The Role of Chemical Potential in the Adsorption of Lysozyme at the Air-Water Interface

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Received October 2, 1991. In Final Form: January 31, 1992

The adsorption of ^{14}C-labeled lysozyme at the air–water interface has been studied at various temperatures using the radiotracer method. It was observed that the surface concentration of lysozyme decreased with time during the first 60–100 min of adsorption, followed by a rapid increase at later stages. Concomitant with the decrease in surface concentration, the surface tension of the protein solution increased during the initial period, followed by only a gradual increase at later stages. The results indicated that at the moment of creation of the interface, the undenatured lysozyme tends to desorb from the interface owing to its high electrochemical potential at the interface. At the subsurface, lysozyme undergoes partial unfolding, which subsequently facilitates positive adsorption at later stages. The effect of temperature on the kinetics of adsorption indicated that the activation energy barrier for adsorption of lysozyme at the air–water interface was about 12 kcal/mol. On the basis of the data presented, a general mechanism for protein adsorption is proposed, which invokes that it is the chemical potential gradient rather than concentration gradient that acts as the driving force for adsorption of proteins at interfaces.

Introduction

It is generally accepted that the adsorption of proteins and small molecular weight surfactants at interfaces is diffusion controlled. A theoretical model for diffusion-controlled adsorption was first proposed by Ward and Tordai[1] and later with several modifications by others.[2-4] Several investigators have applied these models to study the kinetics of adsorption of proteins at interfaces.[5-13] In these studies, any deviation from diffusion-controlled adsorption was explained in terms of surface pressure barrier and/or other activation energy barriers to adsorption.[6-9,14,15]

The basic assumptions involved in the diffusion controlled adsorption theory is that when a fresh interface is created, the molecules at the subsurface instantaneously adsorb to the interface.[1] The depletion of concentration at the subsurface creates a concentration gradient between the subsurface and the bulk phase, which drives molecules from the bulk phase to the subsurface and then to the interface. During the course of adsorption the subsurface concentration is assumed to be close to zero. In the treatment of the diffusion theory of adsorption, it is explicitly assumed that the potential energy of protein molecules is always lower at the surface than at either the subsurface or bulk phase.[1] This may not be true for all amphiphilic molecules, and certainly not for all proteins. Proteins that are highly charged, compact, and structurally stable against surface denaturation forces would behave like macroions and experience higher electrochemical potential at the surface than at the subsurface. In the present study, we show that, during initial stages of adsorption, lysozyme molecules at the freshly created air–water interface actually migrate from the interface into the subsurface. The effect of temperature on adsorption indicated that the activation energy for adsorption is equivalent to breakage of about three hydrogen bonds in the protein.

Materials and Methods

Crystallized and lyophilized chicken egg white lysozyme was purchased from Sigma Chemical Co. Na_2CNBH_3 and ultrapure Na_2HPO_4 and $Na_2H_2PO_4$ were purchased from Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]Formaldehyde was purchased from New England Nuclear Co. (Boston, MA). All other reagents used in this study were of reagent grade.

Radiolabeling of Lysozyme. Lysozyme was radiolabeled with [^{14}C] by reductive methylation of the lysyl residues with [^{14}C]formaldehyde as described elsewhere.[10,16] Briefly, 30 μL of [^{14}C]formaldehyde solution (containing 0.01 mmol of formaldehyde having a total radioactivity of 0.1 mCi) was added to 20 mL of 20 mM sodium phosphate buffer containing 20 mg of lysozyme. A 25-mg portion of Na_2CNBH_3 was added to the above solution, and the reaction mixture was incubated at room temperature for 2 h. The methylated sample was dialyzed against pure water for more than 24 h. After dialysis, the ^{14}C-labeled lysozyme solution was distributed into cryovials (Nalgene) and lyophilized. The dried samples were then stored frozen at −70 °C. The protein concentration of lysozyme stock solution was determined using a $E^{1\%}$ value of 26.3 at 281 nm. The specific radioactivity was determined using a scintillation counter.

Adsorption Studies. Extreme care was taken in purifying water for the adsorption studies. Purified water from a Milli-Q ultrapure water system (water resistivity was 18.2 MΩ cm) was further distilled 2 times from a dilute alkaline permanganate solution using an all-glass distillation unit. Teflon bottles (Nalgene) were used to collect and store distilled water. To check water quality, the surface tension of water was measured at 20 °C. If the surface tension of water was not 72.9 ± 0.1 mN m^{-1} and did not remain constant during 24 h of aging, it was discarded.

