during adsorption.[3]

Before considering the effect an interface may have on the thermal aggregation of globular proteins, the mechanism of the heat set gelation of proteins in bulk solution was studied. The spectra for the thermal denaturation of lysozyme in bulk solution can be seen in Figure 1. During heating of the protein two significant changes to the amide peaks were observed. Initially, the disappearance of the N−H residual amide II peak at 1540 cm^{-1} was seen which indicated unfolding of the protein. At higher temperatures, shoulders began to appear in the amide I peak, one at 1620 cm^{-1} and a less pronounced shoulder at 1684 cm^{-1}. These shoulders, according to the assignation of the Clark et al. studies[9,16,17] (which were also carried out at elevated temperatures), reveal the formation of antiparallel β-sheet structure and indicate the onset of the intermolecular association which leads in the bulk to gelation. These β-sheet peaks remained prominent within the spectra during cooling of the protein solution.

By calculating the integrals of the amide peaks, the rates and the temperatures at which these two transitions occur are revealed. Figure 2 shows peak areas plotted against temperature for the N−H residual amide II peak at 1540 cm^{-1} (revealing protein unfolding) and the 1620

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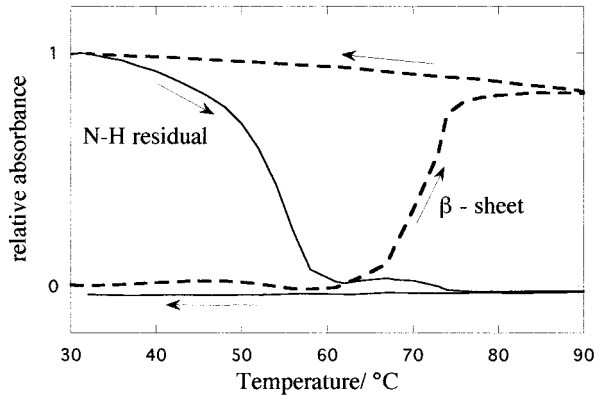

Figure 2. Normalized peak integrals as a function of temperature of the residual N−H amide II peak (solid line) and the 1620 cm^{-1} β-sheet shoulder of the amide I peak (dashed line) for lysozyme in solution on heating to 90 °C and subsequent cooling to 30 °C.

cm^{-1} shoulder of the amide I peak (corresponding to β-sheet formation) for the experiment detailed in Figure 1, where the protein solution is heated to 90 °C and then cooled. Similar experiments were also carried out, in which the protein solution is heated and then incubated at 50 or 60 °C for approximately 8 h before cooling. By comparing all of these data we are able to make deductions about the mechanism of the denaturation process.

Analysis of the data corresponding to the 90 °C experiment revealed that the protein molecules initially unfolded at around 50 °C, seen by a rapid decrease in the peak area of the N−H residual amide II peak at 1540 cm^{-1}. At approximately 70 °C, after completion of the unfolding transition, a second sharp transition was observed indicating the initiation of protein aggregation and seen as an increase in the peak area of the 1620 cm^{-1} β-sheet shoulder of the amide I peak. These data revealed that the two transitions associated with the heat set aggregation of lysozyme were sharp and discrete and that at a temperature of around 60 °C the protein existed in a state where it had substantially unfolded but did not immediately begin to aggregate (the noise in our data and the nonzero background mean that we cannot confidently describe the unfolding as complete). Thus, it may be that an intermediate stable state can exist where the protein molecules exist in an unfolded but structurally intact conformation, and also that the aggregation transition is strongly temperature dependent. Indeed, in further experiments where the heating cycle has been held at 50 or 60 °C for 8 h, complete unfolding occurred as marked by complete H/D exchange, but no significant change in secondary structure was observed. When incubated at 50 °C, complete unfolding of the protein gradually took place over the first 5 h of incubation and at 60 °C the unfolding transition occurred within the first hour during the initial heating of the protein solution. In both cases intermolecular association did not appear to occur as the overall shape of the amide I peak, the secondary structure of the protein, did not change. Thus, it appeared that a fairly stable unfolded intermediate was formed that contained much of its native secondary structure. This behavior suggested that the protein may exist in an intermediate state; unfolded, yet not able to form strong intermolecular associations. It is tempting to associate this state with the "molten globule" state found in other globular proteins such as α-lactalbumin. However, other studies have not revealed such an equilibrium state for hen egg white lysozyme, though studies have suggested the existence of an analogous kinetic intermediate.[29,30]

