한국산학기술학회논문지
Vol. 4, No. 3, pp. 230-235, 2003

수용성 단백질의 계면상 등온곡선의 모델과 실험적 규명

권성현* · 조대철**

Model and Experimental Isotherms of Soluble Proteins at Water Surfaces

Sung Hyun Kwon* and Daechul Cho**

요 약 수용성, 구형의 단백질 분자는 기능성(효소, 유화제, 증화제 등)에 따라 센서나 유사생체기관에 응용성이 크다. 본 연구는 물-공기 계면에서 형성되는 단백질의 표면상태방정식(일명 표면등온선)을 이론적으로 도출하고 그 결과를 실험적으로 확인하여 단백질 분자의 기능적 이용에 활용하고자 한 것이다. 아미노산 부분사슬간, 분자와 물과의 인력, 정전기적 인력을 고려하여 종합적 상태방정식을 도출하였으며 탄소 14로 tagging한 albumin 실험과 비교하여 상당히 일치하는 경향을 확인할 수 있었다.

Key Words : Surface Equation of State, Globular Protein, Lattice Model

1. Introduction

Protein adsorption at fluid-fluid interfaces is important for a proper understanding of its ability to stabilize foams and emulsions in a variety of applications. Adsorption of proteins at the gas-liquid interface leads to a lowering of the surface tension or an increase in the surface pressure. The relationship between the surface pressure and the surface concentration or density is usually referred to as the surface equation of state or surface isotherm. Although this relationship can be determined for some proteins [1], a satisfactory mathematical model did not exist in the literature for handy prediction of protein behavior in solutions because the proteins' properties are not fully understood. Proteins are complex macromolecules formed by the association of a large number of amino acids. In globular proteins, the amino acid residues are folded into spherical shapes. Upon adsorption at an interface these proteins unfold. The degree of unfolding depends on the nature of protein, the interaction between the adsorbed segments and the protein molecule, and the surface concentration. Thus, the behavior of proteins at interfaces is very complicated compared to the behavior of polymers.

Extensive work has been done on the adsorption of flexible polymers at the air-liquid interface. Singer [2] derived an expression for the surface pressure of flexible polymers with all segments adsorbed and no interactions on the basis of statistical thermodynamics. This Singer's model was supplemented by Davies and Lopis, Frisch and Simha [3, 4]. They incorporated segment-segment interactions and formation of loops and trains into the model. The number and size of the loops and trains formed due to the adsorption of a polymer have been shown to depend on the energy configuration of the polymer and are independent of its molecular weight. Though the existing models can fairly describe the surface equation of state of random coiled, flexible proteins such as casein, they are not likely to fit to globular macromolecules such as BSA and lysozyme, model system in this work. The aim of this work is to develop a reasonable surface equation of state describing interfacial behavior of globular type proteins.

*Department of Marine Environmental Engineering, Institute of Marine Industry, Kyeongsang National University, Tongyong 650-160
**Department of Environmental Engineering, Soonchunhyang University, Asan, Chungnam, 336-745, Tel. 041-530-1341, E-mail: daechul@sch.ac.kr

2. Model Development

Globular protein molecule in aqueous solution tend to assume a tertiary structure in which most of the hydrophobic functional groups are buried inside the molecule and the hydrophilic functional groups are exposed to the aqueous medium at the surface since such a conformation is energetically favorable. Adsorption of globular protein at the air-water interface results in partial penetration of the molecule while the rest of the molecule retains more or less its original structure. Upon penetration, the molecule unfolds so that the hydrophobic patches are directed to the aqueous medium. The extent of penetration and subsequent unfolding of the molecule depends on the surface pressure and segment-segment interactions of the molecule. Moreover, the extent of unfolding is hindered by the presence of disulfide bonds within the protein molecule.

