Surface Activity−Compressibility Relationship of Proteins at the Air−Water Interface

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The surface concentration versus surface pressure relationship of 39 proteins under dynamic adsorption conditions at the air−water interface has been studied. It was found that the slope of these plots, Θ (in ergs/mg), which is defined as *surface activity*, showed a positive correlation ($r = 0.86$) with the adiabatic compressibility of the proteins. The linear regression line passed almost through the origin, suggesting that, theoretically, rigid and inflexible globular proteins would not be able to reduce the surface tension of water even at a saturated monolayer coverage. Some empirical equations were derived for the estimation of the Θ values of unknown proteins on the basis of their amino acid compositions as well as secondary structure contents. The compressibility values predicted from these calculated Θ values agreed with the experimental values. The experimental Θ values of globular proteins also showed a negative linear correlation ($r = 0.77$) with the $\Delta G°$ of thermal unfolding of the proteins. The results of this study indicated that the surface activity of globular proteins is intimately related to their molecular flexibility and their susceptibility to conformational changes at the interface. A direct correlation exists between the thermal stability and the interfacial stability of globular proteins.

Introduction

Proteins exhibit a high propensity to accumulate at phase boundaries. This phenomenon is ubiquitous in several biological and chemical processes. Biological processes such as cell adhesion, blood clotting, and so forth are manifestions of the behavior of proteins at interfaces. A majority of intracellular enzymic processes occur mainly at phase boundaries, that is, at the membrane−water interface of the endoplasmic reticulum, the cell membrane, and other organelles. The expansion and contraction of lungs during respiration involve adsorption of pulmonary surfactant, which is a mixture of surfactant-associated proteins, at the air−water interface.[1−3] In technological applications, proteins are being used as macromolecular surfactants in dispersed food systems, such as foam- and emulsion-type foods. In such systems, apart from acting as a surfactant, proteins form a cohesive viscoelastic film around oil droplets and air cells and stabilize these dispersed systems against flocculation and coalescence via electrostatic and steric repulsive forces. Although all proteins are amphiphilic, they differ widely in their surface activity.[4,5] The process of adsorption and film formation of proteins at interfaces can be viewed as a two-step process, namely, the initial anchoring of the protein at the interface and subsequent conformational change and rearrangement of the adsorbed protein to form a cohesive viscoelastic film. The molecular factors affecting the initial anchoring of proteins to an interface are fairly well understood. Studies on the kinetics of adsorption of proteins at the air−water interface have shown that the probability of initial anchoring of a protein at the interface is dependent on the surface hydrophobicity of the protein.[6,7] However, since about 50% of the solvent-accessible surface area of all globular proteins is comprised of nonpolar surfaces,[8] the surface hydrophobicity-dependency of adsorption cannot be related to the average hydrophobicity of the protein surface but rather to the distribution pattern of the nonpolar residues on the surface.[5] That is, for successful anchoring of a protein at an interface, the nonpolar surface should exist in the form of segregated patches on the protein's surface.

Denaturation studies on proteins have consistently shown that a change in the environment of proteins affects their thermodynamic stability. Since the thermodynamic conditions at phase boundaries are quite different from those in the bulk phase, proteins might undergo conformational changes upon adsorption at an interface. In fact, the occurrence of sulfhydryl−disulfide interchange reaction-induced polymerization of proteins at the air−water and oil−water interfaces has been attributed to partial denaturation of proteins at these interfaces.[9−11] The molecular factors affecting the stability of proteins at interfaces however are not well understood.

In this paper we report that the surface activity, defined as the net reduction in surface tension per milligram of protein adsorbed per square meter of the interface (mN·m/mg), of 39 different proteins at the air−water interface under dynamic adsorption conditions is linearly correlated with the adiabatic compressibility of the proteins. In addition, the dynamic surface activity of proteins is also inversely correlated with the free energy change of thermal

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unfolding of the proteins. The results suggest that the flexibility of proteins plays a dominant role in the surface activity of proteins, and protein denaturation at the asymmetrical force field of the air–water interface is directly related to their thermal stability.

Experimental Section

Materials. Actin, myosin, and tropomyosin were kindly provided by Professor Marion Greaser. Fibrinogen was purchased from United States Biochemical Corp. (Cleveland, OH). Bacteriophage T_4 lysozyme was from Epicenter Technologies (Madison, WI). All other proteins used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure NaH_2PO_4 and Na_2HPO_4 were obtained from Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]Formaldehyde was from New England Nuclear Co. (Boston, MA). All other reagents in this study were of analytical grade.

Extreme care was taken in purifying water for adsorption studies. A Milli-Q ultrapure water purification system (Millipore Corp., Bedford, MA) with a Qpak-1 cartridge package capable of removing inorganic and organic impurities was used to purify water. The resistivity of water was usually 18.2 mΩ cm. 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 and did not remain constant during 24 h of aging, it was discarded.

Radiolabeling. The proteins were radiolabeled with ^{14}C-nuclide by reductive methylation of amino groups with [^{14}C]-formaldehyde at pH 7.5, as described previously.[12,13] Since the methylated amino groups retains their positive charge,[14] the net charge of the methylated proteins remain the same. The specific radioactivity of the labeled proteins ranged from 1 to 3 μCi/mg of protein, which corresponded to incorporation of 1–4 methyl groups/mol of protein. At this low level of methylation the conformation of proteins would not be altered significantly.