Ultrapure Na_2HPO_4 and NaH_2PO_4 were used to prepare phosphate buffer, pH 7.0. The ionic strength of the buffer was

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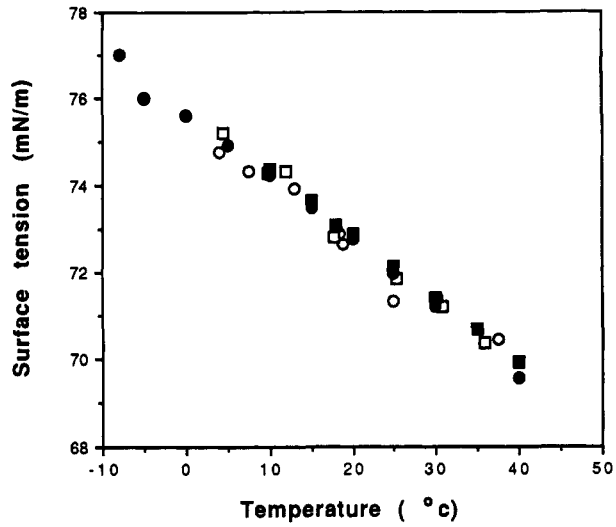

Figure 1. Surface tension of water (or 20 mM phosphate buffer) at various temperatures. The open symbols (○, □) represent data from this study. ● is from Jasper, J. J. *J. Phys. Chem. Ref. Data* **1972**, *1*, 949, and ■ is from *CRC Handbook of Chemistry and Physics*; Chemical Rubber Co.: Boca Raton, FL, 1990; p F-33.

adjusted to 0.1 using NaCl. To ensure that the buffer used in each of the experiments in this study was free of any organic contaminants, the surface tension of the NaCl–sodium phosphate buffer solution was determined before the starting of each experiment. These values are presented in Figure 1 along with the surface tension of pure water at various temperatures reported in the literature. Only the buffer solutions which did not show a decrease in surface tension during aging for at least 12 h was used for adsorption studies.

The rates of change of surface pressure and surface concentration of a protein solution were measured simultaneously using a single experimental setup. The rate of change of surface pressure was monitored by the Wilhelmy plate method[8,9] using a Cahn electrobalance (Cahn Instruments Co., CA). A thin, sand-blasted platinum plate of 1.0 cm width was used as the sensor. The exact width of the sensor was calibrated by measuring the surface tension of Gold Label hexadecane (Aldrich Chemical Co., WI). A Teflon trough of 21 × 5.56 × 1.27 cm interior dimensions was used. The entire assembly was housed in a refrigerated incubator (Ambi-Hi-Lo-Changer, Lab-line). A small plexiglass sliding window (18 × 25 cm) was installed in the door of the incubator to perform operations inside the incubator.

In a typical experiment, an aliquot of the radiolabeled lysozyme stock solution was diluted to the required final concentration with the buffer solution that was preincubated at the experimental temperature for at least overnight. The protein solution (120 mL) was then poured gently into the Teflon trough with the platinum sensor hanging in position. The liquid surface was cleaned by gently sweeping the surface with a clean fine capillary attached to an aspirator until the surface tension of the solution was equal to that of the buffer at the experimental temperature. The protein was then allowed to adsorb from the unstirred bulk phase to the air–water interface. The changes in surface pressure were recorded continuously on a strip-chart recorder.

The rate of change of surface concentration was monitored by measuring the surface radioactivity.[8,9,13] A rectangular gas flow counter with a Mylar window (8 × 4 cm) (Ludlum Instruments, Inc., TX) was set up at the other end of the Teflon trough which was being used for surface pressure measurement. The air space between the Mylar window and the liquid surface was about 7 mm. The carrier gas was 98% argon and 2% propane. The counts per minute were integrated using a rate meter (Model 2200, Ludlum Instruments) and printed out on a strip chart calculator interfaced with the rate meter. The counts per minute measurements were made at 1-min intervals during the initial period and at 10-min intervals at later stages of adsorption. To convert counts per minute into surface concentration in mg m^{-2}, a calibration curve relating counts per minute versus surface concentration was constructed by spreading the ^{14}C-labeled lysozyme on 1 M sodium sulfate solution. In this case, an aliquot (200 μL) of lysozyme stock solution was heated at 75 °C for 30 min and vacuum dried; the residue was dissolved in a spreading solvent made up of $CHCl_3$/methanol/HCl at the ratio of 166:83:1 (v/v/v). To correct for the background radioactivity from the bulk phase, a standard curve relating counts per minute versus specific radioactivty was constructed using $Na_2{}^{14}CO_3$ solutions.

To prevent evaporation and surface cooling during adsorption, the humidity inside the incubator was maintained close to saturation by spreading thick wet paper towels, which were constantly wetted by water in a large beaker. The temperature control inside the incubator was within ±0.5 °C.

Results

The time courses of simultaneous changes in the surface concentration and surface pressure of dilute lysozyme solutions at various temperatures and at two different bulk phase concentrations are shown in Figures 2 and 3. The changes in the surface concentration during the first 60–100 min of adsorption are shown in the inserts of Figures 2 and 3. In these carefully controlled experiments, the adsorption of lysozyme showed some interesting behavior: During the first 60–100 min of adsorption, there was a gradual but significant decrease in surface radioactivity and an increase in surface tension of the lysozyme solutions. After this first phase, the surface concentration remained constant for a brief period of time, followed by a rapid increase. For instance, the surface concentration of lysozyme at $t = 0$ was about 0.3 mg m^{-2}, which decreased to about 0.05–0.1 mg m^{-2} during the first 60–100 min of adsorption. After the lowest point was reached, the surface concentration remained constant typically for a period of about 10–30 min, followed by a rapid increase. In most cases the surface concentration apparently approached a steady-state value after about 1000 min. The apparent steady-state surface concentration was affected by the temperature.