Consider a 2-dimensional lattice consisting of N_s lattice sites. Each lattice is occupied by a segment of an adsorbed protein molecule. In order to calculate the configurational entropy, we first need to calculate the number of ways in which the protein molecules themselves can be placed on the lattice, followed by the number of ways of mixing the adsorbed segments belonging to a protein molecule. The protein has n segments and the number of adsorbed molecules are N_2 whereas the number of solvent molecules (water) are N_1. We know that the segments can be placed in many ways within n lattice sites. If z is the coordination number of the lattice, we have the segment placement ways as

$$w_n(z) = \frac{nz\{(z-1)f\}^{n-2}}{2} \tag{1}$$

where f is a flexibility factor.

In case of a completely inflexible protein molecule, the adsorbed segments can be placed only in one way. Then, the problem reduces to that of evaluating the number of ways of placing the molecules on the super lattice. However, the unfolding of the penetrated segments actually requires some flexibility of the segments so the flexibility parameter never goes to zero. The local ordering of amino acids due to the formation of α-helices and β-sheets, therefore, results in the loss of flexibility of the adsorbed segments [5]. Furthermore, the adsorbed segments which belong to the interior of the globular protein experience an additional loss of flexibility because of strong segment-segment interactions and the presence of disulfide bonds. Consequently, the average flexibility of adsorbed segments is likely to be lower if the fraction of adsorbed segments subject to the interior of the molecule is higher. As a result, the flexibility depends on the extent of penetration and therefore on the number of segments adsorbed.

Hence, the number of ways of putting N_2 molecules (adsorbed protein) in the lattice will be

$$\Omega = \frac{\Pi(N_s/n-i)^{w_n(z)}}{N_2!} \quad \text{for } i = 0, N_2-1 \tag{2}$$

From the entropy of mixing of statistical thermodynamics, we need

$$\Delta S = k\ln\Omega$$
$$= kw\sum_{i=0}^{N_2-1}\ln\left(1-\frac{ni}{N_s}\right) + kN_2w\ln\left(\frac{N_s}{n}\right) - kN_2\ln N_2 \tag{3}$$

Also the enthalpy of mixing can be expressed as

$$\Delta H = \chi\theta N_1 = \chi\frac{N_2 n}{N_s}N_1 \tag{4}$$

where χ is the Flory-Huggins parameter and θ is the fraction of the surface occupied by the segments. Constitution of above two factors gives Gibbs Free Energy of Mixing, ΔF_{mix}.

$$\Delta F_{mix} = \Delta H - T\Delta S \tag{5}$$

The free energy of the unit area of electrical double layer ΔF_{el} is given by [6]

$$\Delta F_{el} = \frac{8\mathrm{kTm}}{\kappa}\left\{\cosh\left[\frac{\Psi e\gamma}{2\mathrm{kT}}\right]-1\right\} \tag{6}$$

where κ is Boltzmann's constant, T is the absolute temperature, m is the number concentration of the electrolyte, κ is the Debye length, e is the electron charge, γ is the valence number of the electrolyte, and Ψ is the potential of the protein layer which is assumed to be charged.

Since the total free energy is the sum of mixing and electrical energy, we now have

$$\Delta F_t = \Delta F_{mix} + \Delta F_{el}N_s\sigma_0 \tag{7}$$

where ΔF_{el} considers Gouy-Chapman model of the electrical double layer and σ_0 is the area of a lattice site.

The surface pressure Π is then related to the free energy with

$$\Pi = -\left(\frac{d\Delta F_t}{dA}\right)_{N_2,T} = f(\Gamma) \quad (8)$$

All the equations can be solved numerically (simultaneously and arithmetically) in the environment of Microsoft Excel 5.0.