Similar experiments have also been performed to investigate the thermal denaturation of BSA in bulk solution. As seen for lysozyme, the unfolding transition was seen as the reduction in the 1540 cm^{-1} amide II peak and protein aggregation observed by the formation of shoulders in the amide I peak. Figure 3 shows the peak integral data for the N−H residual amide II peak and the β-sheet shoulder for BSA during heating to 90 °C. A comparison of Figures 2 and 3 shows that on heating a BSA solution to 90 °C the protein unfolds and aggregates in a manner similar to lysozyme. However, the rates of unfolding and β-sheet formation are slower for BSA, resulting in overlapping of the two transitions at 60 °C. When heated to 60 °C, BSA was able to completely unfold within the first hour of incubation. However, although a slight broadening of the amide I peak was initially observed within the first hour of incubation, suggesting partial protein aggregation, the protein solution did not completely aggregate or gel. No further changes in protein structure were seen during prolonged incubation or during cooling. Therefore, it appears that both lysozyme and albumin form fairly stable intermediates upon incubation at temperatures up to 60 °C where the protein is in an unfolded state with its native secondary structure significantly intact. This suggests that the aggregation transition is predominantly temperature dependent, and that even complete unfolding is not a sufficient condition for intermolecular association to take place.

Adsorption and Thermal Denaturation of Lysozyme to ZnSe. To investigate the possible interfacial effects upon the protein unfolding and aggregation process during thermal denaturation, lysozyme was adsorbed to a ZnSe ATR crystal. Adsorption took place over a 2 h period, after which the system was washed with buffer-only solution and the adsorbed lysozyme layer heated. Spectra were recorded regularly during both the adsorption and heating experiments, allowing the rate of adsorption and conformational changes that occur during both adsorption and thermal denaturation to be considered. These spectra indicated that the adsorption is rapid, quickly leading to saturation of the surface, and that the protein undergoes a relatively small degree of unfolding at the interface. Figure 4 compares the spectra of lysozyme and BSA in bulk and at the interface; changes in the overall shape of the amide I peak during adsorption are apparent, indicating some secondary structure reorientation of the adsorbed protein molecules. This reveals that the structure of the adsorbed lysozyme molecules differs from their native structure in the bulk state.

Graphs of peak integrals versus temperature for the N−H residual amide II peak and the 1620 cm^{-1} shoulder of the amide I peak are shown in Figure 5. During heating to 90 °C the protein layer continued to unfold gradually over the whole temperature range, as manifested by a slow decrease in the 1540 cm^{-1} N−H residual amide II peak. Also, upon heating the shape of the amide I peak began to alter, corresponding to the continuous and gradual formation of antiparallel β-sheet structure. A slight reduction in β-sheet character was detected upon

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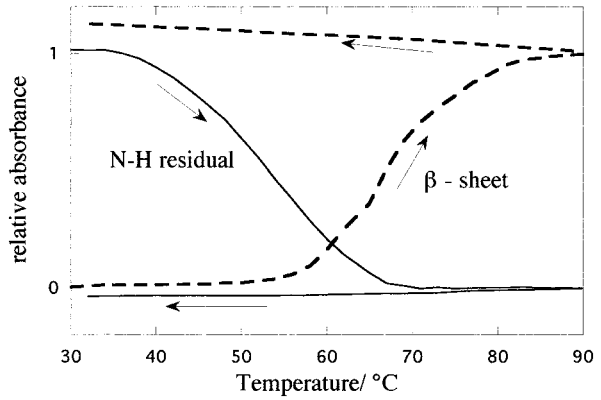

Figure 3. Normalized peak integrals as a function of temperature of the residual N−H amide II peak (solid line) and the 1620 cm^{-1} β-sheet shoulder of the amide I peak (dashed line) for BSA in solution on heating to 90 °C and subsequent cooling to 30 °C.

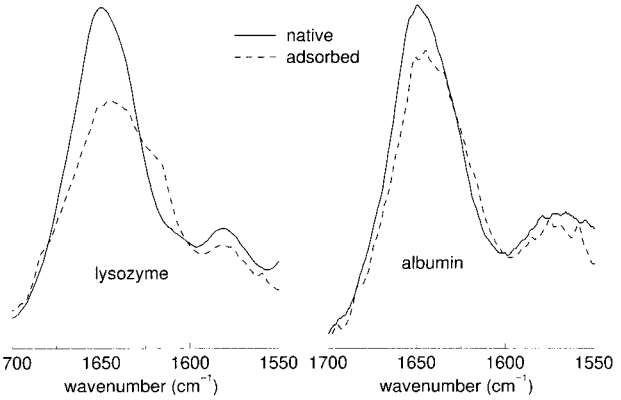

Figure 4. The IR spectra in the amide I region for lysozyme and BSA in bulk and following adsorption to a ZnSe interface.

