Structure–Function Relationship of Proteins: Adsorption of Structural Intermediates of Bovine Serum Albumin at the Air–Water Interface

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Six structural intermediates of bovine serum albumin were prepared. The secondary-structure contents of these intermediates were analyzed by circular dichroism spectroscopy. Adsorption of these intermediates at the air–water interface followed a biphasic first-order kinetics. The first-order rate constants K_1 and K_2 exhibited a bell-shaped relationship with the extent of recovery of folded structure; the maximum occurred when the protein contained about 50% α-helix and 50% random-coil structures. Molecular area calculations indicated two distinct molecular events: the first being the area ΔA_1 cleared for anchoring the molecule in the interface and the second being the area ΔA_2 cleared during rearrangement of the molecule in the interface. Although the ΔA_1 was the same for all the samples, the ΔA_2 exhibited a bell-shaped relationship with recovery of the folded state. These studies have revealed that neither the completely unfolded nor the folded native protein has the ability to occupy a large area at the interface; an optimum ratio of ordered to disordered structure seems to be essential to cause greater change of surface pressure per adsorbed molecule. The relevance of these studies with regard to improving the functional properties of seed storage proteins is discussed.

Adsorption of proteins at liquid interfaces and their behavior in the adsorbed state play an important role in many biological and technological processes. In formulated foods, especially in foam- and emulsion-based products, proteins are often used as functional ingredients to perform the role of a surface-active agent (Kinsella, 1976; Kinsella and Damodaran, 1980; Tornberg, 1978a,b). Although all proteins are amphiphilic, they differ considerably in their surface-active properties. The differences in the surface/interfacial properties of proteins are attributed to conformational and compositional differences (MacRitchie, 1978; Graham and Phillips, 1979a–c; Tornberg, 1978b). Since the molecular properties of proteins differ widely with respect to amino acid composition, sequence, ordered-to-disordered structural segments, and the hydrophobicity/hydrophilicity ratio, no two proteins exhibit similar surface-active properties in food systems.

The important initial step involved in the formation and stabilization of protein-based foams and emulsions is the adsorption and spreading of proteins at the surface/interface. Since adsorption of a macromolecule is influenced by molecular factors such as ionic, hydrophobic, and conformational characteristics, it is fundamentally important to understand how each of these parameters, individually as well as in combination, affect the dynamics of the adsorption and spreading processes. These relationships are not well understood. To elucidate the role of conformation on the surface properties of proteins, Graham and Phillips (1979a–c) studied the adsorption behavior of three different proteins, viz., β-casein, bovine serum albumin, and lysozyme, at fluid interfaces. They concluded that the differences in the surface behavior of the three proteins were manifestations of differences in their conformational characteristics. However, it should be recognized that the differences in the surface behavior of these three proteins cannot solely be attributed to conformational differences alone, because differences in their amino acid composition and distribution as well will influence their surface behavior; at best, the differences in their surface behavior should reflect only the gross differences in their physicochemical properties.

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In order to determine the influence of protein conformation alone on the surface properties, we adopted a different approach: We prepared six structural intermediates of bovine serum albumin and studied their adsorption and molecular behavior at the air–water interface. The rationale in this approach is that because the amino acid composition as well as the sequence of these intermediates are not altered, the observed differences in the surface adsorption behavior of these intermediates can solely be attributed to conformational differences alone.

EXPERIMENTAL SECTION

Materials. Crystallized and lyophilized bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents used in this study were of reagent grade.

Preparation of BSA Structural Intermediates. Structural intermediates of bovine serum albumin were prepared as follows: Albumin was dissolved (0.2%) in 0.2 M Tris-HCl buffer, pH 8.6, containing 10 M urea, 1 mM EDTA, and 50 mM dithiothreitol. The solution was incubated at room temperature for 6 h to facilitate complete reduction and denaturation. The protein solution was then adjusted to pH 4.0 and passed through a Sephadex G-25 column equilibrated with 0.1 N HCl to remove urea and other small molecular weight additives. The reduced and denatured albumin stock solution in 0.1 N HCl was stored at 4 °C and used within 3 days.

