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Thermotropic Behavior of Proteins and Acylated Proteins in Monolayers

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In order to obtain information concerning the effect of proteins on the thermotropic properties of biological membranes, the temperature-dependences of monolayers of different proteins and some acyl derivatives of bovine serum albumin (BSA) have been investigated. The hydrophobic proteins such as hemoglobin, β-lactoglobulin, and β-casein formed dilute monolayers, whereas the hydrophilic proteins such as cytochrome C and lysozyme formed concentrated monolayers. BSA showed an intermediate isotherm between those of dilute and concentrated films. The monolayers of BSA, cytochrome C, and lysozyme were gradually expanded with increasing temperatures. However, the temperature-dependences of the monolayers of hemoglobin, β-lactoglobulin, β-casein, and acyl derivatives were significantly different from those of the monolayers of hydrophilic proteins. In detail, the monolayers of hemoglobin and β-lactoglobulin formed the most compact structures around 20 °C and 30 °C, respectively, while the monolayers of β-casein formed the most expanded structure around 30 °C. In addition, the monolayers of acetyl and propionyl derivatives formed the most compact structures around 30 °C. These results suggest that the hydrophobicity of protein is an important factor relating to thermal stability of proteins in monolayers.

Lipids and proteins are the main constituents of biological membranes. One of the major concerns in membrane biology is to understand how lipids and proteins interact with each other and how such interaction affects the properties of both lipids and proteins in membranes.

Membrane properties are sensitive to environmental influences such as temperature. From this viewpoint, the protozoan *Tetrahymena pyriformis* has been used as the model system for investigating molecular mechanisms involved in cellular acclimation to environmental temperature extremes.[1-3] Furthermore, the effects of lipids on the thermotropic properties of biological membranes have been investigated extensively.[4-11] However, the interpretation of such studies may be complicated by the present lack of detailed information concerning the effect of proteins on the thermotropic properties of biological membranes. One way to gain detailed insight into the molecular aspects of such interactions may be to investigate the thermotropic properties of proteins in membranes.

Because of the strongly amphipathic nature of protein, the change to an extended conformation in which the protein molecule lowers the Gibbs energy of the system by maximizing contact of nonpolar side chains with the nonpolar phase, within steric limitations, is understandable.[12] In biological membranes, integral proteins penetrate the hydrocarbon region (hydrophobic), while peripheral proteins are located adjacent the outer or inner surface (hydrophilic) of the membranes. The integral proteins are arranged in such a manner that their polar groups are either exposed to the aqueous environment or associated electrostatically with the hydrophilic groups of membrane, while nonpolar parts of the protein maintain contact with the inner hydrophobic region.[13] From this viewpoint, Jähnig[14] has analyzed the factors that effect the thermodynamics of the partitioning of proteins between aqueous and membrane phases, and demonstrated that the hydrophobic effect provides the main driving force for insertion into the membrane.

In order to model the physical properties of biological membranes, artificial bilayers such as vesicle, planar bilayers such as black lipid membrane, and monolayers are often used. Although the monolayer does not completely mimic the specific environments of cell membrane surfaces, the study of spread monolayers offers useful insights into the surface conformational behavior of proteins.[15] Another advantage of using a monolayer technique is that the orientation of the protein molecules at the interface is understood precisely and the area occupied by each molecule can be determined over wide limits by careful compression of the film.

With this in mind, the author started the current work to estimate the thermotropic behavior of different native proteins in monolayers. Although the proteins used were not membrane bound but water soluble, they were chosen because of their well-known amino acid sequences and three dimensional structures. On the other hand, the hydrophobicity of proteins can be converted by the technique of selective chemical modification. For example, acylation of lysine side chains in proteins is not only the modification leading to loss of charge, but the reaction leading to significant increase of hydrophobicity. Therefore some acyl derivatives of protein have been used as a model for investigating the hydrophobic effect.[16] The present work also intends to investigate the contributions of hydrophobic effects of proteins in membranes through measurements of the temperature-dependence of monolayers of some acylated proteins.

Experimental

Chemicals. All chemicals were of the highest purity available, used without further purification. Water was distilled once, passed through a mixed ion bed exchanger, and redistilled.

Proteins. The following proteins of high purity were used in this study as obtained from the suppliers: Bovine serum albumin (BSA), bovine hemoglobin (2×crystallized), horse cytochrome C (Type III), β-casein, and β-lactoglobulin (contain variant A and B) were from Sigma Chemical Co., lysozyme (6×crystallized) was a product of Seikagaku Kogyo Co. (Japan).

Acylation of the Proteins. For this study the acetyl, propionyl, butyryl, and palmitoyl derivatives of BSA were prepared by the method of Fraenkel-Conrat.[17] Satisfactory compounds were obtained by reacting with appropriate anhydride in 50% saturated sodium acetate solution at 0 °C. Acetylation required 1 h, whereas propionylation and butyrylation required 2 h. The palmitoyl derivative was prepared by reacting BSA in 50% saturated sodium acetate solution with palmitic anhydride dissolved in ethanol, at 50 °C.[18] Palmitoylation required 10 min. The reaction mixtures were exhaustively dialyzed against water to remove excess reagent. The palmitoyl derivative was then washed with diethyl ether to remove unreactive palmitic anhydride. These derivatives were isolated by lyophilization.