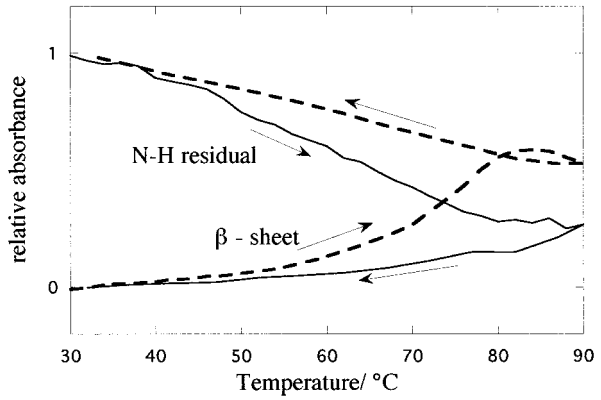

Figure 5. Normalized peak integrals as a function of temperature of the residual N−H amide II peak (solid line) and the 1620 cm^{-1} β-sheet shoulder of the amide I peak (dashed line) for lysozyme adsorbed at a ZnSe interface on heating to 90 °C and subsequent cooling to 30 °C.

heating the protein layer to temperatures above 80 °C, but increased again during cooling. This reduction in β-sheet structure at high temperatures was also observed for lysozyme in solution (pD 2) by Clark et al. and corresponds to "melting" of protein aggregates.[7] The protein aggregates partially melt at temperatures above 80 °C but reform on cooling. Figure 6 shows the total peak areas for the amide I and amide II′ (deuterated amide II) peaks during heating of the adsorbed protein layer. These data show that the sizes of both peaks gradually increase during heating and cooling to approximately three times their original peak areas. Since the adsorbed protein layer

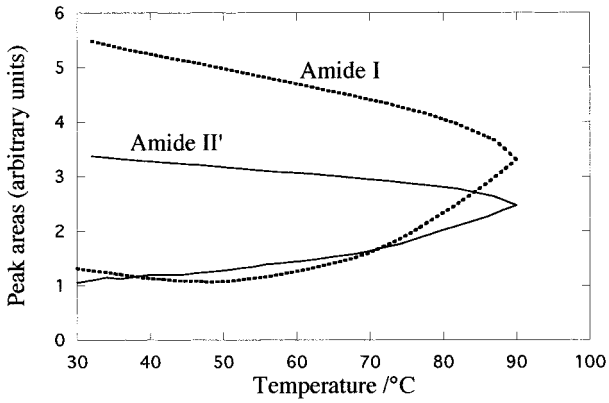

Figure 6. Total peak integrals for the amide I peak and the amide II′ (N−D) peak for lysozyme adsorbed to ZnSe as a function of temperature.

is in contact with a buffer-only solution, it is unlikely that such an increase in peak areas is due to an increase in the concentration of the protein layer. Instead, it seems probable that this intensity increase reflects changes in the orientation and local environment of the amide groups. In particular, changes in the state of hydrogen bonding reflecting heat-induced modifications of secondary structure are likely to lead to substantial changes in the transition moments, as is observed in the IR spectra of water close to interfaces.[31]

We also carried out experiments in which the adsorbed lysozyme was heated to 50 °C and then held at that temperature. These showed that both unfolding and β-sheet formation continue to occur, initially quite rapidly but tailing off as the incubation time increases. After 8 h incubation the amide I peak contained distinct shoulders due to β-sheet formation, but the extent of this intermolecular association appears less than when the system is heated to 90 °C. Both this result and the observation of partial aggregation of the protein molecules during adsorption highlight the significant effect the presence of an interface has on the association process. In bulk solution intermolecular association occurs only at high temperatures. However, when adsorbed at an interface, intermolecular association of proteins commences at much lower temperatures.

Thus the presence of an interface appears to severely alter the structural behavior of the protein. An adsorbed protein layer does not exhibit discrete and sequential transitions corresponding to unfolding and the onset of intermolecular association upon heating. Instead, when adsorbed at an interface, intermolecular association in lysozyme commences at temperatures far lower than observed for bulk aggregation in solution; the unfolding and intermolecular association transitions occur simultaneously but at significantly slower rates than when in bulk solution.