The extent of decrease in surface concentration during the first 60–100 min was apparently dependent on the temperature; the higher the temperature, the greater was the extent of decrease (Figures 2 and 3, insets). After the initial decrease in the surface concentration, positive adsorption of lysozyme at the air–water interface commenced after about 100 min. The time of onset as well as the rate of positive adsorption was affected by the temperature: The higher the temperature, the shorter was the onset time (see the insets in Figures 2 and 3) and higher was the rate of adsorption.

The time course of changes in the surface tension of lysozyme solutions exhibited a behavior similar to that of the changes in surface concentration. There was an initial increase in surface tension concomitant with the decrease in surface concentration. The increase in surface tension ceased approximately at the time of onset of increase in surface concentration and remained constant for more than 1000 min despite increase in surface concentration during that period. The time of onset of increase in surface pressure (i.e., decrease in surface tension) was influenced by the temperature: The higher the temperature, the shorter was the onset time (Figures 2 and 3). However, at all temperatures studied, the onset of increase in surface pressure lagged much behind that of surface concentration. The data suggest that upon adsorption at the air–water interface lysozyme does not immediately affect the force field at the interface; it exerts its influence only when a certain degree of conformation change has taken place at

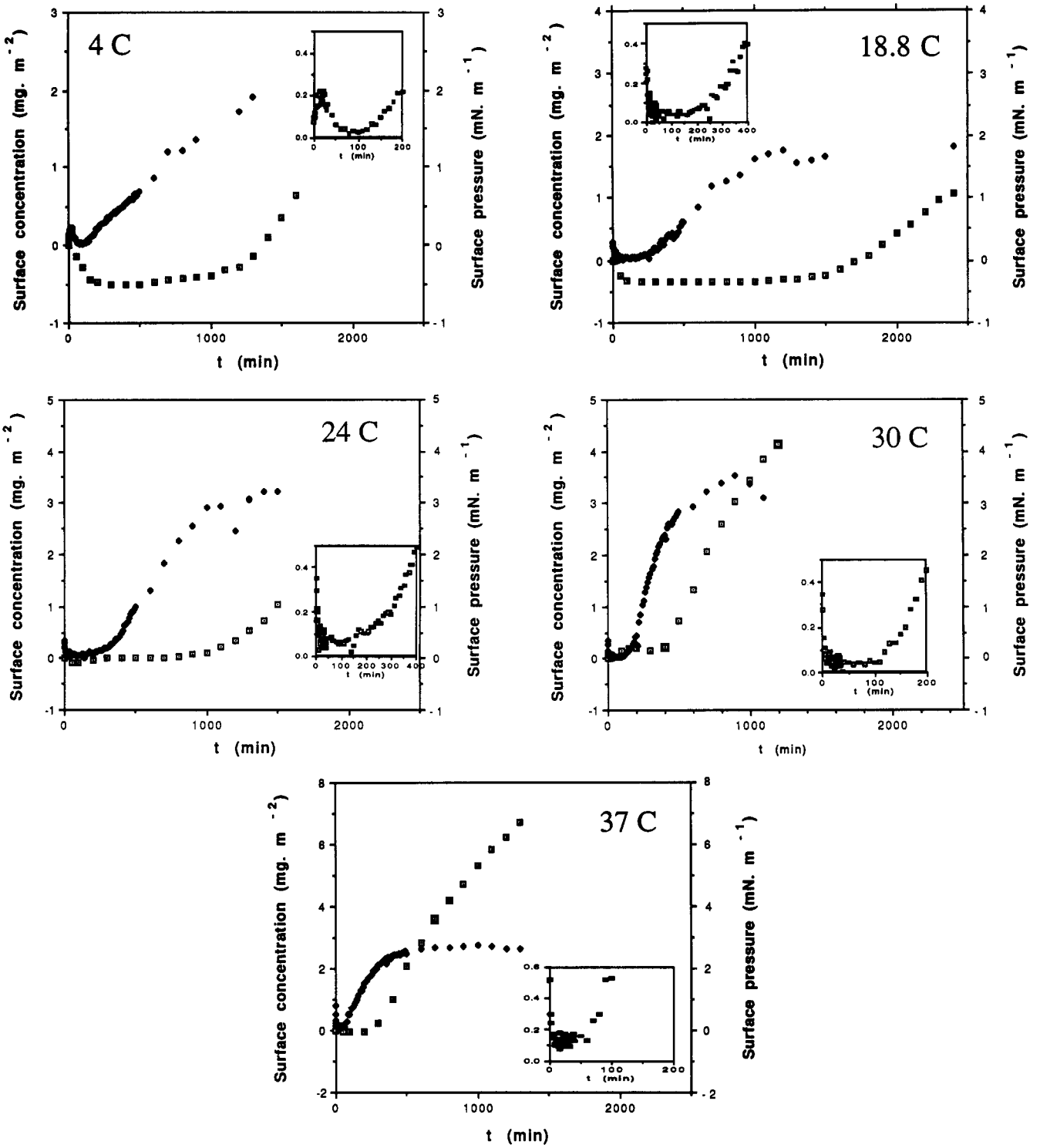

Figure 2. Variation of surface concentration (◆) and surface pressure (□) with time during adsorption of lysozyme at the air–water interface at various temperatures. The bulk phase protein concentration was 0.75×10^{-4}%. The insets show changes in surface concentration during the initial periods of adsorption.

the interface. Similar conclusions have been reached in other studies.[8,17,18]

The net increase in the surface pressure of lysozyme solutions after 1500 min of adsorption ranged from 1 to 7 mN m^{-1} depending upon the temperature and the bulk phase protein concentration. However, at the experimental conditions reported here the surface pressure did not approach a steady-state value even after 24 h of adsorption.