Adsorption Studies. The kinetics of adsorption of radiolabeled proteins at the air–water (20 mM phosphate buffered saline solution, pH 7.0, $I = 0.1$) interface was studied by using a surface radiotracer method, as described elsewhere.[12,13] Briefly, the rate of change of protein concentration at the air–water interface of radiolabeled protein solutions (100 mL) in a Teflon trough (21 × 5.56 × 1.27 cm^3) was monitored by measuring the surface radioactivity using a rectangular gas proportional counter (8 × 4 cm^3) (Ludlum Measurements, Inc., Sweetwater, TX). The gas proportional counter probe was placed at about 4 mm from the surface of the liquid in the trough. The entire experimental setup was housed in a refrigerated incubator maintained at 25 ± 0.2 °C. A carrier gas composed of 98.0% argon and 2.0% propane was passed continuously through the gas proportional counter at the rate 20 mL/min. A calibration curve relating cpm versus surface radioactivity (μCi/m^2), constructed by spreading ^{14}C-labeled β-casein on the air–water interface,[12] was used to convert surface cpm versus adsorption time to μCi/m^2 versus time. The surface concentration (mg/m^2) as a function of adsorption time was then calculated by dividing μCi/m^2 by the specific radioactivity (μCi/mg) of the protein. The rationale for using ^{14}C-labeled β-casein to construct the cpm versus surface radioactivity (μCi/m^2) has been discussed in the previous work.[12] The contribution of bulk radioactivity to cpm was corrected using a standard curve relating cpm versus the specific radioactivity of $CH_3{}^{14}COONa$ solutions. The rate of change of surface pressure was monitored by the Wilhelmy plate method using a thin sand-blasted platinum plate (1 cm width) hanging from an electrobalance (Cahn Instruments, Co., Escondido, CA). Both surface concentration and surface pressure were monitored simultaneously in each experiment.

Protein Concentration Determination. The concentration of proteins used in this study was determined by using their extinction coefficients reported in the literature. The concentration of ovoglobulins was determined according to Lowry et al.[15]

Circular Dichroism. The secondary structure contents of most of the proteins used in this study were from published literature. However, for some of the proteins, for which literature data were not readily available, we estimated the secondary structure from circular dichroic (CD) measurements using a computerized spectropolarimeter (On-Line Instrument Systems, Jefferson, GA). The instrument was calibrated with D-(+)-10-camphorsulfonic acid. A cell path length of 1 mm and protein concentration of 0.01–0.02% in 20 mM phosphate buffer was used. Twenty scans of each sample were averaged and corrected for the appropriate buffer baselines. The relative amounts of secondary structures were calculated according to the method of Chang et al.[16]

Statistical Analysis. Multiple regression analyses were performed using the SAS system.

Results and Discussion

To understand the influence of protein structure on surface activity, the kinetics of adsorption of 39 proteins at the air–water interface was studied. The structural characteristics of these proteins are listed in Table 1. Figure 1 shows the time-dependent evolutions of surface pressure (Π) and surface concentration (Γ) of five proteins, viz., fibrinogen, phosphoglycerokinase, myosin, hemoglobin, and ovalbumin, at the air–water interface. The bulk protein concentration in all experiments was 1.5 mg/mL. All these five proteins, which are shown here as representative examples of the 39 proteins studied, commenced their adsorption from the bulk phase to the air–water interface soon after a fresh interface was created. In all cases, the surface concentration seemed to approach an equilibrium value after about 400 min. The rates of adsorption as well as the final surface excesses at equilibrium of these proteins however were different. For instance, the apparent surface excess at equilibrium of fibrinogen, phosphoglycerate kinase, myosin, hemoglobin, and ovalbumin was about 1.75, 1.9, 1.25, 0.8, and 0.85 mg/m^2, respectively.

The most distinguishing feature of the data presented in Figure 1 is the time lag between the commencement of protein adsorption and surface pressure development. For all the five proteins, the onset of surface pressure development lagged well behind that of surface concentration. For instance, in the case of phosphoglycerokinase, although the surface concentration increased soon after the creation of a fresh air–water interface, there was no increase of surface pressure for about 100 min (Figure 1b). The time lag in surface pressure evolution seems to be related to protein structure. For instance, although the $\Gamma - t$ curves of hemoglobin and ovalbumin were very similar, the lag time for surface pressure development was only about 9 min for ovalbumin, whereas it was about 55 min for hemoglobin (Figure 1d). Further, the surface pressure of hemoglobin tended to reach a saturation value when its surface concentration approached a saturation value; whereas, in the case of ovalbumin, the surface pressure continued to increase even after its surface concentration has reached its saturation value. These results tentatively suggest that structural differences among proteins affect their ability to decrease the free energy of the air–water interface.

The surface pressure of a surfactant film at the air–water interface at any given surface excess is a sum of contributions from three forces.[17]

$$\Pi_{a/w} = \Pi_{kin} + \Pi_{ele} + \Pi_{coh} \tag{1}$$

where Π_{kin}, Π_{ele}, and Π_{coh} are the contributions from

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Table 1. Physicochemical Properties of Various Proteins Investigated in This Study