Conformational Changes of Adsorbed BSA and Lysozyme. Similar experiments for BSA adsorbed to ZnSe suggested that BSA unfolds more readily during adsorption than does lysozyme. Following this observation, experiments were performed for both lysozyme and BSA that allowed significantly longer adsorption times before beginning the heating stage of the experiment. In these experiments adsorption was monitored for 4 h, after which the surface was washed with a buffer-only solution and incubated at room temperature for a further 20 h before heating began. During the first 4 h of protein adsorption

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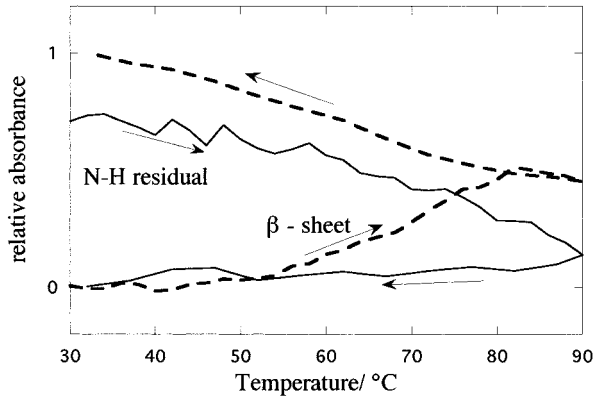

Figure 7. Normalized peak integrals as a function of temperature of the residual N−H amide II peak (solid line) and the 1620 cm^{-1} β-sheet shoulder of the amide I peak (dashed line) for lysozyme adsorbed at a ZnSe interface, having been incubated for 24 h at room temperature prior to heating to 90 °C and subsequent cooling to 30 °C.

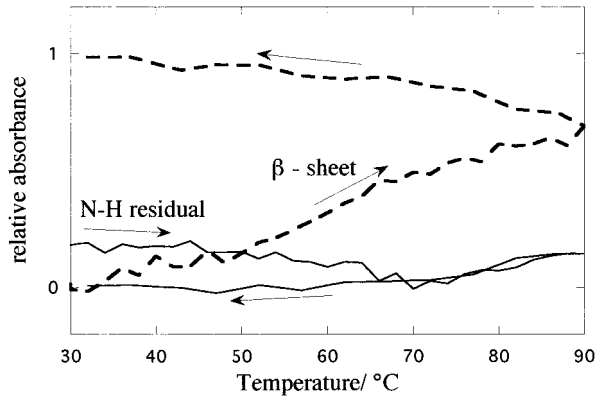

Figure 8. Normalized peak integrals as a function of temperature of the residual N−H amide II peak (solid line) and the 1620 cm^{-1} β-sheet shoulder of the amide I peak (dashed line) for BSA adsorbed at a ZnSe interface, having been incubated for 24 h at room temperature prior to heating to 90 °C and subsequent cooling to 30 °C.

to ZnSe for both lysozyme and BSA, the amide peak areas were monitored; protein adsorption was rapid, and during adsorption some protein unfolding did occur. However, during the 4 h period BSA continued to unfold, whereas lysozyme only partially unfolded during the first 1−2 h, subsequently remaining substantially unchanged. Also during adsorption changes in the shape of the amide I peak were observed as seen from the spectra in Figure 4. Broadening of the amide I peak during adsorption was seen in the spectra of lysozyme and suggests that the protein's secondary structure shifts toward an increased β-sheet content and, therefore, that some intermolecular association between the unfolding adsorbed molecules occurs without the addition of heat. In contrast, the secondary structure of BSA did not show significant changes in the shape of the amide I peak during adsorption, which implies that although the BSA molecules had significantly unfolded the adsorbed layer did not begin to aggregate at room temperature. Incubating the adsorbed protein layers at room temperature for a further 20 h had no appreciable effect on the spectra of either protein. Finally, upon subsequently heating the adsorbed layers to 90 °C (Figures 7 and 8), unfolding and β-sheet formation occurred for lysozyme at rates similar to that described previously for Figure 5. For BSA, the already largely unfolded protein molecules slowly and continually aggregated during heating in a manner similar to lysozyme. However, unlike lysozyme there was no evidence of "melting" at higher temperatures; in BSA aggregation during heating is irreversible.