It should be emphasized that the initial increase in surface tension up to about 100 min of adsorption time cannot be attributed to temperature fluctuations, because the solutions used for adsorption studies were preequilibrated at the experimental temperature for at least 12 h. Furthermore, mixing of the protein stock solution with buffer and pouring of the protein solution into the Teflon trough (which was also preincubated at the temperature of the experiment) were all done inside the incubator through the small window in the door. No increase or decrease in the temperature inside the incubator was observed during these operations. Hence, the increase in

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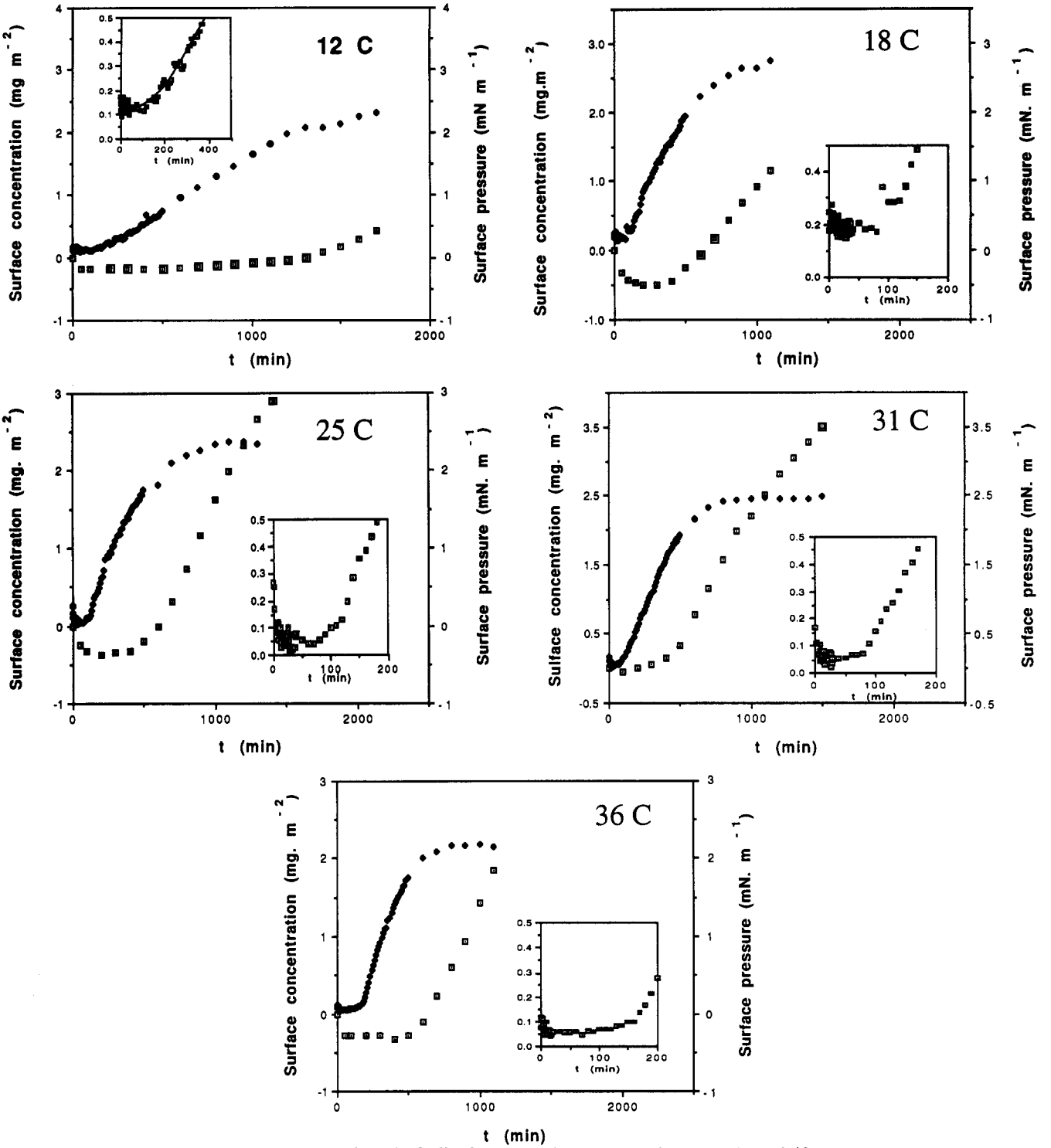

Figure 3. Same as the legend for Figure 1, but the bulk phase protein concentration was 1.5×10^{-4}%.

surface tension during the initial period of adsorption must be related to the decrease in surface concentration during that time.

