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APPROACH FOR AN EQUATION OF STATE FOR ADSORBED PROTEIN SURFACES

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SUMMARY

A surface equation of state for polymer interfaces is obtained by equating the chemical potentials of the solvent in the bulk and surface phases. This equation of state contains the fraction of the surface covered with polymer and the surface activity coefficient as parameters. These parameters may be obtained by measuring the thermodynamic modulus of elasticity. Moreover, if the amount of protein in the interface is known the degree of folding in the surface may be evaluated. The present theory was applied to adsorbed β-lactoglobulin in surfaces. The results are in agreement with qualitative equations and results from other sources.

INTRODUCTION

Protein surfaces play a very important role in biological systems, e.g. as the outer core of membranes. In spite of this, little is known about the constitution of such monolayers. It is expected that when a protein molecule is adsorbed at an interface, some of the intramolecular bonds, which hold the molecule in its native configuration, are broken by surface forces yielding a more or less unfolded molecule. This unfolding or unravelling is called surface denaturation. Surface denaturation is a rather complicated process and depends, among other things, on the surface pressure against which the molecule has to expand in order to unfold, and on the "history" (e.g. previous denaturation in the bulk). The unfolding process requires time, so the surface denaturation should be dependent on the rate at which protein is supplied from the bulk to the interface (adsorption rate).

By the unfolding process, reactive groups inside the native molecules become available to form new intermolecular bonds with other protein molecules. In this way a surface coagulum is formed. This surface coagulum can only be formed when the protein molecules become close enough together, so surface coagulation is not expected to be important at low surface pressure. From a given surface pressure range, the surface coverage becomes high enough for surface coagulation to set in. This pressure is called the coagulation pressures. It should be stressed that the coagulation pressure is not sharp, but rather a transition range. From these arguments it is clear that, even

at low surface pressure, the adsorption of proteins, and polymers in general, is an irreversible process. Hence the Gibbs adsorption law does not apply.

It is also clear that the constitution of an adsorbed protein monolayer is entirely different from that of a spread monolayer, obtained under suitable experimental conditions, where all of the amino acid segments are unravelled and lie flat on the surface. In order to gain some insight into the constitution of an adsorbed protein surface, Mitchell et al. [1–3] spread increasing amounts of protein on a constant area. In this way the protein has to unfold against its own surface pressure. Such protein surfaces are called, following Mitchell, π–c layers. This c bears no relation to the concentration of protein in the bulk. In this notation $c \equiv A^{-1}$ (mg/m^2).

From this argument it becomes clear that the following parameters affect the surface pressure: (i) the surface coverage (ii) the surface active coefficients (iii) the degree of unfolding. The latter appears as an additional parameter for polymer surfaces. The scope of this paper is to give a sound method to evaluate these three parameters. The amount of protein adsorbed at the surface must be evaluated from other data or experiments e.g. diffusion from the bulk to the surface, the above mentioned π–c layers of Mitchell, or radiotracer techniques.

GLOSSARY OF SYMBOLS

A: area per mg protein (Eqn 14);
A_o: limiting area per mg protein (Eqn 14);
a: proportionality constant between surface pressure and elasticity (Eqn 9);
c: concentration;
ρ_o: activity coefficient of solvent in bulk (standard state: infinite dilution (Eqn 1);
k: Boltzmann constant;
T: absolute temperature;
t: time;
p: degree of unfolding (Eqn 15);
s: superscript denotes surface phase;
B: superscript denotes bulk phase;
o: subscript denotes the solvent;
ε: modulus of elasticity (Eqn 7);
π: surface pressure (Eqn 2);
γ: surface activity coefficient (standard state infinite dilution) (Eqn 2);
φ_o: volume fraction of solvent in bulk phase (Eqn 1);
θ_o: fraction of surface covered by solvent (Eqn 2);
θ: fraction of surface covered by polymer (Eqn 4);
μ: chemical potential;
ζ: standard chemical potential (standard state infinite dilution);
ω: limiting molecular area of the solvent (Eqn 2);
σ: surface tension;
σ_o: surface tension of the solvent.