Extent of Acylation. The extent of acylation of BSA was measured by the loss of ninhydrin-positive color in the derivatives compared to BSA.[19]

Apparatus. A Kyowa's surface pressure apparatus (Kyowa Surface Science Co.) was used. The film balance is fully automated and provide with continuous recording of surface pressure versus film area. The inside dimensions of the Teflon-coated trough were 70×14×0.5 cm.

Spreading Solvents. A 50% 1-propanol containing 0.5 mol dm^{-3} sodium acetate was employed as a spreading solvent for BSA, hemoglobin, β-casein, cytochrome C, and lysozyme. Acidic chloroform–methanol (2:1 v:v with 1 cm^3 concd HCl/250 cm^3 solvent)[20] was used for β-lactoglobulin, acetyl-BSA, and propionyl-BSA. A 50% 1-propanol containing 0.05 mol·dm^{-3} sodium carbonate was used for butyryl-BSA and palmitoyl-BSA. These solutions were stored in the refrigerator and used after about 24 h. Comparison of spreading solvents was made by spreading BSA from a 50% 1-propanol containing 0.5 mol·dm^{-3} sodium acetate. No difference was found in the surface pressure–area isotherm.

Surface Pressure. Surface pessure was measured by the Wilhelmy method, using a roughened glass plate. The films were compressed by the moveable Teflon barrier. In the experiments reported below, no buffer was used, in order to provide a very simple subphase. The water was poured into the trough to a height of 1—2 mm above the rim. The surface of the substrate was cleaned by moving the Teflon barrier from one side of the trough to the other. The cleaning of the surface was repeated until the surface pressure of the substrate did not exceed 0.2 mN·m^{-1}. Constant temperature were controlled by running water in the trough surrounded by a water jacket and regulated by a Cool Mate (TE-105 M, Toyo Seisakusho Co.) circulation bath. The temperature was monitored below and as close as possible to the surface of the water phase. The monolayers were made on the surface by using a microsyringe (Hamilton Co.). For the surface pressure–area (π–A) isotherms, exactly 0.05 mg of protein was spread from a 0.05% solution onto an area of 897 cm^2. Monolayer spreading was made by the direct application of numerous small drops (≈0.0006 cm^3) of spreading solution to the surface of the substrate. A time interval of 30 min was allowed for the equilibration of the monolayer before it was compressed. The π–A isotherms for proteins were made by using a compression velocity of 0.0567 $m^2 \cdot mg^{-1} \cdot min^{-1}$. Each part of the experiment was replicated at least three times and then averaged.

Results

Unlike the common substances studied as insoluble monolayers, quantitative spreading of water soluble proteins requires that none is able to dissolve into the bulk solution and diffuse away. For this purpose, two techniques are developed.[21] One is achieved by allowing a very thin layer of the solution to flow down a glass rod before reaching the water surface, this was just introduced by Trurnit.[22] Another satisfactory quantitative spreading technique is also achieved by the direct application of small drops of protein solution which is prepared with 50% 1-propanol containing 0.5 mol·dm^{-3} sodium acetate, as introduced by Ställberg and Teorell.[23] A comparison of the two spreading techniques was made using lysozyme. No difference was found between the two techniques, and the latter was used in this study.

Characteristics of Protein Monolayers at an Air-Water Interface. The wide variety of surface behavior exhibited by proteins at the air–water interface is illustrated by the properties of β-casein, hemoglobin, β-lactoglobulin, BSA, cytochrome C, and lysozyme. Figure 1 shows the π–A isotherms obtained at 25 °C for monolayers of the six proteins spread on water. β-

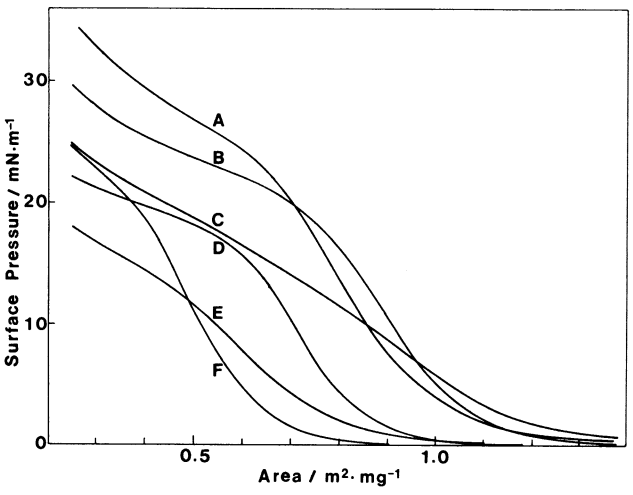

Fig. 1. π–A isotherms for monolayers of different proteins spread on water at 25 °C. A) β-Lactoglobulin, B) hemoglobin, C) β-casein, D) BSA, E) cytochrome C, F) lysozyme.