no.	protein	M, $\times 10^3$	secondary structure, %				$E^{\%}_{1\text{cm}}$	H_ϕ, cal/mol	$10^{12}\beta_s$, cm²/dyn	ΔG, J/g
			α-helix	β-sheet	β-turn	aperiodic				
1	actin	41.7					11.5	990	−6.3	0.66
2	α-amylase (bacillus species)	45.2					26.0	970	5.12	
3	alcohol dehydrogenase (bakers yeast)	36.7	29.1	45.0	18.7	7.2	12.6	1020		
4	bovine serum albumin	65.0	60.0	0	0	40.0	6.67	990	10.5	0.46
5	carbonic anhydrase (bovine erythrocytes)	29.2	15.8	44.7	26.3	13.2	17.0	1029	6.37	1.5
6	carboxypeptidase A (bovine pancreas)	34.4	37.0	15.0	26.0	22.0	18.8	1010		
7	α_s-casein (bovine milk)	23.5	15.0	12.0	19.0	54.0	10.5	1054	5.68	
8	β-casein (bovione serum)	24.0	12.0	14.0	17.0	57.0	4.6	1340	3.8	
9	catalase (bovine liver)	15.0					16.8	1029	5.45	
10	α-chymotrypsin (bovine pancreas)	25.7	9.7	50.0	24.5	15.8	20.0	908	4.15	2.1
11	conalbumin (+Fe)	83.2					11.6	995	4.89	
12	cytochrome *C* (bovine heart)	12.0	38.0	0	17.0	45.0	19.0	1049	0.066	2.9
13	deoxyribonuclease I	31.0					12.3	930		
14	egg lysozyme (chiken)	14.1	45.7	19.4	22.5	12.4	26.3	893	4.67	4.0
15	ferredoxin (red marine algae)	6.5	24.0	29.6	22.2	24.2	16.6	900		
16	fibrinogen	50.5	42.0	7	20.2	30.8	15.1	990		
17	globulin						5.4			
18	11S globulin (6)	16.0	8.5	64.5	0	27.0	8.1			1.9
19	hemoglobin (4)	66.0	87.0	0	7	6	17.15	985	10.9	
20	hexokinase (type III, bakers yeast)		40.4	16.2	24.0	19.4	9.2			
21	human lysozyme	15.1					26.3			
22	α-lactalbumin	14.3	18.5	40.5	1.0	40	20.1	1022	8.27	1.55
23	β-lactoglobulin	18.4	8.0	60.0	2.0	30.0	9.4	1080	8.45	1.5
24	lipase	35.0					13.3	980		0.9
25	myosin	200					5.6	880	−18.0	
26	ovalbumin	46.3	49.0	13.0	14.0	24.0	7.5	992	8.78	0.54
27	ovomucoid	20.5	26.0	46.0	10.0	18.0	4.55	807	3.38	
28	papain	23.4	25	19	26	30	21.5	1030		3.7
29	pepsin (pig)	35.5					14.3	929	8.6	1.2
30	pepsinogen (porcine stomach)	43.0					13.05	960		1.6
31	phage T4L	18.6	55.0	7.0	15.0	23.0	13.06			3.1
32	phosphoglycerokinase (bakers yeast)	46.0	29.0	21.7	20.9	28.4	5.0	980		0.4
33	rhodanase (type I)	33.4	37.0	15.0	24.0	17.5				
34	ribonuclease-A	12.6	23.0	37.0	21.0	19.0	6.95	777	1.12	2.0
35	thermolysin (protease type X)	37.5	39.5	31.3	18.7	10.5	17.6	890		
36	tropomyosin	39.0					3.3		−41.0	
37	trypsin (bovine pancreas)	23.0	10.0	50.0	32.0	8.0	15.6	884	0.92	2.6
38	trypsinogen (bovine pancreas)	23.5					13.9	893	1.34	
39	concanavalin A (4) (jack bean type IV)	25.6	2.0	51.0	9.0	38.0	11.4			
40	κ-casein	19.1	23.0	31.0	14.0	32.0	12.2	1060	7.49	
41	α-chymotrypsinogen	25.7	11.02	49.4	21.2	18.38	19.5	908	4.05	
42	insulin	6.0	60.08	14.7	10.8	13.7	10.0	996	9.25	
43	myoglobin	17.0	79.0	0	5.0	16.0		1038	8.98	
44	subtilisin BPN′	27.5	31.0	10.0	22.0	37.0		859	−1.11	

kinetic, electrostatic, and cohesive forces, respectively. Since proteins are large and therefore the kinetic motion of the molecules at interfaces is slow, the contribution of Π_{kin} to surface pressure usually is very low. The contribution of Π_{ele} to surface pressure has been shown to be appreciable even at high ionic strength.[18] However, Evans et al.[19] reported that, at $I = 0.1$, an increase in the net charge of β-casein from 11 to 27 through succinylation increased the surface pressure only by 1.5 mN/m in the surface coverage range 0.5−0.76 mg·m^{-2}. Since the saturated monolayer coverage for most globular proteins is about 1 mg·m^{-2}, and the net charge is typically in the range ±5 to ±25, differences in the Π_{ele} contribution to the total surface pressure of globular proteins should be only marginal. Thus, in general, Π_{coh}, that is, the cohesive interaction between protein molecules at the interface, is the most important factor that causes variations in the surface pressure of protein films.