3. Materials and Methods

BSA and lysozyme were purchased from Sigma Chemical Co. and stored at 4°C. They were used without further purification. 4×10^{-4} mmoles of BSA powder was dissolved in 12.5 ml of 0.05 M phosphate buffer (pH=7.4). Then, 1.25 ml of 0.1 M $NaCNBH_3$ was added to the solution. 10 ml of 2.1×10^{-4} M $H^{14}CHO$ stock solution (102 μCi) was mixed well with the protein solution. The reaction proceeded for 2 h at room temperature [7,8]. After the reaction, the mixture was immediately put in a dialysis membrane (spectraPOR; MW cut-off, 6000-8000) and was dialyzed for 30 h at 4°C. The dialyzed solution was dehydrated using PEG 8000. The final solution was kept in a freezer at -70°C for preservation. A langmuir trough (330×75×6.5 mm^3 from KSV) was used for adsorption experiments (the entire scheme is shown in Figure 1). The trough was equipped with Wilhelmy plate for surface pressure and a gas proportional detector (Ludlum Model 120 with 2×2 mylar window) for radioactivity detection. The radioactivity was measured under P-10 gas environment (55 ml/min) through the detector chamber and at 1450 V.

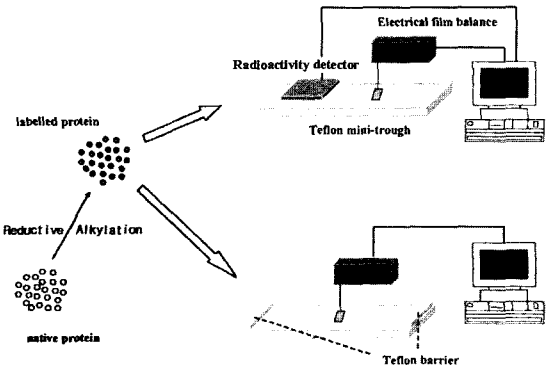

Figure 1. Radiolabelling process and adsorption experiment setup

4. Results and Discussion

As mentioned in the Model Development, the relationship between the number of adsorbed segments n and the surface concentration Γ can be described as

$$\frac{n}{n_0} = f(\Gamma/\Gamma_{max})$$

where n_0 is the total number of segments in the protein molecules and Γ_{max} is the maximum surface concentration at monolayer coverage. This functional relationship can be re-expressed as

$$\frac{n}{n_0} = 1 - a\left(\frac{\Gamma}{\Gamma_{max}}\right)^b$$

where parameter a is related to the average degree of unfolding of the protein molecule. The equation assumes that decreasing values of a manifests protein molecules that unfold more upon adsorption and vice versa. The derived equation of state was fitted to the experimental data for BSA and lysozyme using nonlinear optimization technique. Γ_{max} was determined by experiments monolayer coverage parameter a, b, x and w were summarized in Table 1.

Analysis of the sensitivity of the surface equation of state to the parameter was performed and the

Table 1. Values of model parameters used in the calculation

	BSA	lysozyme
M_w	68,000	14,400
n_0	606	147
w	67.54	24.72
x	0.605	0.695
a	0.709	0.816
b	1.0	-0.05
Γ_{max} (mg/m^2)	2.22	5.0
Π_{max} (mN/m^2)	17	21
σ (m^2)	10×10	17×10

results of this analysis are shown in part in Figures 2 and 3. The effect of parameter *a* on the surface isotherm is shown in Figure 2. The surface pressure for high surface density is found to be very sensitive to the values of *a* (Figure 2). On the other hand, there is negligible variation in the surface pressure for smaller surface densities. As shown in Figure 2, the predictions of Π_{max} decrease as parameter a increases because of the decrease in the degree of unfolding. The effect of parameter *b* on the surface isotherm is shown in Figure 3. The surface pressure is found to be very sensitive to the values of parameter *b* and increases for larger values of *b* over the entire range of surface densities. This may be attributed to the fact that the number of adsorbed segments increases for larger values of *b*. The predicted surface isotherm is not as sensitive to the Flory-Huggins parameter as to the other parameters.