Table 1. Some Structural Parameters, Ratio of Polar to Apolar Amino Acid Residues, and Hydrophobicity Parameters for Various Proteins

Protein	Polar-apolar ratio	Hydrophobicity parameter, $H\phi_{ave}$ /kJ·res.$^{-1}$	Helix content (%)	SS bond content per molecule	Molecular weight
β-Casein	0.86	5.57	10	0	23000
Hemoglobin	1.09	4.65	75	0	64500
β-Lactoglobulin	1.28	4.98	55	2	18400
BSA	1.63	4.35	55	17	66300
Cytochrome C	2.04	4.23	45	0	11700
Lysozyme	2.26	4.06	35	4	14300

Casein, hemoglobin, and β-lactoglobulin formed dilute monolayers, whereas cytochrome C and lysozyme formed concentrated films. BSA showed an isotherm intermediate between those of the dilute and concentrated films. Dilute protein monolayers are supposed to be completely unfolded molecules, whereas concentrated films are considered to contain incompletely unfolded molecules.[24]

Some structural parameters of these proteins are summarized in Table 1, together with the ratio of polar to apolar amino acid residues and the hydrophobicity parameter. The values of the ratio of polar to apolar amino acids were estimated by Hatch's theory.[25] Proteins with a large proportion of apolar residues exhibit the lower values of the ratio of polar to apolar amino acids (below 1.5). The hydrophobicity parameter, the average hydrophobicity ($H\phi_{ave}$), is based on Tanford's Gibbs energies to transfer the amino acid side chains from an organic environment to an aqueous environment.[26] This was calculated by Bigelow's theory.[27] Proteins with high hydrophobicity exhibit the higher values of $H\phi_{ave}$ (over 4.6 kJ·res.$^{-1}$). It is clear from Fig. 1 and Table 1 that proteins such as β-casein, hemoglobin, and β-lactoglobulin, with either a high content of apolar residues or high hydrophobicity, unfold more easily than proteins such as cytochrome C and lysozyme, with either a low content of apolar amino acids or low hydrophobicity.

Effect of Temperature on Various Protein Monolayers at an Air-Water Interface. The π-A isotherms for various protein monolayers over a range of temperature 10—40 °C are shown in Figs. 2—7. Figures 2—4 show the isotherms for the monolayers of BSA, cytochrome C, and lysozyme spread on water at different temperatures, respectively. These monolayers gradually expanded with increasing temperature. Figures 5—7 show the isotherms for the monolayers of hemoglobin, β-lactoglobulin, and β-casein spread on water at different temperatures, respectively. The temperature-dependences of the monolayers of these proteins were considerably smaller than those of the monolayers of hydrophilic proteins such as BSA, cytochrome C, and lysozyme over the whole temperature range measured. Figure 8 shows the plots of area as a function of temperatures for various protein monolayers at (a)

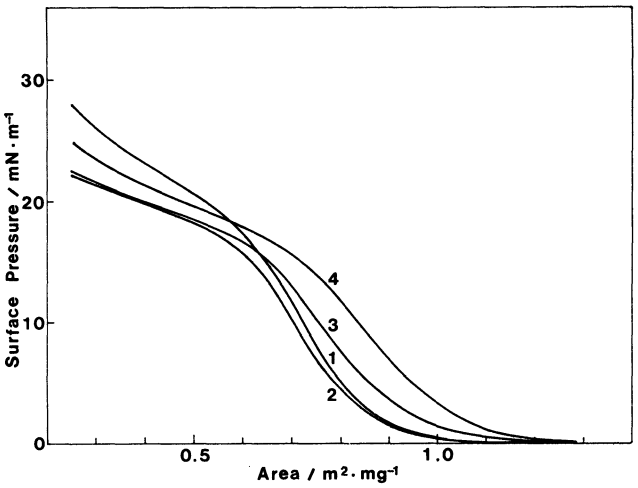

Fig. 2. π-A isotherms for BSA monolayer at different temperatures. Isotherm temperatures are numbered with increasing temperatures: 1) 12.9 °C, 2) 23.1 °C, 3) 33.5 °C, 4) 43.0 °C.