THEORY

Since solvent and protein molecules are very different in size, it seems more realistic to replace the mole fractions by volume fractions in the relation for the thermo-

dynamical chemical potential, μ. In this way the chemical potential of the solvent, μ_o^B, in the bulk phase reads [4]:

$$\mu_o^B = \zeta_o^B + kT \ln \rho_o \varphi_o \tag{1}$$

Similarly, the mole fractions should be replaced by the fraction of the surface covered by the solvent, for the chemical potential in the surface. Hence the chemical potential in the surface phase reads:

$$\mu_o^s = \zeta_o^s + kT \ln \gamma_o \theta_o - \sigma\omega \tag{2}$$

Changing the mole fractions to volume or surface fractions is allowable, since Eqns (1) and (2) still contain activity coefficients as adjustable parameters.

Generally the bulk solution is very diluted, whence:

$$\mu_o^B = \zeta_o^B \tag{3}$$

At chemical equilibrium, the chemical potentials in both phases are equal, hence:

$$\zeta_o^B = \zeta_o^s + kT \ln (1-\theta)\gamma_o - \sigma\omega \tag{4}$$

Here, θ, represents the fraction of the surface covered by the protein molecule $\theta_o + \theta = 1$. If our system contains only solvent, $\sigma = \sigma_o$; $\theta = 0$; $\gamma_o = 1$, whence:

$$\zeta_o^B = \zeta_o^s - \sigma_o \omega \tag{5}$$

In this way Eqn (4) results in:

$$\pi = -\frac{kT}{\omega} \ln (1-\theta)\gamma_o \tag{6}$$

where $\pi = \sigma_o - \sigma$.

It should be stressed that we did not assume equilibrium between the protein molecules in both phases, but only equilibrium between the solvent molecules.

Eqn (6) as it stands, contains two yet unknown parameters, θ and γ_o. Experimental measurements of the slope of the $\pi - \theta$ curve should give additional information. This slope may be obtained by measurement of the modulus of elasticity. By definition the modulus of elasticity, ε, is given by:

$$\varepsilon = -\frac{d\pi}{d \ln A} = \frac{d\pi}{d \ln \Gamma} \tag{7}$$

where A is the area of the surface and Γ the amount of protein adsorbed per unit area. In Eqn (7) it is assumed that $d \ln A = -d \ln \Gamma$. This means that the compression rate is fast compared to back diffusion from the surface to the bulk phase. This implies that the protein surface behaves, in a given frequency range, as an insoluble monolayer. This assumption is confirmed by experiments.

In the surface equation of state (Eqn 6) we did not introduce adsorptions, mole fractions, or molecular areas because, for an equal adsorption, the fraction of the surface covered with protein may be very different, depending on the degree of unfolding of the protein. Since the surface coverage is proportional to the adsorption, and it is assumed that, during a compression and expansion cycle, the conformation

of the protein at the interface is not changed, we have:

$$\varepsilon = \frac{d\pi}{d \ln \theta} \tag{8}$$

We have now to obtain an expression for the modulus of elasticity in terms of the surface coverage.

Elimination of θ between Eqns (6) and (8) results in:

$$\varepsilon = \frac{kT}{\omega} \cdot \frac{\gamma_o \exp\left(\frac{\pi\omega}{kT}\right) - 1}{1 + \frac{kT}{\omega} \frac{d \ln \gamma_o}{d\pi}} \tag{9}$$

From experiments we know that, over a considerable range, there is a linear relationship between the modulus and the surface pressure:

$$\varepsilon = a\pi \tag{10}$$

For many protein systems, the constant of proportionality a is close to 8.

Substitution of Eqn (10) in (9) leads to the differential equation:

$$a\pi \left[1 + \frac{kT}{\omega} \frac{d \ln \gamma_o}{d\pi}\right] = \frac{kT}{\omega} \left[\gamma_o \exp\left(\frac{\pi\omega}{kT}\right) - 1\right] \tag{11}$$

The solution of this differential equation is:

$$\theta = \left(\frac{\pi\omega}{kT}\right)^{1/a} \tag{12}$$

and

$$\gamma_o = \frac{1}{1-\theta} \exp\left(-\frac{\pi\omega}{kT}\right) \tag{13}$$

Hence we get the important result that, if we make a choice for the Gibbs-dividing surface, by assignment of a value for ω, we can, at a given surface pressure, calculate the fraction of the surface covered by the protein. Additionally, we can evaluate the surface activity coefficient.