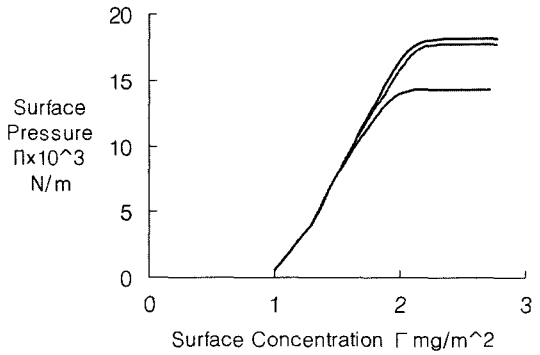

Figure 2. Effect of variation in parameter a, which varies 0.68, 0.70, and 0.85. The other values are from Ref. 1

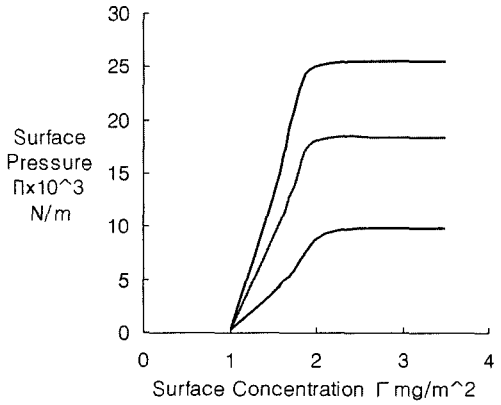

Figure 3. Effect of pH in the surface equation of state. The pH values are 4.0, 5.0, and 6.0 from the top. The other values are from Ref. 1

It is found that parameter *a* for BSA (0.71) is lower than that for lysozyme (0.86; see Table 1). That implies that the average degree of unfolding for lysozyme is lower than that for BSA. Moreover, from the values of parameter *b*, we can say that the number of segments of BSA adsorbed at the air-water interface have a linear relationship with the surface concentration, where as the number of adsorbed segments of lysozyme is more of less independent of the surface concentration. These agree well with the experimental observation that lysozyme is more compact than BSA (Table 1). We also know that the Flory-Huggins values for BSA and lysozyme are 0.605 and 0.695, respectively. The values reveal that the segment-solvent interactions are unfavorable (which means the solvent is poor). This unfavorable interaction results from the exposure of more hydrophobic patches due to the unfolding of the protein molecule.

Also, at monolayer coverage, the neutral adsorbed interfacial protein layer consists of closely-packed islands of segments connected to the part of the protein molecule that extends into the solution. Electrical charging this film leads to a decrease in the cohesiveness of the adsorbed segments because of electrical interactions and consequently to a reduction in the surface density. The effect of charge was negligible at lower surface concentrations but became significant only at near Γ_{max} as observed by Graham and Phillips [1].

4.1. Experimental Π-Γ Relationship and its comparison with the Model

Dynamic Π-Γ data for two species of BSA are compared with the Π-Γ isotherms obtained by spread monolayer using Trurnit's method (Figure 4). The values of Π and Γ during the adsorption of BSA and hydrophobically modified BSA for different bulk concentrations and pH values are shown. The data for c_b=10 ppm deviate much more from the data at the lower bulk concentrations, possibly due to some adsorption additional to monolayer at this higher concentration. For both proteins, the surface pressure during adsorption is found to be smaller than that given by the spread monolayer isotherm

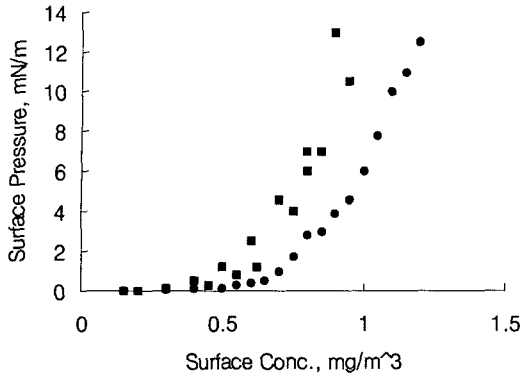

Figure 4. Experimental surface equation of states (or dynamic Π-Γ relationship) for BSA (circle) and hydrophobically modified BSA (rectangle)