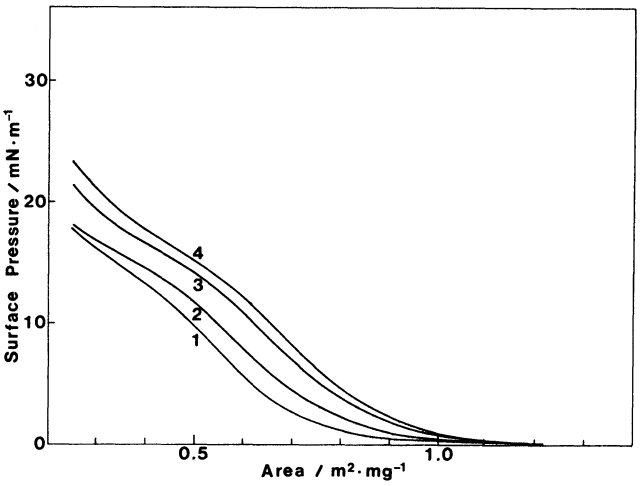

Fig. 3. π-A isotherms for cytochrome C monolayer at different temperatures. 1) 11.9 °C, 2) 24.1 °C, 3) 32.8 °C, 4) 41.0 °C.

π=5 mN·m^{-1} and (b) π=15 mN·m^{-1}, derived from Figs. 2—7. The result indicates that BSA, cytochrome C, and lysozyme at the interface exhibit the extended conformations with increasing temperatures, while

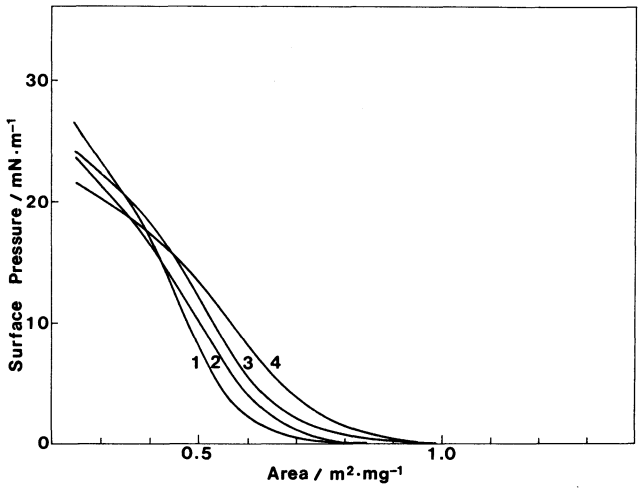

Fig. 4. π-A isotherms for lysozyme monolayer at different temperatures. 1) 10.6°C, 2) 15.4°C, 3) 25.1°C, 4) 34.5°C.

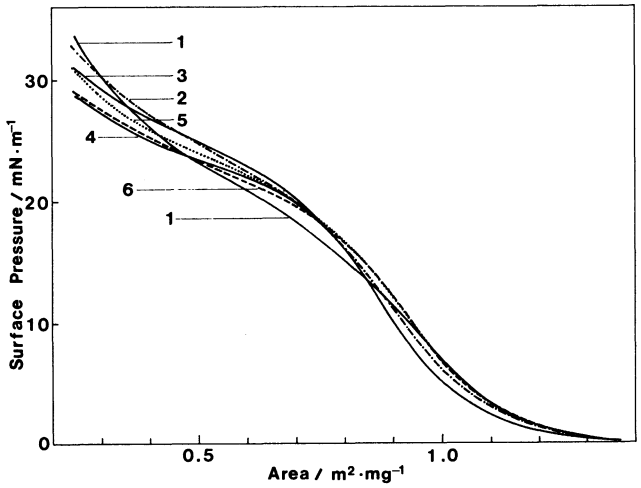

Fig. 5. π-A isotherms for hemoglobin monolayer at different temperatures. 1) 7.2°C, 2) 11.2°C, 3) 19.8°C, 4) 24.4°C, 5) 32.7°C, 6) 41.8°C.

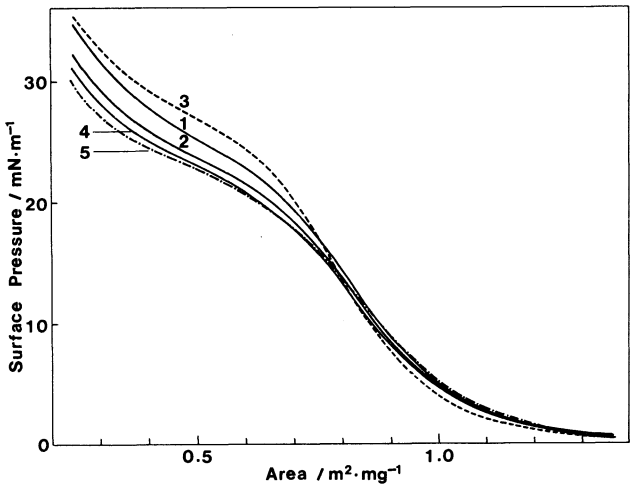

Fig. 6. π-A isotherms for β-lactoglobulin monolayer at different temperatures. 1) 11.6°C, 2) 16.3°C, 3) 26.7°C, 4) 39.8°C, 5) 45.9°C.