The relative adsorption of a surfactant, Γ_{i1}, with respect to a given component, preferably the solvent (noted by the subscript 1), is given by [5]:

$$\Gamma_{i1} = \Gamma_i - \Gamma_1 \frac{c_i}{c_1}$$

where Γ_{i1}: the relative adsorption of i with respect to water. Γ_i: the adsorption of component i. c_i: concentration of i (moles $\cdot$ cm^{-3}). c_1: concentration of the solvent ($\approx$ 0.055 moles $\cdot$ cm^{-3}). The position of the dividing surface is assigned by the value of ω. For an insoluble monolayer $c_i = 0$ and the relative adsorption is independent of the position of the dividing surface. Even for a soluble monolayer any reasonable

value of Γ_1 would result in practically the same value of the adsorption of the surfactant, provided the concentration of the surfactant is small in comparison with c_1. In the convention used here $\omega = 10\,A^2$ (or $\Gamma_1 = 1.67 \cdot 10^{-9}$ moles $\cdot$ cm^{-2}) is very near the actual cross section of the water molecule.

EXPERIMENTAL

The protein, bovine β-lactoglobulin, was purchased from Koch Light, ($3\times$ crystallized). Solutions of this protein were made in phosphate buffer ($Na_2HPO_4 \cdot 2H_2O$: 3.6434 g/l, and KH_2PO_4: 0.6176 g/l). The protein solutions were poured in a Langmuir trough (area 250 cm^2). After the surface was swept with the barrier, the modulus of elasticity and the surface tension were measured as functions of time using a Wilhelmy plate suspended on an electrobalance. The modulus of elasticity was measured by creating longitudinal waves on the surface by means of a barrier performing a sinusoidal compression and expansion cycle. The resulting surface tension variation was recorded and the elasticity, ε, calculated from the relation:

$$\varepsilon = \frac{\Delta\sigma}{\Delta \ln A} \tag{14}$$

The amplitude of the barrier motion was less than 1 %. At the same time the phase difference between the barrier motion and the surface tension variation was measured.

It was argued that an adsorbed protein surface can be compared to a layer, where increasing amounts of protein were spread on an aqueous surface at constant area. The area was 250 cm^2, protein solution (0.03 %) was spread in steps of 0.01 ml using the method of Trurnit [6]. The frequency was $67 \cdot 10^{-3}$ Hz. At this frequency, the elasticity is within experimental error, depending on the frequency and the fact that the viscous phase angle is small (less than 5 °).

RESULTS AND DISCUSSION

The modulus of elasticity, ε, as well as the surface pressure, π, was measured as a function of time for different concentrations of protein. As is seen from Fig. 1, the modulus of elasticity attains a maximum value. From the two graphs: π–t and ε–t, the modulus of elasticity is plotted as a function of the surface pressure (see Fig. 2). In Fig. 2 the results for the π–c layers are also given (spreading increasing amounts of protein at constant area). From this figure the conclusion may be drawn that the ε–π curve is nearly independent of the protein concentration, and also that the same results for the π–c layers were obtained. Moreover, over a considerable range of surface pressures (till about 8–9 dyne/cm) a linear relationship between surface pressure and elasticity is found. This experimental fact was used in Eqn 10. It has to be proved that the modulus of elasticity has been measured at a frequency range where it becomes independent of frequency (thermodynamical modulus of elasticity). This was done for a π–c layer at a surface pressure of 4.6 dyne/cm. The results are given in Table I, and show that the modulus is indeed, for this range, independent of the frequency. We are now allowed to use these values of ε in our calculation.

One would expect that the modulus of elasticity can be evaluated from the

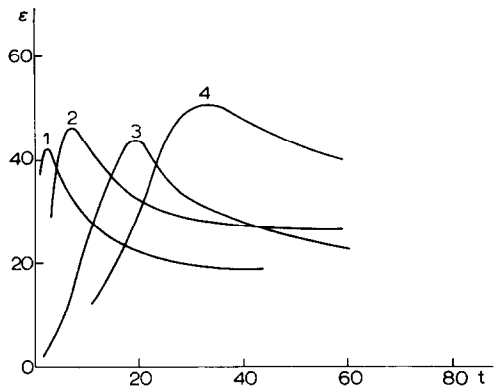

Fig. 1. Surface elasticity, ε as a function of time, t, for various protein concentrations (t in minutes). (1) 20 mg/l, (2) 10 mg/l, (3) 5 mg/l, (4) 3 mg/l.