To prepare structural intermediates, an aliquot of the denatured albumin stock solution was added to 2 L of regeneration buffer (0.1 M Tris-HCl, pH 8.0, containing 1 mM EDTA, 1 mM reduced glutathione, and 0.1 mM oxidized glutathione) so that the final albumin concentration was about 0.5–1.0 μM. Under these conditions, re-formation of disulfide bonds and refolding of serum albumin have been shown to occur with time (Johanson et al., 1981; Damodaran, 1986). In order to trap structural intermediates of the albumin, refolding was stopped at predetermined time intervals by blocking the free sulfhydryl groups in the protein (Creighton, 1977). This was accomplished by adding iodoacetamide to the regeneration solution to a final concentration of 0.1 M. The solution was incubated at room temperature for 30 min, then dialyzed against water at pH 7.0 for 48 h with frequent changes, and then lyophilized. Six structural intermediates

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of bovine serum albumin were prepared in similar manner by blocking refolding at 0, 30-min, 2-h, 3-h, 6-h, and 24-h time intervals.

Circular Dichroism Measurements. Circular dichroic measurements were made with a Jasco Model J-41C spectropolarimeter. A cell path length of 1 mm and a protein concentration of 0.01% were used. Mean residue ellipticity, $[\theta]$ (deg·cm^2/dmol), was calculated from

$$[\theta] = 100dM_r/nC \tag{1}$$

where d is the rotation in degrees per 1.0-cm path length, M_r is the molecular weight of the protein [67 000 for bovine serum albumin (Brown, 1977)], n is the number of amino acid residues [582 for bovine serum albumin (Brown, 1977)], and C is the concentration of protein (mg/mL). The mean residue ellipticity values of the intermediates at 221 nm were taken as the criteria for determining the extent of refolding and secondary-structure content of these intermediates. These data are presented in Table I.

Disulfide Content. The disulfide content of the albumin intermediates was determined by the 2-nitro-5-thiosulfobenzoate method (Thannhauser et al., 1984) as modified by Damodaran (1985).

Surface Adsorption Measurements. The surface adsorption behavior of the albumin intermediates at the air–water interface was studied by the Wilhelmy plate technique (Graines, 1966; Graham and Phillips, 1979a) with a Cahn electrobalance, Model 2000, equipped with a dynamic surface tension accessory and a strip-chart recorder (Cahn Instruments Co.). A thin platinum plate of 1-cm width, suspended from the arm of the electrobalance, was used as the sensor.

In a typical experiment, albumin stock solution (0.1%, w/v) was prepared in 20 mM sodium phosphate buffer, pH 7.0. The solution was centrifuged at 12000g for 10 min to remove insoluble particles. An aliquot (1.0 mL) was diluted to 100 mL with the buffer to obtain a final protein concentration of 10^{-3}% (w/v). For surface pressure measurements, 90 mL of the diluted sample was poured gently into the Teflon-coated Langmuir trough with the platinum sensor plate hanging in position. The trough was placed in a circulating water bath, which was maintained at 25 ± 0.2 °C. To remove the protein film initially adsorbed to the surface, the surface was cleaned by sweeping with a fine capillary tube attached to an aspirator. The surface sweeping was repeated until the surface tension of the protein solution was equal to that of the buffer (72.0 mN/m). The protein was then allowed to adsorb from the subphase to the air–water interface. The change in surface pressure, π, was recorded continuously on the recorder.

Spread Monolayer. The surface pressure–area isotherms (π vs. A) of spread monolayers of albumin samples were studied by the method described elsewhere (Trurnit, 1960). In these experiments the buffer was placed in the trough first, with the platinum plate hanging in place. The Teflon barriers of the dynamic surface tension accessory were lowered into the trough. A thin glass rod clamped to a jack was dipped up to 5 mm into the subphase at the center of the surface area between the Teflon barriers. An aliquot (50 μL) of albumin solution (in the phosphate buffer) containing 10 μg of albumin sample was slowly run onto the glass rod from a Hamilton glass syringe. The rod was then removed from the surface, and the film was allowed to equilibrate for at least 10 min. The film was then compressed stepwise to various surface areas and allowed to equilibrate for 10–20 min, and the corresponding surface pressures were measured. Knowing the total amount of protein loaded onto the surface, the π vs. A isotherms were converted to π vs. Γ isotherms, where Γ is the surface concentration.