Thus it appears that the effect of adsorption at an interface is broadly similar for both BSA and lysozyme. Both proteins begin to aggregate at lower temperatures, but at slower rates, than when in bulk solution. However, BSA is a less rigid molecule than lysozyme and is not as stable; it unfolds almost completely upon adsorption. Thus the process of surface unfolding differs between the two molecules, occurring mostly during heating for lysozyme and during adsorption for BSA. There are differences, too, between the two proteins with regard to the process of intermolecular association when adsorbed at a surface. During adsorption, the secondary structure of lysozyme changes suggesting an increase in β-sheet formation and the existence of aggregation at room temperature. Also, during heating, lysozyme loses β-sheet character at temperatures above 80 °C, which it is able to reform on cooling. Neither of these trends is observed during the adsorption and thermal denaturation of BSA.

Lysozyme at Polymeric Interfaces. So far the effect that the presence of an interface has on the conformations of lysozyme and BSA has been considered and compared with the corresponding conformational changes observed in the bulk state. However, since the properties of different interfaces vary considerably, it is also important to investigate the effects different surface properties have on the adsorption and thermal denaturation of proteins. To compare well-characterized surfaces with distinct surface properties, we coated ZnSe crystals with thin polymer layers. We investigated the effect of hydrophobicity by comparing the effect of a poly(methyl methacrylate) (PMMA) surface, which is relatively hydrophilic and has a contact angle with water of 60°, to the much more hydrophobic polystyrene surface, with a water contact angle of 90°. Both PMMA and polystyrene are glassy at room temperature and thus present a solid, unyielding surface to the solution. We compared these results to results obtained from a surface coated with an ethylene−propylene copolymer (EPDM). This material forms an amorphous layer which at room temperature is well above its glass transition temperature. Therefore, although its bulk viscosity is high enough for it to form a stable coating on the crystal, on a local scale the polymer segments have liquidlike local mobility. Thus these EPDM layers may be considered as a model for an interface between water and a hydrophobic liquid.

Adsorption of lysozyme to each of the polymeric interfaces was rapid and quickly resulted in saturation of the surface and a plateau in adsorption, observed by the peak area of the amide I peak (data not shown). During adsorption changes in the protein's secondary structure and partial unfolding of the protein were observed. The extent of unfolding of the lysozyme molecules is shown in Figure 10, which compares the changes in peak integrals for the N−H residual amide II peak during adsorption to each of the polymeric surfaces. During adsorption to the hydrophilic interface, PMMA, only a small reduction in the size of the N−H residual peak is seen. For both the hydrophobic polymers, PS and EPDM, the final degree of unfolding is greater than for PMMA; both polymers show an initial fast unfolding on a time scale of minutes which is not apparent on the PMMA interface. The greatest reduction of size occurs for adsorption to the EPDM coated surface, presumably reflecting the greater ease of reorientation on a liquidlike hydrophobic surface than on a glassy, rigid surface of comparable hydrophobicity. As noted for the ZnSe interface, the shape of the amide I

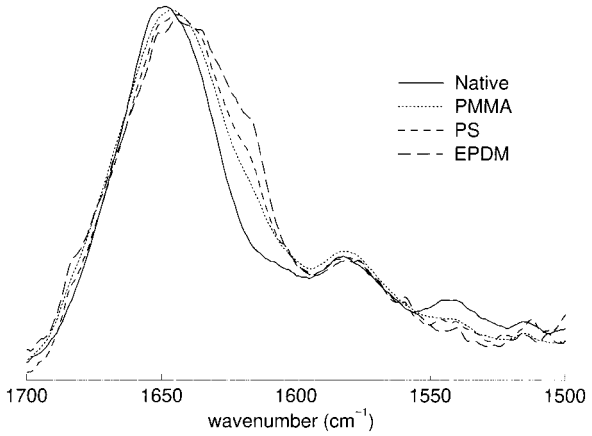

Figure 9. The IR spectra in the amide I and amide II region for lysozyme in bulk and after 2 h adsorption to interfaces with polystyrene (PS), poly(methyl methacrylate) (PMMA), and EPDM rubber.