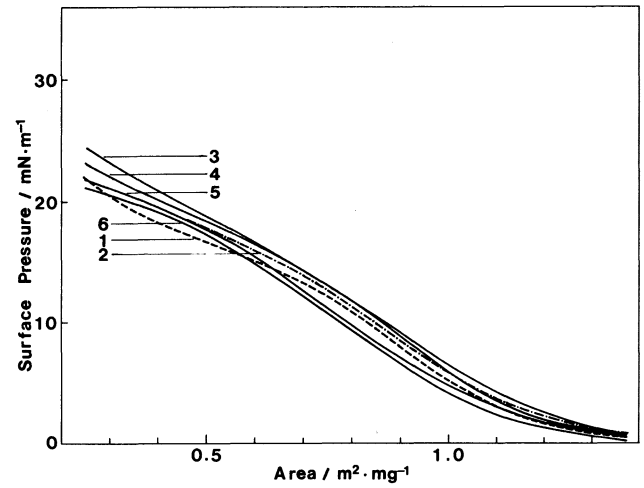

Fig. 7. π-A isotherms for β-casein monolayer at different temperatures. 1) 9.5°C, 2) 16.6°C, 3) 24.5°C, 4) 29.4°C, 5) 36.8°C, 6) 39.5°C.

hemoglobin and β-lactoglobulin at the interface exhibit the most compact conformations around 20 °C and 30 °C, respectively, and β-casein exhibits the most extended conformation around 30 °C.

Characteristics of Monolayers of BSA and Its Derivatives at an Air-Water Interface. On acylation the amino groups involved were principally the ε-NH_2 of the lysines and the terminal amino group of the protein.[16] Tanford[26] has shown that the Gibbs energy change to be expected when methylene or methyl groups are transferred from organic solvent to water is +3.06 kJ·mol^{-1} at 25 °C. By using this value, acylated lysines, i.e., acetyl, propionyl, butyryl, and palmitoyl lysines were assigned hydrophobicities of 15.3, 18.4, 21.4, and 64.1 kJ·mol^{-1}, respectively. $H\phi_{ave}$ is the total hydrophobicity divided by the number of residues.[27] $H\phi_{ave}$ of acyl derivatives of BSA in this way could be calculated. Table 2 shows the extent of substitution of amino acid residues and $H\phi_{ave}$ of BSA and its derivatives.

Figure 9 shows the isotherms obtained at 25 °C for monolayers of BSA and its derivatives spread on water. An interesting feature of these isotherms is the expansion as the length of alkyl side chain on the modifying acyl group was increased up to n=2 (propionyl) but the condensation as it was further increased up to n=15 (palmitoyl), compared to the isotherm of BSA.

Effect of Temperature on Monolayers of Acyl Derivatives of BSA at an Air-Water Interface. The π-A isotherms for the monolayers of acyl derivatives of BSA over a range of temperature 10—40 °C are shown

Table 2. Degree of Substitution of Amino Acid Residues and Hydrophobicity Parameters for BSA and Its Derivatives

BSA and its derivative	Degree of substitution of amino acid residue (%)	Hydrophobicity parameter, $H\phi_{ave}$/kJ·res.$^{-1}$
BSA	—	4.35
Acetyl-BSA	66.9	4.98
Propionyl-BSA	67.4	5.19
Butyryl-BSA	63.9	5.36
Palmitoyl-BSA	98.5	10.17

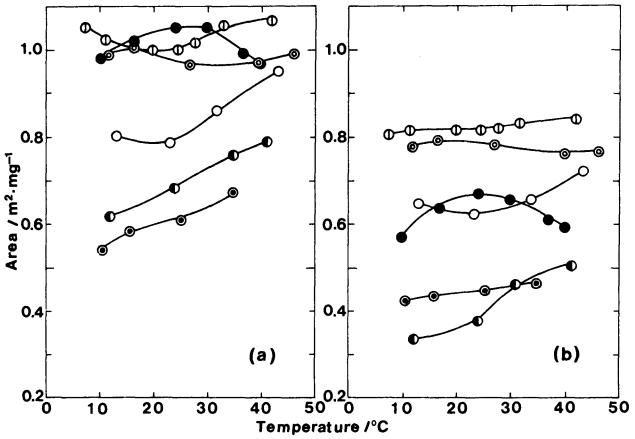

Fig. 8. Plots of area as a function of temperature for various monolayers at (a) π=5 mN·m^{-1} and (b) π=15 mN·m^{-1}, derived from Figs. 2—7. The symbols indicate ⓓ, hemoglobin; ◎, β-lactoglobulin; ●, β-casein; ○, BSA; ◐, cytochrome C; ⊙, lysozyme.