Against this background, the lag time between the onset of surface concentration and surface pressure developments (as shown in Figure 1), defined as the induction period Δt, might be attributed to two interrelated factors: First, for each protein there may exist a critical surface concentration only above which it can engage in intermolecular cohesive interactions at the interface and cause a greater reduction in surface tension. To determine the critical surface concentration at which the onset of intermolecular interactions occurs under dynamic adsorption conditions, the data in Figure 1 were analyzed by plotting instantaneous Γ against instantaneous Π, as shown in Figure 2. For the proteins shown in Figure 2, the critical surface concentration, Γ_0, at which the surface pressure started to increase was in the range 0.5−1.2 mg·m^{-2}. Below Γ_0, where no measurable surface pressure is detected, the protein molecules at the interface might exist as individual molecules and are probably in a gaseous state. The average distance between the protein molecules might be so great that the potential energy of cohesive interactions between the molecules may be negligible. The Γ_0 and Δt values for the 39 proteins investigated in this study are given in Table 2. For these proteins, the Γ_0 values range from 0.12 mg/m² for ferredoxin to 1.21 mg/m² for phosphoglycerokinase. The differences in Γ_0 were not related to the size of the proteins. For instance, the Γ_0 values of myosin and ovomucoid were the same (Table 2), even though their molecular weights were different at least by 1 order of magnitude (Table 1). In fact, a plot of molecular weight versus Γ_0 of various proteins showed no

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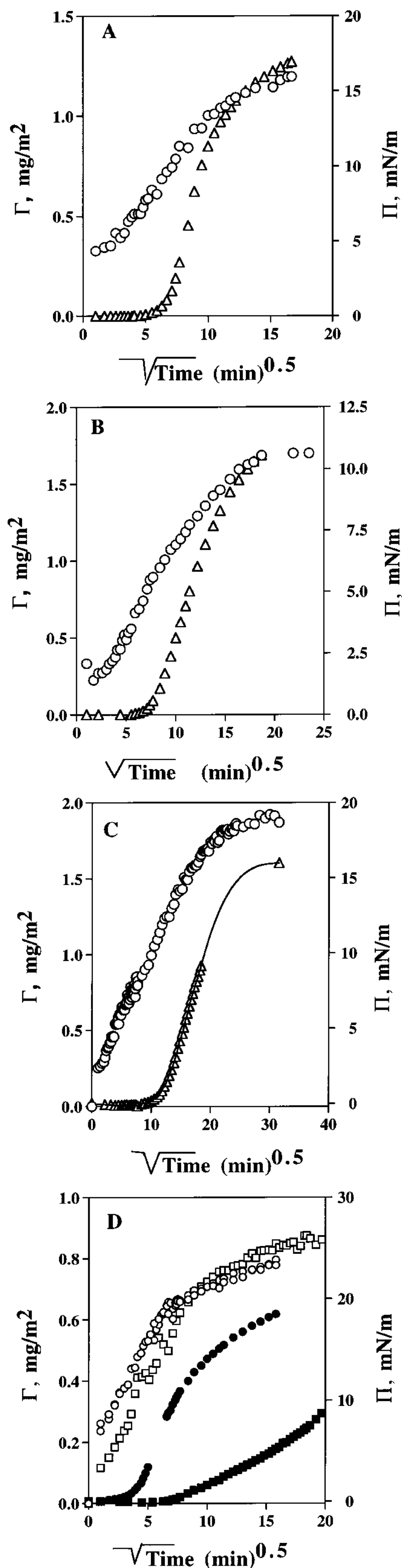

Figure 1. Time-dependent increase of surface concentration (○) and surface pressure (△) during adsorption of fibrinogen (A), phosphoglycerokinase (B), and myosin (C) at the air−water interface. Shown in part D are surface concentration (open symbols) and surface pressure (filled symbols) development of hemoglobin (circles) and hen egg-white ovalbumin (squares). The bulk protein concentration in all cases was 1.5 mg/mL in 10 mM phosphate-buffered saline solution, pH 7.0, $I = 0.1$ at 25 °C.

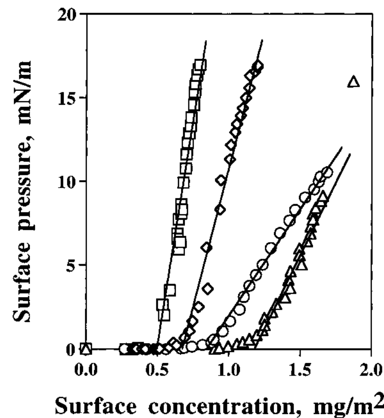

Figure 2. Surface concentration versus surface pressure relationships for fibrinogen (○), phosphoglyerokinase (△), hemoglobin (□), and myosin (◇).

Table 2. Experimental Values of Θ, Γ0, and Δ*t* of Various Proteins at the Air−Water Interface

no.	protein	Θ_{exp}, mN·m/mg	Γ_0, mg/m^2	Δ*t*, min
1	actin	41.3	0.83	55
2	α-amylase[a]			
3	alcohol dehydrogenase	44.2	0.44	351
4	BSA	37.4	0.74	19
5	carbonic anhydrase	18.4	0.60	37
6	carboxypeptidase A	17.1	0.84	277
7	α-casein	16.3	0.53	24
8	β-casein	14.3	0.48	13
9	catalase	25.7	0.64	560
10	α-chymotrypsin	23.9	0.59	23
11	conalbumin	33.1	0.66	42
12	cytochrome *c*	13.3	0.49	292
13	deoxyribonuclease	18.7	0.36	80
14	egg lysozyme	23.0	0.41	400
15	ferredoxin	5.8	0.12	106
16	fibrinogen	13.2	0.91	44
17	globulin	34.0	0.55	35
18	11S globulin	30.3	0.81	93
19	hexokinase	29.4	0.53	144
20	hemoglobin	56.4	0.49	21
21	human lysozyme	29.9	0.55	307
22	α-lactalbumin	38.6	0.47	16
23	β-lctaoglobulin	42.0	0.62	25
24	lipase	29.3	0.55	12
25	myosin	34.9	0.73	45
26	ovalbumin	31.3	0.69	61
27	ovomucoid	24.0	0.73	42
28	papain	8.4	0.89	550
29	pepsin	31.3	0.16	43
30	pepsinogen	25.8	0.56	88
31	phage T4L	28.0	0.47	50
32	phosphoglycerokinase	21.8	1.21	151
33	rhodanase	21.2	0.86	147
34	ribonuclease A	16.0	0.23	990
35	thermolysin	24.4	0.46	152
36	tropomyosin	36.0	0.51	32
37	trypsin	5.9	0.64	90
38	trypsinogen	10.1	0.48	24
39	concanavalin A	35.7	0.73	136