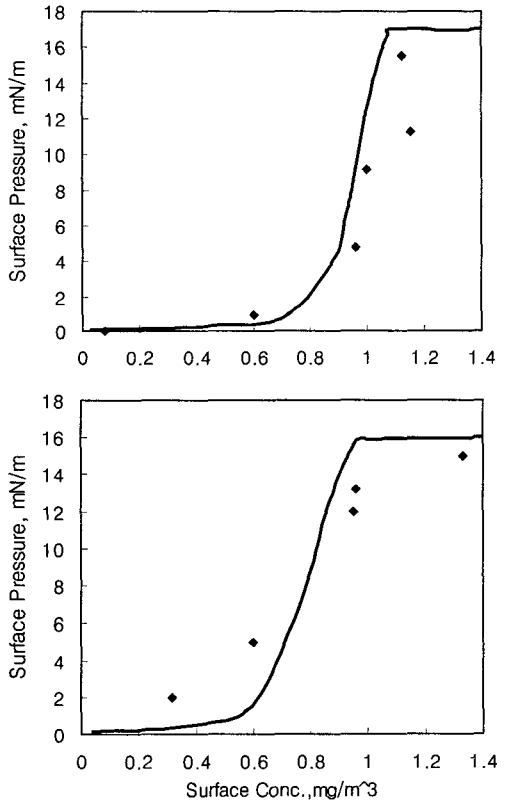

Figure 5. Surface equation of states for BSA in a form of dynamic Π-Γ relationship (top) and in a form of pseudo-equilibrium state (bottom)

for the same surface concentration. In other words, for the same surface pressure, the area occupied by the protein molecule during adsorption is smaller than that for the spread monolayer. Consequently, the protein molecules in the spread monolayer may be more unfolded. Figure 5 shows the comparison of the theoretical isotherm (the surface equation of state) with some experimental data obtained from a radiolabelled protein. It is of interest to compare the spread monolayer Π-Γ isotherm with the plot of the steady-state values of Π and Γ obtained after the protein from the bulk is allowed to adsorb onto the air-water interface for sufficiently long time (20 h or more) (Figure 5). The steady-state Π-Γ isotherm agree fairly well with the isotherm of the spread monolayer. This supports the hypothesis that the adsorbed BSA molecule at the air-water interface unfolds more completely after sufficiently long times.

5. Conclusion

A surface equation of state for globular proteins at air-water interface accounting for the molecular structure, segment-segment, segment-solvent, and electrostatic interactions was proposed and compared to C-14 isotope experiments. This lattice model comprised a simplifying assumption that all adsorbed segments are in the form of trains. The number of segment adsorbed per molecule in case of bovine serum albumin linearly depended on the surface concentration whereas the lysozyme segments adsorbed at the interface were independent of surface concentration. The segment-solvent (water) interaction for both of proteins were found to be unfavorable owing to the proteins unfolding. From comparison of model computation and experimental data, BSA unfolded more than lysozyme because of the larger surface area of contact.

References

[1] Graham, D.E. and Phillips, M.C., "Proteins at liquid interfaces II. Adsorption isotherms," J. Colloid Interf. Sci., 70, pp. 415-426, 1979.

[2] Singer, S.J., "Polymer lattice theory," J. Chem. Phys., 16, pp872-885, 1948.

[3] Davies, J.T. and Lopis, J.L., "Configurations and dimensional analysis of polymer solution," Proc. R. Soc. London A 227, pp537-549, 1955.

[4] Frisch, H.L., and Simha, R., "Polymer and solvent in-

teractions from statistical thermodynamics," J. Chem. Phys., 27, pp702-715, 1957.

[5] Lehninger, A.L., "Biochemistry," p. 151, 2nd Ed., Worth, NY, 1975.

[6] Verwey, E.J.W. and Overbeek, J. th. G., "Theory of the Stability of Lyophobic Colloids," p. 62, Elsevier, NY. 1948.

[7] Jencroft, N. and Dearborn, D.G., "Protein labeling by reductive alkylation," Methods in Enzymol. 91, pp. 570-579, 1983.

[8] Means, G.E., "Reductive alkylation of amino groups," Methods in Enzymol., 91, pp.469-478, 1983.