Table I. Structural Properties of Native and Refolded Bovine Serum Albumin Intermediates

intermed no.	refolding time, h	$[\theta]_{221}$, deg·cm^2/dmol	% $[\theta]_{221}$ rec	no. S–S bonds regained
1	0	−7 693	36.0	0
2	0.5	−10 496	49.5	6.5
3	2.0	−15 300	72.0	12.7
4	3.0	−19 160	90.0	13.3
5	6.0	−20 090	94.5	13.7
6	24.0	−20 410	96.2	15.2
native		−21 220	100.0	17.0

Data Treatment. From the π vs. time curves and the π vs. Γ curves obtained for various albumin intermediates, various molecular and kinetic parameters were obtained. The rate of adsorption, i.e., the number of molecules arriving at the air–water interface per unit time is (MacRitchie and Alexander, 1963a)

$$\ln \frac{d\Gamma}{dt} = \ln (KC_0) - \frac{\pi \Delta A}{kT} \tag{2}$$

where K is the rate constant for adsorption, C_0 is the protein concentration in the bulk phase, k is the Boltzmann constant, T is the absolute temperature, and ΔA is the area at the interface that has to be cleared against the interfacial pressure, π, for the protein molecule to adsorb. Since $d\Gamma/dt = (d\pi/dt)(d\Gamma/d\pi)$, eq 2 can be written as

$$\ln \frac{d\pi}{dt} = \ln \frac{KC_0}{d\Gamma/d\pi} - \frac{\pi \Delta A}{kT} \tag{3}$$

The area cleared for the protein molecule during adsorption can be calculated from the slope of the linear plot of $\ln (d\pi/dt)$ vs. π. Knowing $d\Gamma/d\pi$ (the slope of π vs. Γ curves for spread monolayers) and C_0, the rate constant for adsorption can be calculated from the intercept.

The rate constant of adsorption, K, was also estimated by an alternate approach. Since the change of surface pressure is related to the number of molecules adsorbed to the interface, the rate of adsorption can be expressed in the form of a first-order equation (Graham and Phillips, 1979a)

$$\ln \frac{\pi_{ss} - \pi_t}{\pi_{ss} - \pi_0} = -Kt \tag{4}$$

where π_{ss}, π_t, and π_0 are surface pressures at steady-state conditions, at time t, and at $t = 0$, respectively, and K is the first-order rate constant for adsorption. The K values for albumin intermediates were estimated from the slopes of $\ln [(\pi_{ss} - \pi_t)/(\pi_{ss} - \pi_0)]$ vs. time plots.

RESULTS AND DISCUSSION

The mean residue ellipticity values at 221 nm and the disulfide content of the six intermediates and the native bovine serum albumin are given in Table I. Both these values increased with refolding time and approached those of the native albumin. Since the negative ellipticity at 221 nm is indicative of the content of α-helical structure (Johanson et al., 1981), the increase in the percent $[\theta]_{221}$ recovery may be regarded as the extent of refolding and secondary-structure formation in these intermediates. Although intermediate 1 was trapped at zero refolding time, nearly 30% $[\theta]_{221}$ recovery suggests that some refolding of the polypeptide had occurred very rapidly in the regeneration medium. About 90% of $[\theta]_{221}$ was recovered within 3 h. Comparison of the ellipticity values of inter-

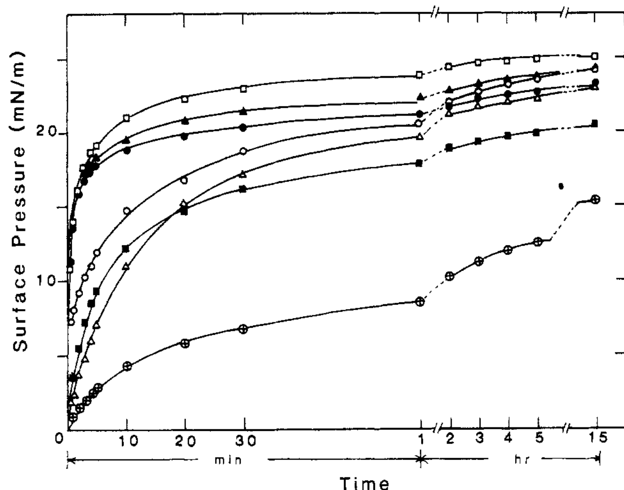

Figure 1. Rate of development of surface pressure during the adsorption of albumin intermediates at the air–water interface. Key: ⊕, native albumin; ○, intermediate 1; △, intermediate 2; □, intermediate 3; ●, intermediate 4; ▲, intermediate 5; ■, intermediate 6.

mediates 5 and 6 suggests that the difference in the secondary-structure content of these intermediates should not be very significant. However, the large difference between and uniform increase in the ellipticity values of intermediates 1–5 suggest that these should serve as the prime candidates for getting some insight into the role of conformation on adsorption and behavior of proteins at interfaces.