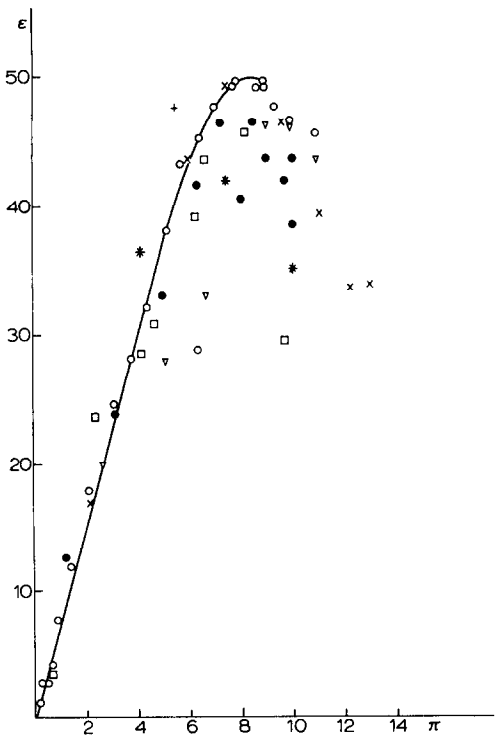

Fig. 2. Surface elasticity, ε, as a function of surface pressure π. ⊙, π-c layer; +, π-c layer after compression; ⊙, π-c layer after expansion; protein concentration (mg/l), ●, 1; ×, 3; ⊡, 5; ▽, 10; *, 20.

TABLE I

DEPENDENCE OF ε ON THE FREQUENCY ν

ν (Hz)	ε (dyne/cm)
$67 \cdot 10^{-3}$	39.2
$33 \cdot 10^{-3}$	40.6
$13.4 \cdot 10^{-3}$	40.6
$6.7 \cdot 10^{-3}$	42.0
$3.3 \cdot 10^{-3}$	42.7
$1.34 \cdot 10^{-3}$	42.0
$0.67 \cdot 10^{-3}$	40.6
$0.49 \cdot 10^{-3}$	40.6

slope of the π–c layer (see Fig. 3). This is not true. At a given surface pressure, we found a modulus of about 40 dyne/cm experimentally whilst the value calculated from the π–c curve only amounts to 7 dyne/cm. This was further investigated in the following way: a π–c layer was obtained by spreading increasing amounts of protein at constant area. When the surface pressure was 5.5 dyne/cm, this π–c layer was compressed, the result is given in Fig. 4. The modulus of elasticity calculated from the slope of this compression curve is 47.5 dyne/cm, a value closer to the experimental one, obtained by a sinusoidal compression–expansion cycle (40 dyne/cm.) In another experiment this π–c layer was expanded, and the elasticity calculated from the slope amounts to 28.8 dyne/cm. This value is lower than that obtained from the compression experiment, but is still higher than the value obtained from the π–c curve (7 dyne/cm). Actually the direct measured modulus lies between these two moduli (experimentally, 40 dyne/cm; compression, 47.5 dyne/cm; expansion, 28.8 dyne/cm; π–c layer slope, 7 dyne/cm). From this, it may be concluded that after a compression–expansion cycle the surface tension has increased, which was indeed found experimentally. This "pumping effect" was also observed by Blank [7]. The difference between these two

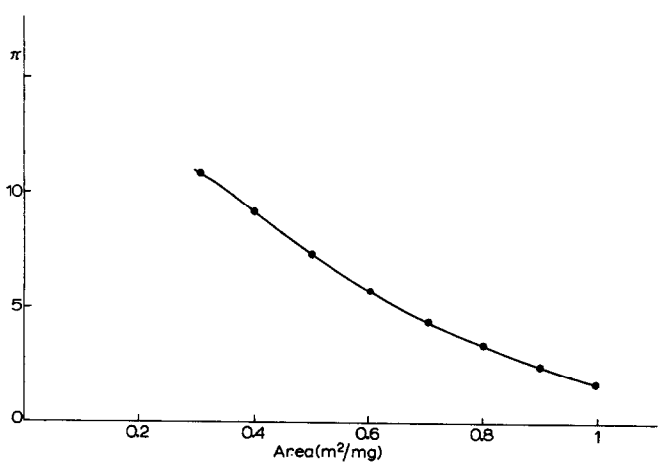

Fig. 3. π–c layer of β-lactoglobulin.