[a] Surface pressure was very low.

correlation. Thus, it appears that Γ_0 is related to the extent of cohesive interactions (i.e., intermolecular hydrogen bonding, electrostatic, hydrophobic, and van der Waals interactions) between the adsorbed protein molecules. The greater the extent of such interactions, the lower would be the critical concentration Γ_0 needed to initiate surface pressure evolution.

Second, the induction period might be related to the time needed for the adsorbed molecules to unfold at the

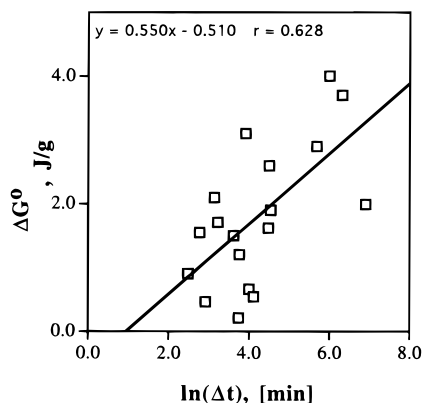

Figure 3. Relationship between ΔG° of thermal unfolding of proteins and the logarithm of induction time for the evolution of surface pressure.

interface[20,21] in order to initiate cohesive interactions. If this is the case, then the induction time Δt should be dependent on protein stability. Figure 3 shows that $\ln(\Delta t)$ is positively correlated with the ΔG° of thermal unfolding of the proteins listed in Table 1. That is, the greater the themal stability, the longer is the induction time. Although the correlation coefficient is only about 0.63, the data do suggest that the induction time is at least partly related to the stability of the proteins.

Taken together, the induction time for the evolution of surface pressure might depend on both the rate of unfolding of the protein at the interface and a minimum surface concentration to initiate cohesive interactions in the surface film. However, the weak correlation between $\ln(\Delta t)$ and ΔG° suggests that, among these two factors, the evolution of surface pressure might be more dependent on a critical minimum surface concentration requirement. This is evident from the data presented in Table 2. For instance, β-casein has a disordered structure and is known to possess no conformational constraints to unfold at interfaces.[22,23] Thus, it is reasonable to assume that as soon as a β-casein molecule adsorbs at the air−water interface, it would assume an equilibrium unfolded state. Yet, its surface pressure evolution starts only after the surface concentration reaches a critical value of 0.48 $mg \cdot m^{-2}$. Thus, at least for β-casein, the induction time Δt therefore must be attributed only to the time required to attain this critical concentration and not to the rate of unfolding at the interface. For other globular proteins with highly ordered structures, the Γ_0 value may depend on the rate of adsorption and the rate of unfolding.

Relationship between Surface Activity and Compressibility. Above Γ_0 the surface pressure increased almost linearly with Γ under dynamic adsorption conditions (Figure 2). This was the case for all the 39 proteins investigated in this study. The slope of the $\Gamma-\Pi$ curve was different for various proteins, indicating that the contribution of Π_{coh} to the surface pressure under dynamic adsorption conditions was protein specific. For example, the slope of the $\Gamma-\Pi$ curve was 13.2, 21.8, 31.3, 34.9, and 56.4 mN·m/mg for fibrinogen, phosphoglycerokinase, ovalbumin, myosin, and hemoglobin, respectively. The slope, Θ (in units of energy per mass), which we define as "*surface activity*" under dynamic adsorption conditions, reflects an instantaneous reduction in the free energy of the *interface* (not the protein) as a result of adsorption of 1 mg of protein under dynamic adsorption conditions. Thus, differences in Θ values may reflect differences in the magnitude of cohesive interactions among protein molecules as a function of protein concentration in the film. The greater the Θ value, the greater are the cohesive interactions in the protein film.

The experimental Θ values of the 39 proteins investigated in this study are given in Table 2. The Θ values ranged from 5.8 mN·m/mg for ferredoxin to 56.4 mN·m/mg for hemoglobin. Intuitively, the Θ values must be related to molecular flexibility of globular proteins. The reasoning for this is as follows: If a globular protein is highly flexible, then its tendency to unfold at the interface also will be high. Upon unfolding, the newly exposed functional groups, such as hydrophobic and hydrogen bonding groups, will promote cohesive interactions between the adsorbed proteins, leading to formation of a cohesive film. Thus, the extent of cohesive interactions in the film and the consequent reduction in interfacial free energy as a function of Γ may be related to the molecular flexibility and therefore to the susceptibility of the protein conformation at the interface.