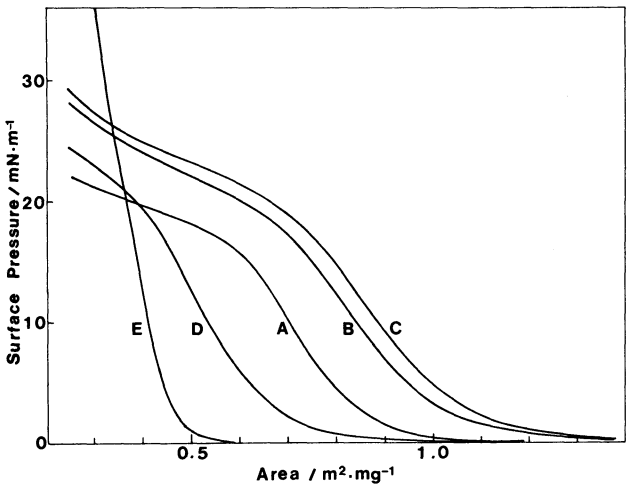

Fig. 9. π-A isotherms for monolayers of BSA and its derivatives spread on water at 25 °C. A) BSA, B) acetyl-BSA, C) propionyl-BSA, D) butyryl-BSA, E) palmitoyl-BSA.

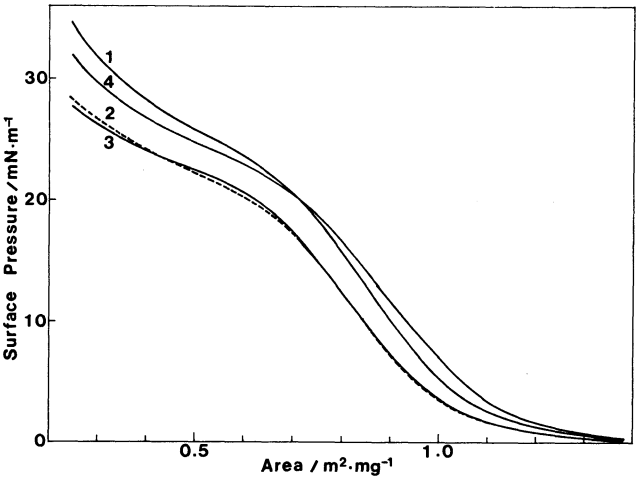

Fig. 10. π-A isotherms for acetyl-BSA monolayer at different temperatures. 1) 9.8 °C, 2) 23.5 °C, 3) 31.7 °C, 4) 41.2 °C.

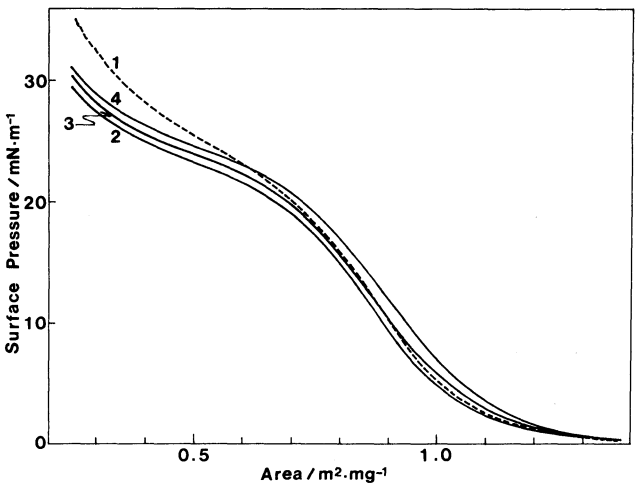

Fig. 11. π-A isotherms for propionyl-BSA monolayer at different temperatures. 1) 11.1 °C, 2) 24.0 °C, 3) 35.1 °C, 4) 41.3 °C.

in Figs. 10—13. Figures 10 and 11 show the isotherms for acetyl-BSA and propionyl-BSA monolayers spread on water at different temperatures, respectively. The isotherms of their derivatives showed a decremental shift with increasing temperatures up to 30 °C but an incremental one with increasing temperatures above 30 °C, indicating that the temperature-dependences of their derivatives were essentially different from that of BSA in Fig. 5. Figures 12 and 13 show the isotherms for butyryl-BSA and palmitoyl-BSA monolayers spread

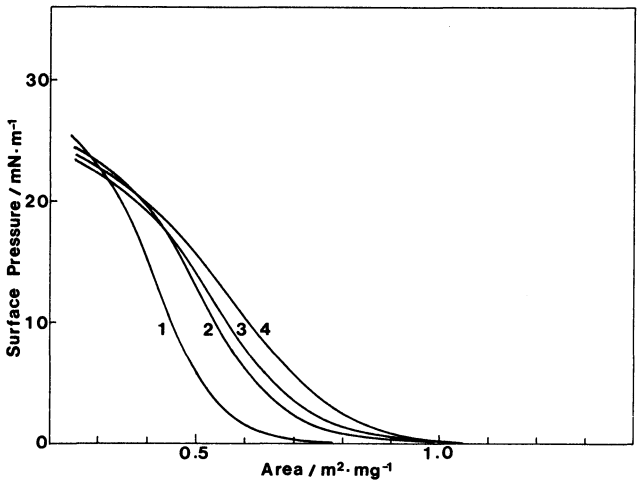

Fig. 12. π-A isotherms for butyryl-BSA monolayer at different temperatures. 1) 8.6°C, 2) 23.6°C, 3) 30.9°C, 4) 42.9°C.