Adsorption and Rearrangement. The changes in surface pressure of albumin solutions over a period of 15 h are shown in Figure 1. Except for intermediates 1 and 2, the subphase protein concentrations in all cases were between 0.9×10^{-3} and 1.0×10^{-3}% (w/v). Since the solubilities of intermediates 1 and 2 were poor, the adsorption experiments for these intermediates were done at 0.3×10^{-3}% and 0.55×10^{-3}% subphase concentrations, respectively. It is apparent that, in the case of the albumin intermediates, steady-state π values were attained within 6 h, whereas the native albumin required about 15 h. Both the rate of increase of π and the steady-state π values for the intermediates were greater than those for native albumin. For example, at about 0.9×10^{-3}% subphase concentration, the steady-state π value for intermediates 3 was about 25 mN/m, whereas that for native albumin was about 15 mN/m.

Among the intermediates, the steady-state π values apparently decreased with the extent of refolding of the molecule. For example, at comparable subphase concentrations the steady-state π values of intermediates 3–6 decreased in the order $3 > 4 \approx 5 > 6$. It must be pointed out that if the adsorption experiments for intermediates 1 and 2 were performed at concentrations equal to those of other intermediates, the steady–state values would have been greater than those of the other intermediates (Figure 1). Although the steady-state π values were inversely related to the extent of refolding, they were not linearly related to the ellipticity values of the intermediates. For example, the percent $[\theta]_{221}$ recoveries for intermediates 5 and 6 were almost the same, indicating that the α-helical contents (secondary structure) of these intermediates were probably the same; however, the steady-state π values for these intermediates differ greatly (Figure 1). This suggests that the tertiary structures, i.e., the way in which the secondary structure segments are arranged in the three-dimensional space, of these intermediates are significantly different; this affects the degree of penetration and orientation of the amino acid residues or polypeptide segments at the interface.

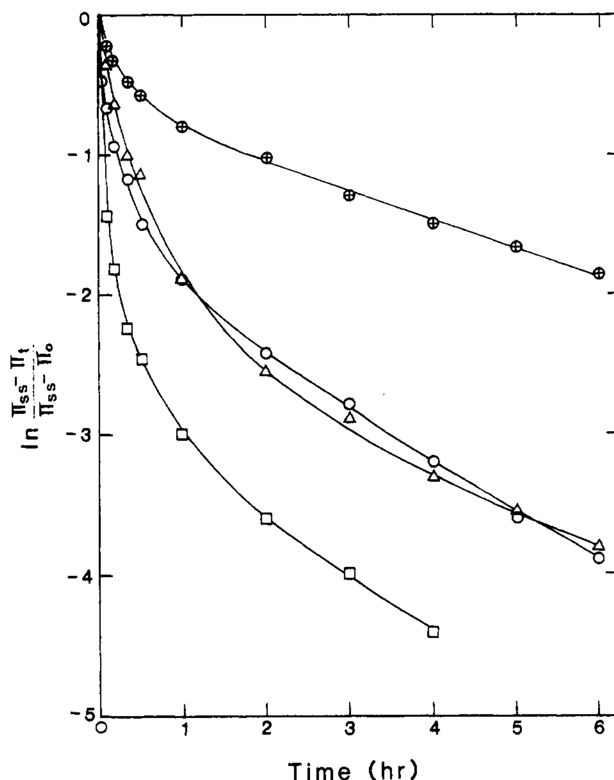

Figure 2. First-order kinetic plots of the data from Figure 1 according to eq 4. (See text for details.) Key: ⊕, native albumin; ○, intermediate 1; △, intermediate 2; □, intermediate 3.