The rate of arrival of protein molecules at an interface from a dilute bulk phase is considered to be a diffusion controlled process[5-8,11,19] and follows the relationship

$$\Gamma = 2C_0(D/3.1416)^{1/2}t^{1/2} \quad (1)$$

where Γ is the surface concentration, C_0 is the bulk phase protein concentration, D is the diffusion coefficient, and t is time. A plot of Γ versus square-root-of-time would be linear for a diffusion-controlled process. To elucidate the influence of temperature on the rate of adsorption of lysozyme at the air–water interface, the data in Figures 2 and 3 were plotted in the form of Γ–$t^{1/2}$ plots and the *apparent* diffusion coefficients were obtained from the linear portions of the plots. An example of the analysis is shown in Figure 4, and the *apparent* diffusion coefficients are given in Table I. The *apparent* diffusion coefficient increased with increase of temperature. At any given temperature, the *apparent* diffusion coefficient determined from surface adsorption was significantly greater than the calculated solution diffusion coefficient of the protein at the respective temperature (Table I).

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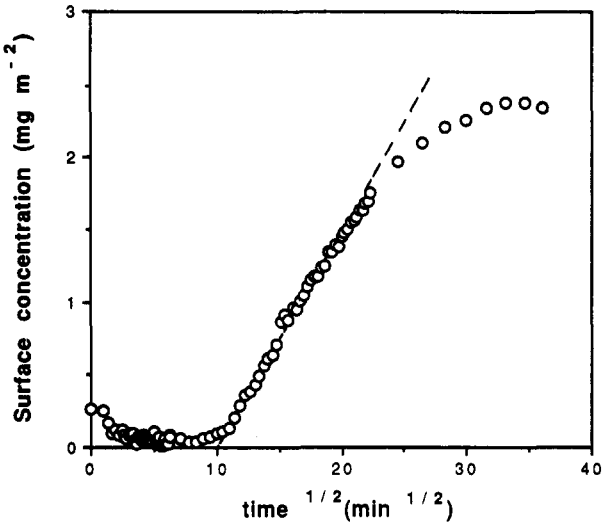

Figure 4. Surface concentration versus square-root-of-time plot for the adsorption of lysozyme at the air–water interface at 18 °C. The bulk phase protein concentration was 1.5×10^{-4}%. The diffusion coefficient was calculated from the slope of the dotted line.

Table I. Apparent Diffusion Coefficient of Lysozyme at Various Temperatures

	diffusion coefficient × 10^6 (cm^2 s^{-1})		
temp (°C)	$C_0 = 0.75 \times 10^{-4}$%	$C_0 = 1.5 \times 10^{-4}$%	in water[a]
4.0	0.714		0.629
4.5		0.669	
10.0		0.591	0.770
12.0		1.934	
18.0		3.57	0.983
18.8	0.795		1.010
20.0	1.945		1.040
24.0	2.611		1.160
25.0	2.723	4.884	1.190
30.0	3.660		1.351
31.0		6.044	1.385
36		8.606	1.558

[a] Calculated using the equation $D_T = D_{20,w}(T/293)(\eta_{20,w}/\eta)$, where $D_{20,w}$ is the diffusion coefficient of lysozyme in water at 20 °C (=1.04 × 10^{-6} cm^2/s), T is the temperature, and $\eta_{20,w}$ and η are viscosities of water at 20 °C and the solvent at temperature T, respectively.[27]

This might be partially attributed to convection, even though the temperature and humidity inside the incubator had been carefully controlled. The Arrhenius plot, i.e., ln D vs $1/T$ plot of the data, is shown in Figure 5. The relationship between the calculated solution diffusion coefficient of lysozyme and temperature is also shown in Figure 5. The apparent activation energy of adsorption, obtained from the slopes of the curves, were 11.3 and 13.9 kcal/mol at $C_0 = 0.75 \times 10^{-4}$ and 1.5×10^{-4}%, respectively, with a mean activation energy of about 12.6 kcal/mol. The calculated energy barrier for solution diffusion alone, calculated from the slope of the dotted line in Figure 5, was 4.8 kcal/mol. This suggests that, in addition to the viscosity barrier for diffusion of the protein molecules, an additional energy barrier of about 8 kcal/mol was involved in the adsorption of lysozyme at the air–water interface.