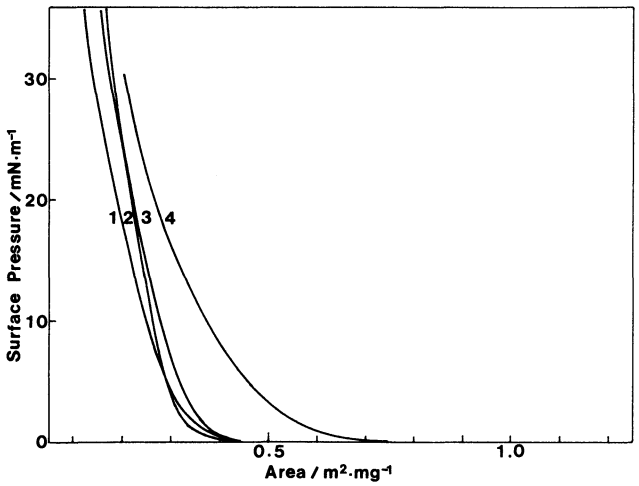

Fig. 13. π-A isotherms for palmitoyl-BSA monolayer at different temperatures. 1) 12.5°C, 2) 23.0°C, 3) 32.5°C, 4) 42.2°C.

on water at different temperatures. These monolayers were gradually expanded with increasing temperatures, although the hydrophobicities of their derivatives were higher than those of acetyl and propionyl derivatives. Figure 14 shows the plots of area as a function of temperature for the monolayers of BSA and its derivatives at (a) π=5 mN·m^{-1} and (b) π=15 mN·m^{-1}, derived from Figs. 2 and 10—13. The result indicates that acetyl and propionyl derivatives at the interface exhibit the most compact conformations around 30°C, while that butyryl and palmitoyl derivatives exhibit the extended conformations with increasing temperatures.

Discussion

It is assumed that proteins at an air-water interface lose their tertiary structure. They exist in extended

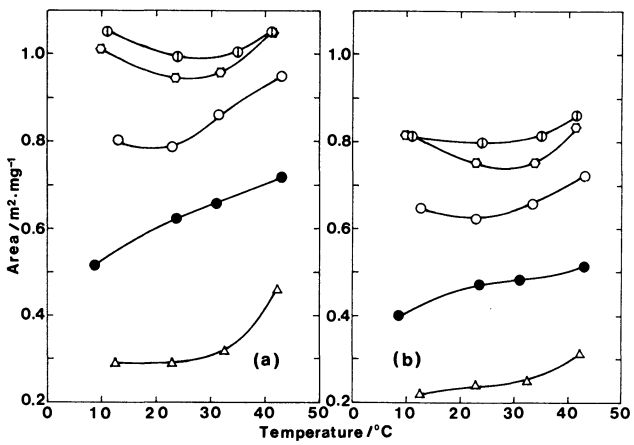

Fig. 14. Plots of area as a function of temperature for monolayers of BSA and its derivatives at (a) π=5 mN·m^{-1} and (b) π=15 mN·m^{-1}, derived from Figs. 2 and 10—13. ○, BSA; ○, acetyl-BSA; ⊕, propionyl-BSA; ●, butyryl-BSA; △, palmitoyl-BSA.

conformations with hydrophobic domains oriented at the interface and hydrophilic domains directed toward the aqueous phase. However, the unfolding of a protein molecule at the interface is not infinite but within steric limitations such as intramolecular disulfide bonding. Accordingly, the unfolding of proteins at the interface is considered as follows. The hydrophobic proteins such as β-casein, hemoglobin, and β-lactoglobulin unfold more easily than the hydrophilic proteins such as cytochrome C and lysozyme. Particularly, β-casein is a flexible more or less random coil molecule without any disulfide bond and with a low helix content, while lysozyme is a rigid globular molecule, the peptide chain being crosslinked by four disulfide bonds (Table 1). In this way, the degree of unfolding may be influenced not only by the hydrophobicity but by the flexibility of the molecule. Similar results have been obtained by Evans et al.,[16] Birdi,[28] and Graham and Phillips.[29]

Privalov et al.[30–33] has extensively studied the stability of the native conformation of proteins in an aqueous phase. The stability of the protein is usually described in terms of the denaturation Gibbs energy for the unfolded and native conformations. That is, the Gibbs energy of protein unfolding, $\Delta G^u(T)$, can be expressed as

$$\Delta G^u(T) = \Delta H^u(T) - T\,\Delta S^u(T) \qquad (1)$$

where $\Delta H^u(T)$ and $\Delta S^u(T)$ are the net change of enthalpy and entropy of protein unfolding, respectively. $\Delta G^u(T)$ is generally shown as a function of temperature. According to Privalov and Khechinashvili,[30] $\Delta G^u(T)$~T curves for lysozyme and ribonuclease decrease steeply with increasing temperatures, respectively. However, $\Delta G^u(T)$~T curves for myoglobin and trypsin