The surface-active properties of proteins depend on their ability to rapidly adsorb, unfold, and occupy a greater area at the interface (Graham and Phillips, 1979a,b). These attributes are closely related to the protein conformation in the subphase. It is generally accepted that a highly flexible random-coil protein with no disulfide linkages, e.g., β-casein, will readily unfold and reorient itself under the prevailing thermodynamic conditions at the interface and hence will exert greater surface pressure, whereas a highly ordered compact protein with disulfide linkages, e.g., lysozyme, will have greater resistance to unfolding and reorientation at the interface and hence will exert lower surface pressure. In the present study, in order to determine the relationship between protein conformation and the kinetics of adsorption at the interface, the rates of adsorption and rearrangement of albumin intermediates were analyzed with use of eq 4. The first-order kinetic plots are shown in Figure 2. All albumin samples exhibited curvilinear first-order plots, which could essentially be resolved into two first-order kinetic phases. For all the intermediates and the native albumin the first kinetic phase, having rate constant K_1, occurred between 0 and 10 min; during this period about 80% of the total change in surface pressure was effected (Figure 1). The second kinetic phase, having rate constant K_2, apparently occurred between 1 and 6 hr (Figure 2). Previously, it had been shown that the first kinetic phase, which occurs rapidly within a short period of time (up to π = 15 mN/m), corresponds to initial adsorption and penetration of protein molecules at the interface; and the second kinetic phase, which occurs at the latter stages (>15 mN/m), corresponds to rearrangement and reorientation of the adsorbed molecules (Graham and Phillips, 1979a).

The first-order rate constants for adsorption and rearrangement, K_1 and K_2, respectively, of albumin interme-

Table II. First-Order Rate Constants for Adsorption (K_1) and Rearrangement (K_2) of Albumin Intermediates at the Air-Water Interface

intermed no.	K_1, min^{-1}	$K_2 \times 10^3$, min^{-1}	intermed no.	K_1, min^{-1}	$K_2 \times 10^3$, min^{-1}
1	0.070	6.25	5	0.103	4.63
2	0.064	5.26	6	0.084	5.50
3	0.194	8.40	native	0.030	3.14
4	0.168	4.07			

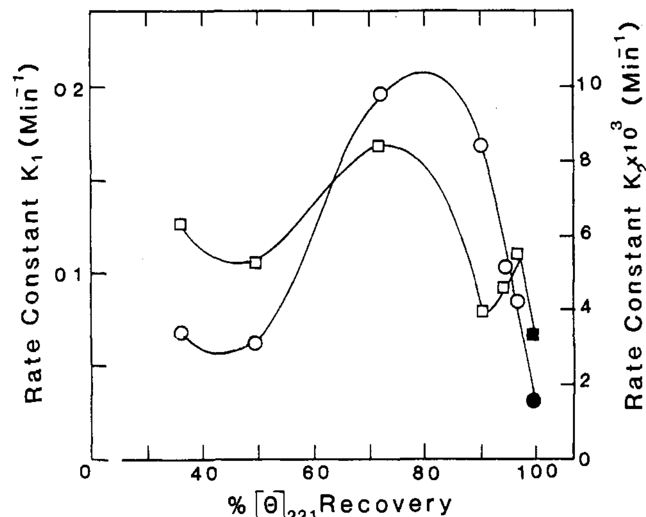

Figure 3. Variation of the rate constants K_1 (○) and K_2 (□) with percent recovery of mean residue ellipticity at 221 nm of the albumin intermediates. Filled symbols indicate corresponding values for native albumin. (See text for details.)

diates are summarized in Table II. The relationships between the rate constants and the secondary-structure content of the intermediates are shown in Figure 3. It is apparent that both K_1 and K_2 are dependent on the solution conformation of the intermediates. The bell-shaped curves indicate that both K_1 and K_2 apparently increase with the extent of refolding of albumin and reach maxima at around 70–80% recovery of $[\theta]_{221}$. Since native albumin contains about 60% α-helical structure (Peters, 1975), the 80% $[\theta]_{221}$ recovery would correspond to an intermediate tertiary structure containing about 50% α helix and 50% random coil. In other words, the data suggest that the surface adsorptivity of bovine serum albumin is maximum when the molecule possesses a partially folded tertiary structure containing about 50% α helix and 50% random coil. Since the tertiary structure is not quantifiable, from this study it is difficult to describe in unambiguous terms the role of tertiary conformation in the adsorption process.

It must be pointed out that according to reports in the literature (Graham and Phillips, 1979a,b) one would expect that the rate constants of adsorption and rearrangement should be greater for intermediate 1 than those of other albumin samples. Because intermediate 1 is highly unfolded and contains no disulfide bonds, it should have the ability to readily spread and rearrange at the interface and thus be able to cause greater change of surface pressure than any other intermediates. However, the results presented here suggest that neither the completely unfolded nor the compact, folded native albumin has the ability to rapidly adsorb and undergo rapid rearrangement at the interface; apparently an optimum ratio of ordered to disordered structure seems to be essential to cause a greater rate of change of surface pressure.