Discussion

The results presented here suggest that the mechanism of adsorption of proteins in general, and lysozyme in particular, might be more complex than previously assumed. The data indicate for the first time that during the initial stages, lysozyme molecules at the freshly formed air–water interface actually migrate away from the interface and into the subsurface, rather than the molecules at the subsurface instantaneously adsorbing to the interface. Several studies on the adsorption of lysozyme at the air–water interface have been reported.[8,11,13,20] However, in none of these studies have the changes in the surface concentration of lysozyme during the first 60 min of adsorption been carefully studied under controlled temperature and humidity conditions. Graham and Phillips[8] studied the kinetics of adsorption of ^{14}C-labeled lysozyme at the air–water interface. The results of those studies cannot be compared with the results presented here, because the authors had used acetylated lysozyme which might have a conformation different from that of reductively methylated lysozyme; furthermore, in the previous study, the bulk phase was stirred during adsorption. In recent studies carried out by Hunter et al.,[13,20] the time $t = 0$ was not well-defined because the authors did not clean the protein solution surface after injecting the protein stock solution into the bulk phase; no attempt had been made to measure the surface pressure in those experiments to determine the cleanliness of the surface. De Feijter and Benjamins[11] reported that at $C_0 = 10^{-4}$% and at 22 °C, the surface pressure development was noticeable only after 10 h of adsorption, while the surface concentration did not increase for more than 30 min; no data, however, were reported for the first 30 min of adsorption.

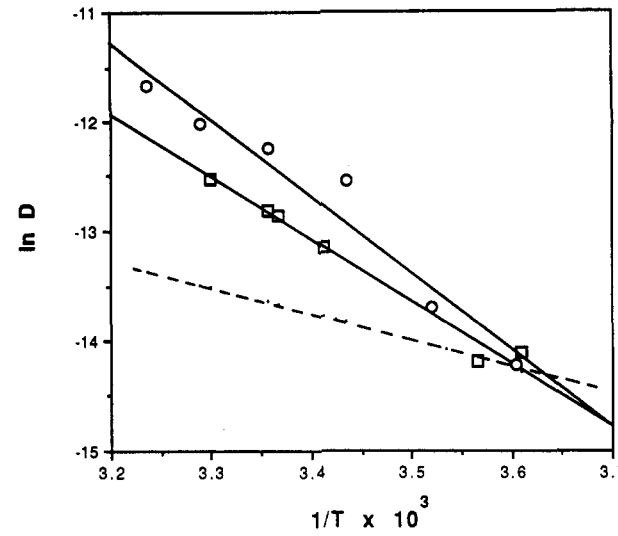

Figure 5. Plot of ln D versus $1/T$ at $C_0 = 0.75 \times 10^{-4}$% (□) and 1.5×10^{-4}% (○). The dotted line represents the Arrhenius plot for the diffusion of lysozyme within solution.

Ward and Tordai[1] first proposed that adsorption of amphiphilic molecules at interfaces is a diffusion-controlled process. The basic assumptions involved in the diffusion-controlled adsorption theory is that when a fresh interface is created, the molecules at the subsurface instantaneously adsorb to the interface. The depletion of concentration at the subsurface creates a concentration gradient between the subsurface and the bulk phase, which allows diffusion of molecules from the bulk phase to the subsurface. However, once the molecules reach the subsurface, they are immediately adsorbed to the interface; during the course of adsorption the subsurface concentration is assumed to be close to zero.

In the treatment of the diffusion theory of adsorption, it is explicitly assumed that the potential energy of protein molecules is always lower at the surface than either at the subsurface or bulk phase. This might not be true for all

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amphiphilic molecules, and certainly not for all proteins. In phenomenological terms, the tendency of a protein to adsorb at an interface should be dependent upon its chemical potential, μ, at a distance ξ from the surface. The driving force for mass transfer either from the surface to the subsurface or from the subsurface to the surface should be fundamentally related to the chemical potential gradient $\delta\mu/\delta\xi$, rather than the concentration gradient. The latter one is not a necessary requirement for mass transfer to the interface.

The chemical potential of an ideal solution is given by

$$\mu_{ideal} = \mu^\circ + RT \ln C \quad (2)$$

where μ° is the chemical potential of the solute in the ideal solution at the standard state and c is the concentration of the solute. In the absence of an external force, mass transfer within a solution is dependent upon concentration gradient, and follows the phenomenalogical linear relationship

$$J_i = -L_i(\delta\mu_{ideal}/\delta\xi)_T \quad (3)$$

where J_i is the flux, ξ is the distance, and L_i is a phenomenological coefficient which is a function of the diffusion coefficient, concentration, and the temperature.[21] The mass transport of proteins from bulk phase to an interface, however, is far from ideal. In this instance the chemical potential that drives the mass transport must include those arising from surface hydrophobicity, surface hydrophilicity, and conformational flexibility/stability of the protein. That is, the chemical potential may be expressed as

$$\mu = \mu_{ideal} + \mu_{conf} + \mu_{H\phi} + \mu_{ele} \quad (4)$$

where μ_{conf} is the contribution from the conformational entropy of the protein, $\mu_{H\phi}$ is the contribution from surface hydrophobic forces of the protein molecule, and μ_{ele} is that from electrostatic forces at the protein surface. The sign and magnitude of μ_{conf}, $\mu_{H\phi}$, and μ_{ele} should be dependent on the location of the molecule from the surface force field: For example, let us assume that e is the net charge of the protein and ϵ_0 and ϵ are the dielectric constants of the aqueous and the gas (air) phases, respectively. The electrostatic theory[22] stipulates that as the protein approaches the air–water interface, an image charge, $e' = e(\epsilon_0 - \epsilon)/(\epsilon_0 + \epsilon)$, would appear in the low dielectric gas phase. If d is the distance of the protein from the air–water interface, the electrostatic repulsive potential at that location from the surface would be

$$\mu_{ele} = ee'/2d\epsilon_0 = (e^2/2d\epsilon_0)\{(\epsilon_0 - \epsilon)/(\epsilon_0 + \epsilon)\} \quad (5)$$