inhibitor have the maximum values at about 30 °C and decrease again with decreasing temperatures, indicating so-called cold denaturation.[34] A similar result has been obtained by Ooi and Oobatake.[35] The extent of protein unfolding may be correlated to the Gibbs function as follows. The native protein progressively unfolds with diminishing the Gibbs energy. In this way, the hydrophilic proteins such as lysozyme (see Table 1) and ribonuclease (polar–apolar ratio of 2.64, average hydrophobicity of 3.68 kJ·res.$^{-1}$) tends to unfold with increasing temperatures, while the hydrophobic proteins such as myoglobin (polar–apolar ratio of 1.26, average hydrophobicity of 4.84 kJ·res.$^{-1}$) and trypsin inhibitor (polar–apolar ratio of 1.57, average hydrophobicity of 4.94 kJ·res.$^{-1}$) aquire the most compact structures around 30 °C.

The thermodynamics of protein adsorption at an interface has been noted by MacRitchie,[36] indicating that the entropy gain due to hydrophobic domains is the dominant factor governing protein adsorption at an interface. Therefore, the above phenomenon in the aqueous phase would apply to protein unfolding at the interface, i.e., the temperature-dependence of unfolding of protein in membranes would be dependent on the degree of hydrophobicity of the protein. The hydrophilic proteins such as BSA, cytochrome C, and lysozyme at the interface exhibited the extended conformations with increasing temperatures (Figs. 2—4 and 8). In addition, the hydrophobic proteins such as hemoglobin and β-lactoglobulin at the interface exhibited the most compact conformations around 20 °C and 30 °C, respectively (Figs. 5, 6, and 8). It is particularly interesting that hemoglobin which is similar to myoglobin in structure and function exhibits the behavior of cold denaturation at the interface. These results suggest that Privalov's thermodynamics may be applied to the mechanisms of protein unfolding not only in the aqueous phase but also at an interface.

However, the thermotropic behavior of β-casein monolayer is in conflict with the above consideration. This may be explained as follows. The decrease in the Gibbs energy is attributed to a hydration effect due to hydrated water around the protein molecule.[35] In this sense, the hydration effect of the protein at the interface will be diminished compared to that of the protein in the aqueous phase. β-Casein, a flexible more or less random coil molecule, may exist in a completely extended conformation with its hydrophobic domains oriented at the interface and its hydrophilic domains directed toward the aqueous phase. Accordingly the thermotropic behavior of β-casein at the interface may be significantly different from that of β-casein in the aqueous phase.

The above speculation was confirmed by investigating the thermotropic behavior of monolayers of acyl derivatives of BSA. The monolayers of acetyl-BSA and propionyl-BSA, where the hydrophobic domains were introduced by acylation of the lysine side chains, were more expanded than that of unmodified BSA (Fig. 9). Furthermore, the isotherms for the acetyl and propionyl derivatives showed distinct decremental shifts with increasing temperatures up to 30 °C, but an incremental one with further increasing temperatures, i.e., these derivatives exhibited the most compact conformations at the interface around 30 °C (Figs. 10, 11, and 14).

On the other hand, the monolayers of butyryl and palmitoyl derivatives, where the hydrophobicities were higher than those of acetyl and propionyl derivatives, were more condensed than that of unmodified BSA. In addition, the monolayers of the butyryl and palmitoyl derivatives were simply expanded with increasing temperatures, despite their significantly high hydrophobicities (Figs. 12—14, Table 2). Particularly, both the shape of the isotherm and the temperature-dependence of the monolayer of the palmitoyl derivative were similar to those of dipalmitoylphosphatidylcholine (DPPC). Phillips[4] has discussed the temperature-dependence of lipid monolayers in the following terms. When the hydrocarbon chains are sufficiently long, condensed monolayers are formed, whereas with shorter chains, liquid-expanded films occur. When the temperature is greater than the phase transition temperature T_c (41 °C in the case of DPPC), the monolayer is completely expanded and the π–A isotherm does not show an intermediate region. When the temperature is considerably lower than T_c, the monolayer will be fully condensed. At intermediate temperatures, the π–A isotherms show the well-pronounced change of slope due to the onset of two-dimensional crystallization. It is seen from Fig. 12 and Table 2 that the apparent area occupied per palmitoyl side chain in the monolayer of palmitoyl-BSA is 7.0×10^{-19} m^2 at 23 °C. When DPPC is spread on water, the area per molecule in the monolayer is about 4.8×10^{-19} m^2 below T_c.[4] Since palmitoyl side chains therefore comprise about 34% of the total area at the air–water interface, the thermotropic behavior of the palmitoyl side chains will become pronounced rather than that of hydrophobic domains in protein.

In conclusion, the results obtained in this study suggest that the hydrophobicity of the protein is the dominant factor determining the thermal stability of proteins in monolayers. This study is being continued to obtain further information about the hydrophobic effect of proteins in membranes through the measurements of the temperature-dependence on mixed monolayers constituted of proteins and lipids.

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