Surface Penetration and Molecular Area. At initial stages of adsorption, the rate of adsorption is considered to be diffusion-controlled (MacRitchie and Alexander, 1963a,b; Graham and Phillips, 1979a). However, once a protein film is formed at the interface, the subsequent rate of adsorption progressively decreases below the rate of diffusion (MacRitchie and Alexander, 1963b). The decrease in the rate of adsorption is considered to be due to an energy barrier at the interface, which is related to the energy needed to clear an area, ΔA, against the surface pressure, π, in order for the molecule to adsorb (Ward and Tordai, 1946; MacRitchie, 1978). In other words, in order for a protein molecule to clear and occupy an area, ΔA, against the surface pressure, π, it should possess a molecular energy equal or greater than $\pi\Delta A$. To elucidate the influence of solution conformation of the albumin intermediates on their ability to overcome the surface energy barrier, the adsorption data were analyzed by use of eq 3. The ΔA values were calculated from the slopes of ln $(d\pi/dt)$ vs. π plots (Figure 4).

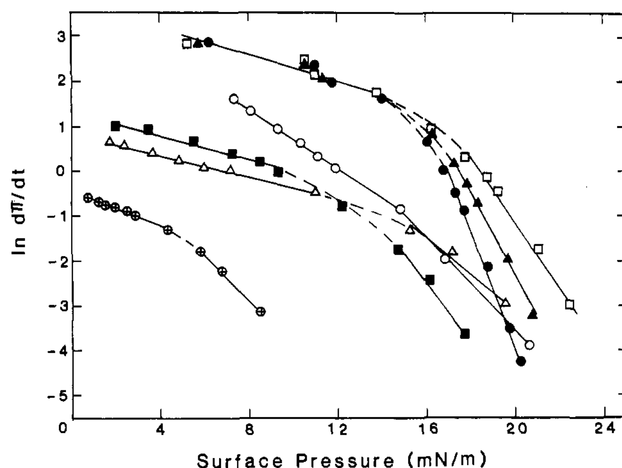

Figure 4. Effect of surface pressure on the rate of change of surface pressure of albumin intermediates. The $d\pi/dt$ values were calculated from the data in Figure 1 by a nonlinear curve-fitting procedure. Key: ⊕, native albumin; ○, intermediate 1; △, intermediate 2; □, intermediate 3; ●, intermediate 4; ▲, intermediate 5; ■, intermediate 6.

Table III. Surface Area Cleared per Protein Molecule during Initial Adsorption (ΔA_1) and Subsequent Rearrangement (ΔA_2) at the Air-Water Interface

intermed no.	ΔA_1, Å^2	ΔA_2, Å^2	intermed no.	ΔA_1, Å^2	ΔA_2, Å^2
1	135.4	212.7	5	56.8	401.6
2	52.6	161.0	6	60.5	259.2
3	48.9	297.3	native	77.3	204.5
4	64.7	534.0			

The ln $(d\pi/dt)$ vs. π plots for all the intermediates and the native albumin were nonlinear (Figure 4). However, the nonlinear curves could be divided into two linear portions. The first linear region for all the intermediates occurred in the range of 3–12 mN/m surface pressure. This region basically corresponds to the first kinetic phase of adsorption (Figures 1 and 2). The second linear region in the ln $(d\pi/dt)$ vs. π curves was observed in the range of 16–25 mN/m surface pressure, which corresponds to the second kinetic phase of rearrangement/reorientation at the interface (Figure 2). Two ΔA values, i.e, ΔA_1 and ΔA_2, corresponding to 3–15 and 16–25 mN/m surface pressure ranges, respectively, were obtained from the slopes of the curves in Figure 4. These values are presented in Table III. The ΔA_1 values correspond to the initial area that has to be created at the surface against the surface pressure for the molecule to adsorb. The ΔA_2 values correspond to the surface area cleared during the subsequent rearrangement of the molecule at the interface.