In the initial stages of adsorption, i.e., when the surface is clean, this repulsive potential would act against adsorption of the protein at the air–water interface.[23] Conversely, if protein molecules were present initially at the freshly formed interface, in the absence of any other attractive potential it would tend to desorb away from the interface. Assuming that the net charge of lysozyme at pH 7.0 is about +9,[19] the minimum distance from the surface at which the electrostatic repulsive potential is equal to the thermal energy, kT, of the molecule at 25 °C would be 277 Å. In other words, because of the electrostatic repulsive potential, the molecule would spontaneously desorb to a distance of 277 Å from the surface. It should be emphasized that this is only an approximate estimation based on a simple calculation using the net charge of the protein. It is possible that other hydrophilic and/or hydration repulsion forces also might exist, which might also further influence the desorption distance from the interface.

Proteins contain several apolar amino acids in its primary sequence. Although there is a general propensity for hydrophobic residues to be buried in the interior of the protein, in many globular proteins about 40–50% of the protein surface accessible to solvent is found to be made up of nonpolar patches, distributed uniformly on the surface.[24] Because of the unfavorable association of water with these hydrophobic surfaces, proteins would be attracted to the air–water interface which acts as a potential energy sink for hydrophobic surfaces. Recently, it has been shown that the hydrophobic potential of a nonpolar molecule of radius R (in nanometers) at a distance d from a flat nonpolar surface (e.g., the air–water interface) is[25,26]

$$\mu_{H\phi} = -84R \exp(d/d_0) \text{ kJ/mol} \quad (6)$$

where d_0 is the decay length, 1 nm. If the radius of curvature of nonpolar patches on the protein surface is 2.0 nm (which is the hydrodynamic radius of lysozyme),[27] then the distance from the air–water interface at which the hydrophobic attractive potential is equal to the thermal energy of the molecule at 25 °C would be about 42 Å. Conversely, when the protein molecule in the bulk phase approaches a distance closer than 42 Å from the air–water interface, it would experience an attractive potential from the air–water interface. It should be borne in mind, however, that proteins are not perfectly spherical, and in most cases the radius of curvature of the hydrophobic patches that bind to the flat interface is several orders of magnitude greater than the mean hydrodynamic radius of the protein. Hence, the actual distance from the interface at which $\mu_{H\phi} > kT$ might be orders of magnitude greater than 42 Å.

The conformation of a protein is the product of various intramolecular interactions and interactions with the surrounding medium. Any change in the environment of the protein would cause de facto changes in the protein conformation. The changes in protein conformation not only would change its inherent entropy-dependent chemical potential, μ_{conf}, but also would affect the hydrophobic chemical potential via exposure of buried hydrophobic surfaces to the surrounding solvent.

On the basis of the above arguments, the adsorption behavior of lysozyme at the air–water interface (Figures 2 and 3) may be explained in terms of the chemical potential gradient from the air–water interface. When a fresh air–water interface is created, the concentration of lysozyme at the surface ought to be the same as that in the bulk. The gross chemical potential, however, would be dependent on the sum of μ_{ideal}, μ_{conf}, $\mu_{H\phi}$, and μ_{ele} at a given location from the surface. Since the concentration gradient is zero at the moment of creation of the interface, adsorption/desorption of the protein would depend only on the relative magnitude of contributions from μ_{conf}, $\mu_{H\phi}$, and μ_{ele}: While μ_{conf} and $\mu_{H\phi}$ would be expected to positively contribute to the adsorption, μ_{ele} would negatively contribute to adsorption. It is known that lysozyme is a compact, stable, highly hydrophilic and positively charged protein with very low surface hydrophobicity. It undergoes very slow denaturation and retains much of its

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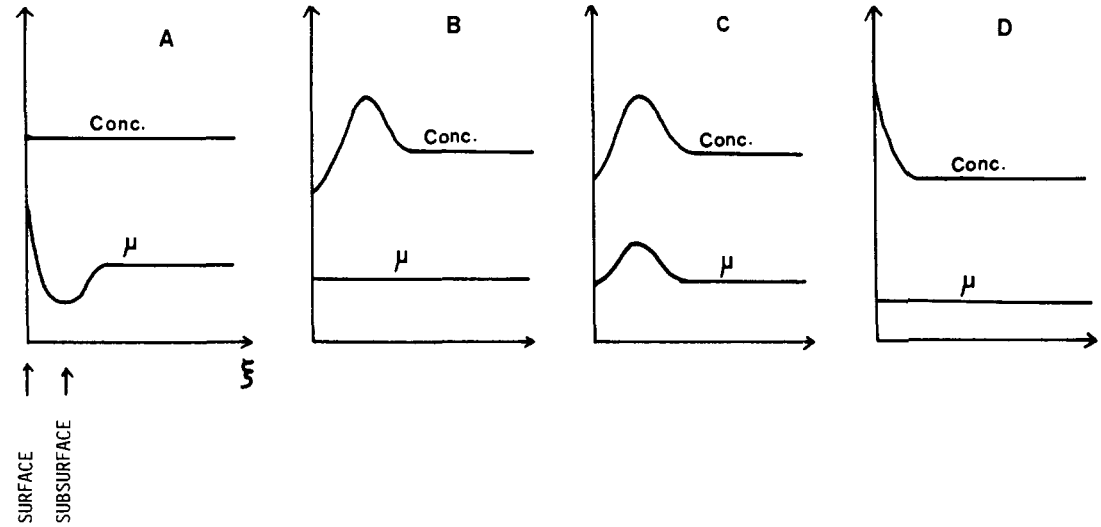