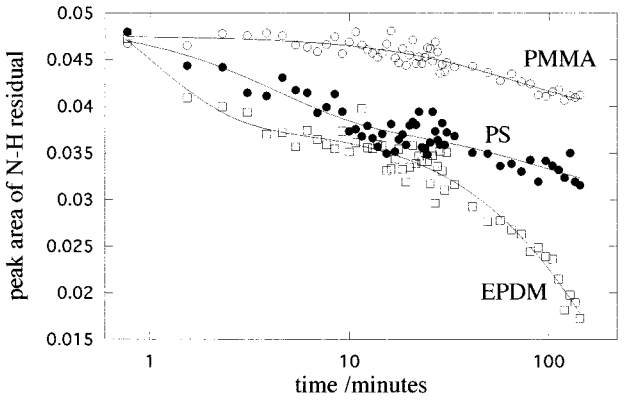

Figure 10. The peak area of the residual N−H amide II peak as a function of time following the adsorption of lysozyme to interfaces with polystyrene (PS), poly(methyl methacrylate) (PMMA), and EPDM rubber.

peak broadens during lysozyme adsorption, suggesting a small shift toward β-sheet structure. This observation was also observed during adsorption to each of the polymeric interfaces (Figure 9). Once again, changes in protein secondary structure during adsorption were most prominent when the absorbing interface was the liquidlike, hydrophobic polymer, EPDM, and least prominent for the relatively hydrophilic, solid PMMA interface.

Upon heating the adsorbed protein layer at each of these interfaces, both the rate of unfolding and intermolecular association were significantly affected by the character of the surface. The peak integrals for the N−H residual amide II peak (unfolding transition) and the β-sheet shoulder of the amide I peak (aggregation transition) during the thermal denaturation of lysozyme adsorbed at each of the interfaces are shown in Figure 11.

At a polystyrene interface (Figure 11a) β-sheet formation began to occur immediately upon initiation of heating and continued to increase as heating proceeded in a manner similar to that seen for lysozyme adsorbed to ZnSe. A slight reduction in the 1620 cm^{-1} shoulder was observed at temperatures above 80 °C; as discussed previously for lysozyme adsorbed to ZnSe, we associate this with a reversible "melting" of the aggregated protein molecules. The rate of unfolding of the protein, which had begun during adsorption, was initially quite slow during heating, but appeared to increase at higher temperatures, as observed by the reduction in the N−H residual amide II peak.

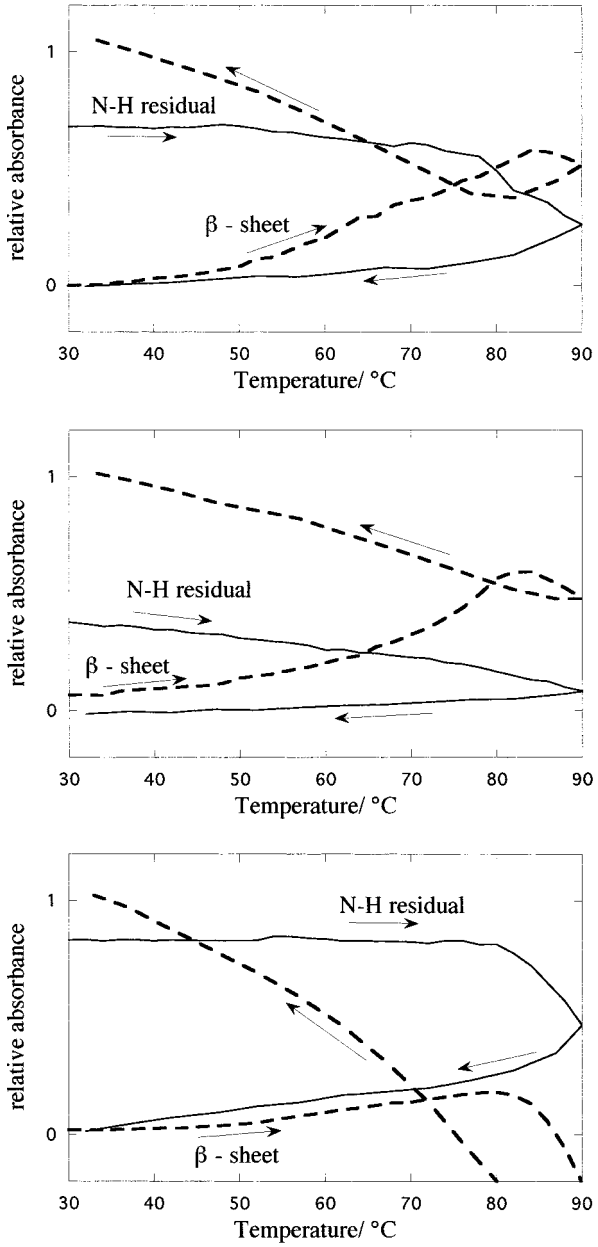

Figure 11. Normalized peak integrals as a function of temperature of the residual N−H amide II peak (solid line) and the 1620 cm^{-1} β-sheet shoulder of the amide I peak (dashed line) for lysozyme adsorbed at (a) an interface with polystyrene, (b) an interface with EPDM rubber, and (c) an interface with poly(methyl methacrylate), on heating to 90 °C and subsequent cooling to 30 °C.