If the above argument is correct, then one should expect a correlation between experimental Θ values and the molecular flexibility of proteins. Molecular flexibility is not a readily quantifiable parameter. However, because the molecular flexibility of proteins manifests itself in dynamic fluctuations in their volumes,[24] it can be indirectly assessed from the compressibility of proteins. The partial specific adiabatic compressibility, β_s, is defined as

$$\beta_s = (\beta_0/v^{\circ}) \lim_{c\to 0} [(\beta/\beta_0 - V_0)/c] \tag{2}$$

where $V_0 = (d - c)/d_0$, β and β_0 are the adiabatic compressibilities of the solution and the solvent, respectively, d is the density of the solution, d_0 is the density of the solvent, c is the concentration of the protein in grams per milliliter, V_0 is the apparent volume fraction of the solvent in solution, and v° is the partial specific volume of the solute. The values of β and β_0 can be experimentally determined from sound velocity using the relation

$$\beta = 1/du^2 \tag{3}$$

where u is the sound velocity. Knowing the values of β, β_0, and V_0, one can determine the limit term of eq 2 from the intercept of a plot of c versus $(\beta/\beta_0 - V_0)/c$. Gekko and Hasegawa[25] and Gekko[26] reported β_s values for some of the proteins investigated in this study. These values are shown in Table 1. To elucidate if the surface activity Θ is related to molecular flexibility, we analyzed the correlation between Θ and β_s of 18 globular proteins for which experimental β_s values are available. Figure 3 shows the correlation between β_s and Θ. The least-squares linear regression of the plot is

$$\beta_s = (0.223\Theta - 0.308) \times 10^{-12}\ \mathrm{cm^2/dyn} \tag{4}$$

with a correlation coefficient of 0.86. However, the surface activity of fibrous proteins, viz., actin, tropomyosin, and myosin, which exhibit negative compressibility values

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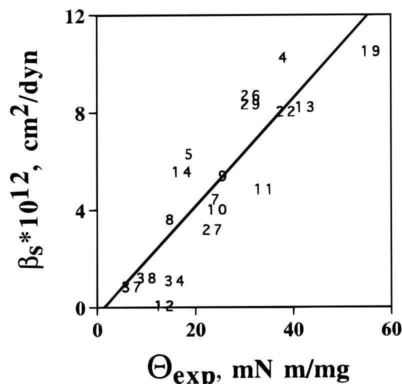

Figure 4. Plots of adiabatic compressibility, β_s, against Θ values of various proteins. The numbers of the data points refer to the identities of proteins in Table 1. The solid line represents the least-squares linear regression.

(Table 1), showed no correlation with β_s. The strong correlation between Θ and β_s of globular proteins clearly supports the proposed hypothesis that the equation of state, that is, the $\Gamma-\Pi$ relationship, of globular proteins under dynamic adsorption conditions is intimately related to their molecular flexibility and thereby their susceptibility to conformational changes at the interface. It should be noted that the regression line in Figure 4 nearly passes through the origin. This implicitly suggests that, theoretically, rigid and inflexible globular proteins would not be able to reduce the surface tension of water even at a saturated monolayer coverage. This further emphasizes the quintessential role of molecular flexibility in surface activity.

Relationship between Amino Acid Composition and Θ. Because volume fluctuations in globular proteins are the result of molecular flexibility,[24] β_s is positively correlated with the partial specific volume of globular proteins.[25] The partial specific volume of a protein can be regarded as the sum of three components, that is,

$$\nu^\circ = V_c + V_{cav} + \Delta V_{sol} \tag{5}$$

where V_c is the sum of the atomic volumes, V_{cav} is the sum of the volumes of void spaces in the interior of the protein, and ΔV_{sol} is the volume change due to hydration. Globular proteins with large ν° generally have large void spaces in the interior. The void spaces in globular proteins arise mainly from imperfect packing of hydrophobic residues in the protein interior. As a corollary, it can be expected that, since the surface to volume (interior) ratio of proteins is related to their hydrophobic content, a fundamental relationship must exist between the amino acid composition and the flexibility of proteins. Since Θ is linearly correlated with flexibility, it may also be dependent on the amino acid composition of proteins. That is, the dynamic surface activity Θ of a protein can be expressed as the sum of contributions from amino acid residues,

$$\Theta_j = \sum n_i \theta_i \tag{6}$$

where n_i is the mole fraction of the ith type amino acid residue and θ_i is the partial specific Θ value of the ith type amino acid residue. The θ_i values of the 20 amino acid residues (unknown variables) were determined from multiple regression of 34 equations representing 34 proteins for which Θ_j values (known variable) have been experimentally determined. The amino acid compositions of the 34 proteins used in this analysis were taken from the Brookhaven protein data bank and other published literature.[27–35] The partial specific θ_i values of the twenty amino acid residues determined from this analysis are given in Table 3. The least-squares multiple regression correlation coefficient of this analysis was 0.983. It is interesting to note that while the θ_i values of a majority of hydrophobic amino acid residues are positive, those of the charged and polar residues (Arg, Asn, Lys, Ser, and Tyr) are negative. This tentatively suggests that whereas the nonpolar amino acids promote cohesive interactions in the protein film at the air–water interface, the polar amino acids seem to interfere with formation of a cohesive film. However, there are several exceptions. Although both isoleucine and valine are hydrophobic, their θ_i values are slightly negative. This might be attributed to branching at the β-carbon atom of these residues, which renders them less flexible and may also introduce steric hindrance for formation of a cohesive film. Similarly, although proline is considered to be hydrophobic, it exhibits a large negative θ_i value. This is unexpected because proline is a helix breaker, and the ability of the Pro–X peptide bonds in proteins to undergo *cis–trans* isomerization is expected to increase the flexibility of the polypeptide chain at the air–water interface. However, it has been shown that, because of lack of rotational freedom of the dihedral angle ϕ of the N–C$_a$ bond of proline residues, polypeptide segments containing proline residues in native proteins tend to be stiff and less flexible.[36] Recently, it has been shown that introduction of proline residues at appropriate places in proteins improved their thermostability.[37–39] This