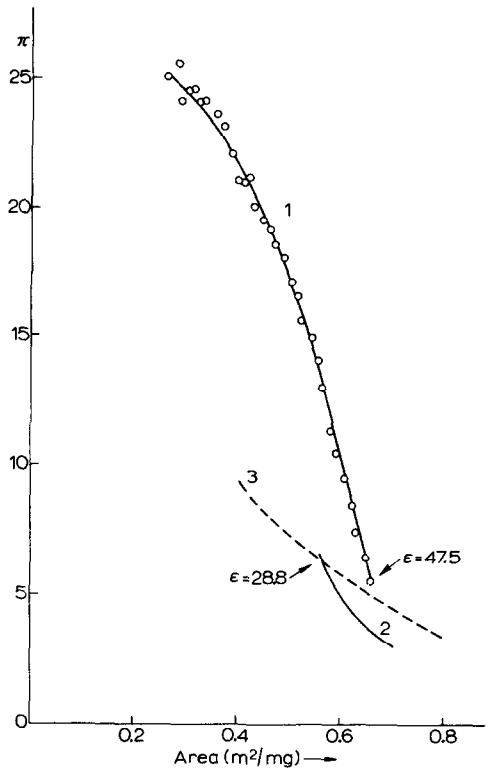

Fig. 4. Compression (1) and expansion (2) of π–c layer. (3) π–c layer of Fig. 3.

moduli (compression and expansion) is believed to be due to some irreversible process. It is noteworthy that the elasticity of a spread unfolded monolayer is comparable to that for an adsorbed monolayer.

At a surface pressure of 8–9 dyne/cm, a maximum is found for the elasticity. It was thought that this is due to a surface coagulation observed by McRitchie and Owens [8]. These authors plotted the surface pressure of a protein solution as a function of the logarithm of time and found a break-point in the plot. This break-point corresponds to a coagulation pressure, above which the protein surface is in coagulated state. We performed the experiment of McRitchie and found a coagulation pressure of 8.1 dyne/cm, a value in close agreement with the pressure at which the elasticity has a maximum value (8–9 dyne/cm). At present we leave out of discussion the state of our β-lactoglobulin surface at pressures exceeding 8 dyne/cm. The present theory seems only to be valid for surface pressures not exceeding the coagulation pressure. For the π–c layer of β-lactoglobulin, where the amount of protein in the surface is known, we are now able to calculate the fraction of the surface covered, θ, using Eqn (12), and the surface activity coefficient γ_o, by means of Eqn (13). The value of ω is still arbitrary but we assumed a value, of 10 A^2, defining in this way the position of the Gibbs dividing surface. The coefficient of proportionality, a, is 7.2. Since, for the π–c layers, we know the amount of protein in the surface, expressed by the area per mg protein, A, we can calculate the limiting area A_o, when the monolayer

TABLE II

CHARACTERISTICS OF π–c LAYER OF β-LACTOGLOBULIN

π	A	θ	A_o	γ_o	p
1	1.20	0.59	0.71	2.39	1.00
2	0.95	0.66	0.63	2.80	0.88
3	0.83	0.69	0.57	3.00	0.80
4	0.74	0.72	0.53	3.23	0.74
5	0.65	0.74	0.48	3.40	0.67
6	0.57	0.76	0.43	3.58	0.60

is closely packed using the relation:

$$\theta = \frac{A_o}{A} \tag{15}$$

The results are given in Table II.

The limiting area, A_o, depends on the surface pressure, but at zero pressure this area amounts to 0.71 m^2/mg. This value is comparable with that found by Mitchell [1–3] for spread and unfolded monolayers. From this data we conclude that at low pressure β-lactoglobulin is completely unfolded. At higher pressures, the monolayer becomes more and more folded. The degree of unfolding, p, at a given pressure may be expressed by:

$$p = \frac{A_o}{0.71} \tag{16}$$

In principle, the same procedure may be followed for a mixed monolayer (e.g. protein–liquid monolayer) to evaluate the surface activity coefficient. This surface activity coefficient yields evidence about interaction at the interface.

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