At an EPDM rubber interface, the structure of lysozyme had significantly altered during adsorption and the protein was already in a largely unfolded state. During heating, as shown in Figure 11b, the protein completed its unfolding transition slowly over the whole temperature range seen by the gradual decrease in the N−H residual amide II peak. Protein aggregation, observed by monitoring the β-sheet shoulder of the amide I peak, continued to form with increasing temperature following a trend similar to that seen when lysozyme is adsorbed to both polystyrene and ZnSe.

Similarly, at a PMMA interface (Figure 11c) a gradual increase in the peak area of the β-sheet shoulder was observed upon initiation of heating. However, its peak area dropped dramatically at temperatures above 80 °C (the apparent negative value of the peak integral is an

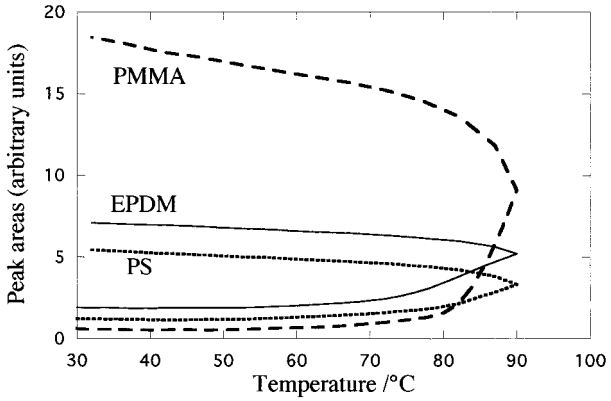

Figure 12. Total peak integrals as a function of temperature for the amide I peak for lysozyme adsorbed to interfaces with with polystyrene (PS), poly(methyl methacrylate) (PMMA), and EPDM rubber.

artifact arising from the method of background subtraction), corresponding to complete loss of aggregation of the protein molecules. The shoulder then increased considerably as the temperature was cooled and intermolecular association reformed. This melt phase also coincided with the unfolding of the protein molecules since the residual N−H peak at 1540 cm^{-1}, only slightly reduced during adsorption, remained very prominent upon heating and did not disappear until a temperature above 80 °C was reached.

Also, as seen during the thermal denaturation of lysozyme at ZnSe, the overall peak areas of the amide I and the amide II′ peaks increased when adsorbed at the polymer interfaces, an observation that we suggested is likely to be due to large shifts in the orientation or local of environment of amide groups within the protein molecules at the interface. Figure 12 directly compares the peak integral changes with increasing temperature of the amide I peak for lysozyme adsorbed at each of the polymeric interfaces. These results show that an increase of approximately 5 times the original size of the amide I peak occurs at both the polystyrene and EPDM rubber. However, the peak increases to over 10 times its original size when the interface is PMMA, the increase in size occurring at temperatures above 80 °C and, thus, coinciding with the "melting" of lysozyme aggregation.

These increases in the sizes of the amide I and II′ peaks seen on heating in adsorbed (ATR) spectra cannot reflect changes in the adsorbed amount, since the adsorbed protein layer is in a buffer-only solution. The depth of penetration for ATR analysis is dependent on the refractive index of the substrates and the adsorbates. Therefore, an increase in the apparent intensity of the protein peaks could conceivably be due to significant temperature dependent changes to the substrate optical properties. The control experiments, where the substrate alone is heated and cooled, show that any changes to the spectra due to the substrate are small and largely reversible. Also, in bulk solution the sizes of these peaks are not significantly affected during heating. This suggests that the intensity changes observed in the adsorbed ATR spectra are surface induced as a result of considerable structural or alignment differences between adsorbed lysozyme and lysozyme in solution.

When we compare all of the above results obtained for lysozyme adsorption and thermal denaturation at a variety of interfaces, it is clear that the character of the interface does significantly alter the behavior of the adsorbed protein layer. The protein's structure barely changed during adsorption to the relatively hydrophilic polymer, PMMA, with only a small amount of unfolding and secondary structural change seen. However, to the hydrophobic interfaces structural changes were far more extensive; this is perhaps as expected since these surfaces may promote stronger interactions between the protein and the interface via hydrophobic interactions. The EPDM rubber, used here to model the hydrophobic liquid surface of an oil/water interface, facilitated further changes in the protein's structure during adsorption, allowing considerable unfolding and β-sheet formation.