Table 3. Predicted θ_i Values of Amino Acid Residues

amino acid	θ_i, mN·m/mg
alanine	1.01
arginine	−1.22
aspartic acid	1.31
asparagine	−1.55
cysteine	2.18
glutamic acid	0.63
glutamine	3.14
glycine	1.47
histidine	5.32
isoleucine	−0.08
leucine	2.22
lysine	−2.72
methionine	4.26
phenylalanine	1.23
proline	−2.73
serine	−2.14
threonine	1.04
tryptophane	0.47
tyrosine	−1.43
valine	−0.6

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has been attributed to a decrease in the entropy at the proline sites. In other words, proteins containing a large number of proline residues tend to be less flexible. A case in point is β-casein: Although β-casein contains very few α-helix and β-sheet structures and has a high content of aperiodic structure, it has a low β_s value compared to those of other proteins, owing to its high (17%) proline content (Table 1). The lack of chain flexibility may probably introduce steric hindrance for cohesive interactions between polypeptide segments at the air−water interface. Thus, proteins with a high proline content may not be able to form a cohesive film. This may also explain the relatively low experimental Θ value of β-casein (Table 2).

Among the amino acids, histidine exhibited the highest positive θ_i value, suggesting that proteins with a high histidine content should be able to form a highly cohesive film at the air−water interface. This may be attributed to the highly polarizable two nitrogen atoms of the imidazole ring. These may promote polar dispersion interactions between protein molecules at the air−water interface.

Relationship between Secondary Structure and Θ. Fundamentally, the flexibility of a protein also must be related to its secondary structure content and the spatial organization of the secondary structure elements in the tertiary structure. For example, generally, proteins with a high β-sheet content tend to be more rigid and thermostable than those containing high α-helix structure. Since Θ of proteins is related to flexibility, it is logical to expect that the surface activity Θ of proteins must also be related to their secondary structure contents. Phenomenologically, in terms of secondary structure contribution, Θ_j can be expressed as

$$\Theta_j = f_h\theta_h + f_b\theta_b + f_t\theta_t + f_{rc}\theta_{rc} \quad (7)$$

where f_h, f_b, f_t, and f_{rc} are the fractions of α-helix, β-sheet, β-turn, and aperiodic structure content, respectively, and θ_h, θ_b, θ_t, and θ_{rc} are the partial specific θ values of the α-helix, β-sheet, β-turn, and aperiodic structure contents, respectively. The partial specific θ values (unknown parameters) of the secondary structures were determined from multiple regression of 26 equations representing 26 proteins for which experimental Θ values have been determined, and the secondary structure contents (known variables) are available from the literature.[40−55] The least-squares multiple regression with a correlation coefficient of 0.933 was

$$\Theta = 0.612f_h + 0.504f_b - 0.662f_t + 0.127f_{rc} \quad (8)$$

Equation 8 suggests that, among the secondary structures, the α-helix plays a dominant role in the surface activity of proteins, followed by the β-sheet structure and to a lesser extent by the aperiodic structure. The β-turn acts as an antagonist. This analysis indicates that α-type proteins should be more surface active than β-type and random-coil-type proteins. The generality of this prediction is borne out by the fact that BSA, which is an α-type protein, is in fact more surface active than soybean globulins, which are β-type proteins. The greater positive contribution of the α-helix to the surface activity of the protein may arise from the fact that the α-helix is amphiphilic in character. That is, half of the α-helix surface is predominantly hydrophobic and the other half is hydrophilic.[56] Because of this amphiphilic character, the α-helix can lie flat at the air−water interface with the hydrophobic surface toward the gas phase and the hydrophilic surface toward the aqueous phase. In addition, generally, α-type proteins are thermally more unstable than β-type proteins, and this may also be true of their relative structural stability at the air−water interface. The antagonistic influence of β-turns is reasonable because these involve a region of four consecutive residues folding back on itself by nearly 180° and therefore are considered to be highly inflexible regions with low entropy.[41]

It is interesting to note that, according to eq 8, the positive impact of aperiodic structure on Θ is only marginal. This is contrary to the general notion that, because of high flexibility and lack of intramolecular constraints, a random-coil-type protein would occupy a greater surface area at the interface and thus be more surface active than a highly ordered globular protein.[4,57] However, on the basis of the adsorption behavior of structural intermediates of BSA, Damodaran and Song[58] showed that neither a completely unfolded nor a compact folded protein structure has the ability to occupy a large area at the air−water interface; in order for a protein to occupy a greater area at the air−water interface and exert a greater surface pressure, it should possess a partially folded structure. The results of the multiple regression analysis are in agreement with those findings.

Figure 5 shows the correlation between the $\Theta_{AA,cal}$ values of proteins calculated from their amino acid compositions and the partial specific θ values of amino acid residues (Table 4) and the $\Theta_{St,cal}$ values calculated from their secondary structure contents and the partial specific θ values of secondary structure elements (eq 8). A slope of 0.988 with a correlation coefficient (r) of 0.886 indicates very good agreement between these values. This also indicates that the partial specific θ values of amino acid residues (Table 3) or those of the secondary structural elements (eq 8) can be used to predict the flexibility of proteins using the linear regression equation of the data in Figure 4. We have carried out such an analysis on five proteins for which experimental adiabatic compressibility, β_s, values are available. The results are shown in Table 4. Among the proteins shown in Table 4, the experimental β_s values of α-chymotrypsinogen and myoglobin, which

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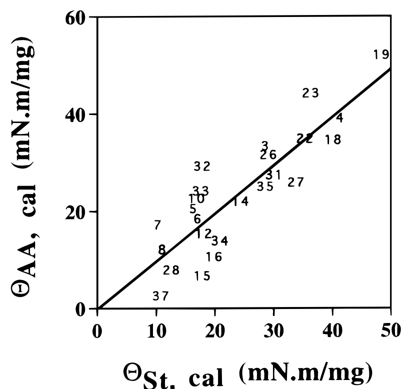

Figure 5. Plots of $\Theta_{st,cal}$ against $\Theta_{AA,cal}$.