Figure 6. Schematic representation of changes in the chemical potential and concentration at various locations during the course of adsorption of lysozyme at the air–water interface: (A) at the moment of creation of a fresh interface; (B) after the initial desorption of the protein into the subsurface and before commencement of positive adsorption; (C) partial unfolding of the protein at the subsurface and at the moment of commencement of positive adsorption; (D) at equilibrium adsorption.

globular form at the air–water interface.[18] Because of these molecular characteristics, it is logical to speculate that at the moment of creation of a fresh air–water interface, the unfavorable μ_{ele} of lysozyme molecules at the interface would be much greater than the sum of the favorable μ_{conf} and $\mu_{H\phi}$. In contrast, for the molecules in the subsurface region, the μ_{conf}, $\mu_{H\phi}$ would be greater and μ_{ele} would be smaller than the molecules at the interface. However, the net chemical potential of the molecules at the interface would be higher than those at the subsurface. Because of this chemical potential difference between the surface and the subsurface, the molecules at the surface would move toward the subsurface, resulting in depletion of the surface and concomitant development of a concentration gradient. As the molecules move downward, the μ_{ideal} (related to concentration gradient), μ_{conf}, and $\mu_{H\phi}$ would gradually increase and μ_{ele} would decrease. The downward migration would continue up to a distance from the surface at which the sum of the gradients of the chemical potentials μ_{ideal}, μ_{conf}, $\mu_{H\phi}$, and μ_{ele} is zero. If the molecules migrate beyond this region, their chemical potential would increase because of an increase in μ_{ideal} and $\mu_{H\phi}$. This means that, in phenomenalogical terms, there exists a chemical potential valley for proteins between the surface and the bulk phase, as shown in Figure 6A. This also means that in the absence of any other change in the state of the system, there will be an accumulation of molecules in this region with time. This region of the bulk phase where the chemical potential is apparently zero may be referred to as the subsurface.

It should be pointed out that the subsurface region is not a geometrically fixed distance from the surface, but depends on the chemical potential of the solute. If $\mu_{H\phi}$ of a protein is much greater than the sum total of μ_{ideal}, μ_{conf}, and μ_{ele}, then the subsurface region would lie almost at the interface, in which case one would not observe initial desorption of the molecules at the surface. Similarly, if μ_{ele} is far greater than the sum of other potentials, then the subsurface would lie deep in the bulk phase, and one would observe desorption, instead of adsorption during initial stages. The experimental observation of this transient desorption phenomenon is possible only when the relaxation time is longer than the time scale of measurement.

As the protein moves toward the subsurface, the following changes would occur: (1) The macroion–dipole interaction between the protein molecules at the subsurface and the surface water molecules would increase the surface tension (see Figures 2 and 3). (2) The concentration gradient between the surface and the subsurface would increase, resulting in an increase in μ_{ideal}. Accumulation of the protein would continue until the chemical potentials at the surface, subsurface, and the bulk phase are equal, as shown in Figure 6B. At this stage the system would exist in a metastable equilibrium state since any change in any one of the potentials at the subsurface would set up a chemical potential gradient. (3) Since the thermodynamic conditions at the subsurface are different from that of the bulk phase, protein molecules at the subsurface would undergo partial unfolding/denaturation. The unfolding is caused by the reaction of high energy water molecules in the subsurface[15] with hydrogen bonds in lysozyme. The exposure of nonpolar residues to the surrounding aqueous medium would increase the hydrophobic chemical potential of the protein. Consequently, a chemical potential gradient would form between the surface and the subsurface which would act as the driving force for the adsorption of the protein from the subsurface to the surface (Figure 6C,D).

From the foregoing arguments, it can be surmised that the activation energy for the adsorption of the protein from the subsurface to the surface is in fact related to the activation energy for partial unfolding of the protein at the subsurface. The temperature studies on the rate of adsorption indicate that the activation energy for adsorption from the subsurface to the surface is about 12 kcal/mol (Figure 5). This corresponds to the energy required to break about three to four hydrogen bonds in lysozyme. Previously, Ter-Minassian Saraga[15] predicted that the energy barrier for adsorption of lysozyme at the air–water interface would be approximately equal to reaction of about 6–12 high energy water molecules in the subsurface with the hydrogen bonds in lysozyme. This prediction agrees well with the results of this study.

Acknowledgment. Financial support from the National Science Foundation (Grant No. BCS 8913053) is gratefully acknowledged.

Registry No. Lysozyme, 9001-63-2.