The type of interface also had a considerable effect on the rate of both unfolding and aggregation during heating. Despite this, the appearance of the protein spectra after cooling suggested that the final structure of the adsorbed protein layer was very similar at each of the polymeric interfaces. The most prominent difference between each of the interfaces was seen when considering the rate of intermolecular association of the protein. For direct comparison, Figure 10 overlays the integral data for the β-sheet shoulder of the amide I peak for lysozyme at each of the interfaces. This shows that initially the changes in the shape of the amide I peak were very similar for lysozyme adsorbed to all three polymers, with the β-sheet structure increasing gradually as the temperature was increased. However, significant differences were observed when considering the extent of loss of β-sheet character during "melting" of the protein aggregates at high temperatures. For lysozyme adsorbed to PMMA, a complete loss of aggregation at temperatures above 80 °C was seen, compared to the more hydrophobic interfaces where only a partial loss of aggregation occurred. Also, when adsorbed to PMMA, the temperature at which this loss of protein aggregation occurred coincided with both the unfolding of the protein molecules and the observed increase in the sizes of the amide I and amide II′ peaks. This result suggests that the hydrophobicity of the interface controls the ability of the aggregated proteins to "melt" at high temperatures. The more hydrophilic interface binds the protein less tightly which reduces the structural reorientations of the protein at low temperatures but facilitates relatively rapid conformational changes at elevated temperatures. An additional complication for PMMA, which may account for its anomalous high temperature behavior, is that the bulk glass transition temperature of PMMA is around 110 °C. In a thin film somewhat swollen by water the transition temperature may be reduced, and it is possible that these conformational changes in the protein are coupled with the onset of increased mobility in the polymer film.

Finally, it is worth pointing out similarities between this work and other interfacial studies of gelation in synthetic polymer systems. Here, a thin layer of gelled material may form at an interface, even when the bulk concentration is too low, as long as the local surface concentration exceeds the threshold required for gelation. Surface gelation was initially observed by Kim et al.[26] in polystyrene sulfonate solutions, and since then its potential importance for protein systems[7,27] has been pointed out.

Conclusion

The proximity of an interface has both qualitative and quantitative effects on the structural changes of globular proteins during heat denaturation. In the bulk state the heating of both lysozyme and BSA resulted in heat induced intermolecular association via two discrete and sequential transitions. The protein molecules initially undergo an

unfolding transition at around 50 °C, and then at around 70 °C intermolecular association commences leading to aggregation. Protein molecules held at elevated temperatures below 70 °C (50−60 °C) are able to complete their unfolding transitions, as marked by complete exchange of labile hydrogen for deuterium, while undergoing only relatively minor changes in secondary structure. This suggests that in the bulk the onset of intermolecular association and aggregation is a rather sharp transition and that there is an intermediate state where the protein is unfolded but remains with its native secondary structure largely intact and is not yet susceptible to the formation of intermolecular associations.

In contrast to the bulk case, when adsorbed at an interface discrete unfolding and aggregation transitions of the protein molecules were not observed. Some partial unfolding of the proteins occurs during adsorption and thus the initial structures upon initiation of heating differed significantly from that of their native structure in the bulk state. In fact, during adsorption, changes in the secondary structure of lysozyme were also observed suggesting that the protein molecules already undergo some intermolecular association as well as unfolding even under ambient temperatures. During heating, instead of sharp unfolding and aggregation transitions, both proteins gradually and simultaneously unfolded and aggregated over a wide temperature range.

The character of the adsorbing interface affects the extent and rate of conformational rearrangements of the adsorbing protein. At a hard, hydrophilic interface, obtained by coating the ATR crystal with PMMA, there was very little structural change during adsorption; a liquidlike, hydrophobic interface, provided by an EPDM coating, facilitated considerable protein unfolding and secondary structural changes during adsorption, while a hard hydophobic interface, polystyrene, provided an intermediate situation.

Thus the conformational transitions undergone on heating by the proteins studied in these experiments were different in both quality and quantity for proteins adsorbed at interfaces when compared to proteins in bulk. What is a sharp unfolding transition in bulk solution, followed sequentially by the relatively rapid onset of intermolecular association, is replaced for adsorbed proteins by the gradual and simultaneous processes of unfolding and association. The degree to which an interface promotes or retards these conformational transitions is a function of both the hydrophobicity of the interface and whether it is solidlike or liquidlike in character.

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