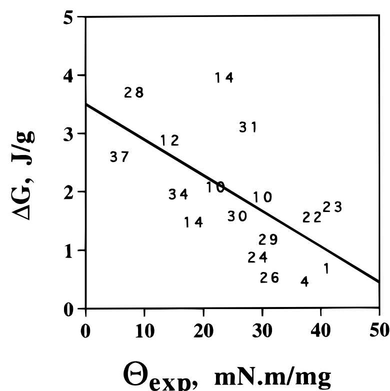

Figure 6. Plots of the free energy change for thermal unfolding ($\Delta G°$) against experimental Θ values of various proteins. The numbers of the data points refer to the identities of proteins in Table 1.

Table 4. Calculated Values of Θ and β_S of Some Proteins

protein	$\Theta_{AA,cal}$, mN·m/mg	$\Theta_{St,cal}$, mN·m/mg	$10^{12}\beta_S$, cm²/dyn	$10^{12}\beta_S$,cal, cm²/dyn
κ-casein	2.8	24.7	7.49	0.3/5.2
α-chymotrypsinogen	20.9	20.1	4.05	4.3/4.2
insulin	80.3	38.5	9.25	17.6/8.3
myoglobin	54.4	46.7	8.98	11.8/10.1
subtilisin BPN′	10.7	14.8	−1.11	2.1/3.0

are truly globular proteins, were very close to the β_s values predicted from their $\Theta_{AA,cal}$ and $\Theta_{St,cal}$ values. On the other hand, the experimental β_s values for κ-casein and insulin agreed very well only with the β_s predicted from $\Theta_{st,cal}$ but not with that predicted from $\Theta_{AA,\ cal}$ values. For example, for insulin, a $\beta_{s,cal}$ value of 8.3×10^{-12} cm²/dyn predicted from $\Theta_{st,cal}$ was the same as the experimentally determined value of 9.25×10^{-12} cm²/dyn, whereas a $\beta_{s,cal}$ value of 17.6 $\times\ 10^{-12}$ cm²/dyn predicted from $\Theta_{AA,cal}$ was not. The probable reason for this might be the fact that both κ-casein and insulin are not typical globular proteins. Insulin contains two short polypeptides connected by two disulfide bonds, whereas κ-casein is a glycoprotein with a unique amphiphilic character. Thus, because of these attributes, the surface activity of these two proteins may hinge more on their structural characteristics rather than on their amino acid composition.

Although subtilisin BPN′ is a globular protein, it has a negative β_s value.[26] However, its β_s values predicted from $\Theta_{AA,cal}$ and $\Theta_{St,cal}$ values were positive but very close to the experimental β_s value.

Correlation between Θ and Structural Stability. The fact that Θ shows a good correlation with β_s suggests that Θ is affected by the protein's ability to unfold at the air–water interface. In thermodynamic terms, unfolding of the protein at the interface, that is, interfacial denaturation, is caused by a change in the thermodynamic environment at the interface compared to that in the bulk phase. When a protein adsorbs at an interface, the change in its environment affects the stabilities of intramolecular hydrogen bonding, hydrophobic, and electrostatic interactions. In this respect, the mechanism of interfacial denaturation of a protein may be similar to its thermal denaturation characteristics. If the contention that Θ is related to the susceptibility of a protein to conformational changes at the interface followed by cohesive interactions between molecules, then one should expect a correlation between Θ and $\Delta G°$ of thermal denaturation of proteins. Figure 6 shows a plot of Θ versus $\Delta G°$ of thermal denaturation for 17 proteins for which $\Delta G°$ values are available in the literature.[59–67] The correlation coefficient for this limited data set is only about 0.63. The correlation coefficient however increases to 0.77 when the data points for hen egg-white lysozyme and phage T_4 lysozyme are omitted. The correlation between $\Delta G°$ and Θ is similar to that between $\Delta G°$ and $\ln(\Delta t)$ (Figure 3), suggesting that both Θ and Δt are affected by the same molecular factors, namely protein stability and cohesive interactions between protein molecules. The data in Figure 6 nevertheless suggest that proteins with low $\Delta G°$ of thermal unfolding tend to be more surface active, providing an indirect evidence that interfacial denaturation of proteins is similar to thermal denaturation. Kato and Yutani[68] reported that the thermal stability of mutants of tryptophan synthase α-subunits influenced their surface tension, foaming, and emulsifying properties. Mutants that were less thermostable were more surface active. This agrees with the correlation shown here.

The results of this study show that the surface activity Θ of proteins, determined from their dynamic equation of state at the air–water interface, is correlated with their compressibility (or flexibility). The partial molar θ values of amino acid residues and secondary structure elements, derived from multiple regression of data sets for 34 and 26 proteins, respectively, can be used to predict the compressibility of proteins from their amino acid composition or from their secondary structure content. It was also found that Θ is negatively correlated with ΔG of thermal unfolding of proteins, providing an evidence that denaturation of proteins at the air–water interface is similar to thermal denaturation in solution.

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