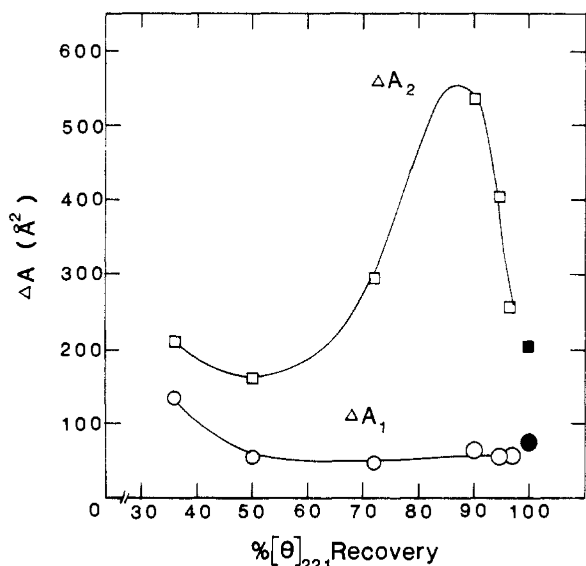

Figure 5. Variation of ΔA_1 (surface area cleared during adsorption) and ΔA_2 (during rearrangement) with percent recovery of mean residue ellipticity at 221 nm of albumin intermediates. Filled symbols indicate corresponding values for native albumin.

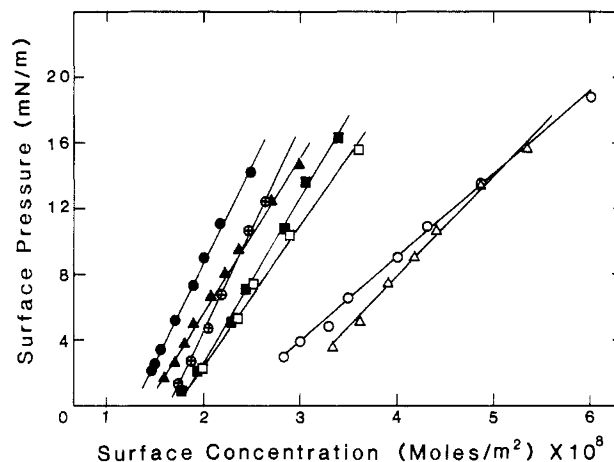

Figure 6. Surface pressure–surface concentration (π vs. Γ) relationship of the spread monolayers of albumin intermediates. Key: ⊕, native albumin, ○, intermediate 1; △, intermediate 2; □, intermediate 3; ●, intermediate 4; ▲, intermediate 5; ■, intermediate 6.

The relationship between solution conformations of the intermediates and the ΔA_1 and ΔA_2 values are shown in Figure 5. It may be noted that except for intermediate 1, the ΔA_1 values of all the other intermediates and the native albumin were almost the same, i.e., about 50 Å^2. This value is consistent with the results of Graham and Phillips (1979a). Further, the data also imply that the initial work, $\pi\Delta A_1$, at any given π, needed for the molecules to penetrate and become anchored in the interface is independent of the solution conformation of the protein. Previously it was reported that, for the various proteins studied, the ΔA values were within the range of about 100–175 Å^2 and showed no dependence on the molecular size or shape (MacRitchie, 1978; Ter-Minassian-Saraga, 1981). The results presented here are in accord with those findings. However, on the other hand, the ΔA_2 values were greatly affected by the solution conformation of the intermediates (Figure 5). The ΔA_2 values increased with the extent of refolding of albumin and reached maximum value at about 80% $[\theta]_{221}$ recovery; further folding caused lower ΔA_2 values.

It is generally considered that a highly unfolded and disordered protein would occupy a greater surface area than a compact folded protein (Graham and Phillips, 1979a,b). However, our results apparently do not support this view. Intermediate 1, which is highly unfolded and contains no disulfide linkages, actually occupies less surface area (ΔA_2) compared to other folded intermediates. In order to confirm whether this indeed is the case, we studied the behavior of spread monolayers of the albumin intermediates. The data in Figure 6 show the π vs. Γ curves for spread monolayers. It should be noted that the surface concentration, Γ, required to exert a given surface pressure was the highest for intermediate 1 and lowest for intermediate 4 (Figure 6). Since $1/\Gamma$ is the surface area occupied by 1 mol of protein, it is evident that intermediate 1 occupied less surface area than intermediate 4 at any given surface pressure (Figure 6). Furthermore, it can be seen that the relationship betwen $1/\Gamma$ and the percent $[\theta]_{221}$ recovery of the intermediates at any given π value apparently has the same trend as that of the data shown in Figure 5 for the adsorbed films. These results strongly indicate that in the completely unfolded state serum albumin actually occupies a lesser area at the air–water interface than the partially folded intermediates. The data also indicate that, in order for a protein to rapidly adsorb and occupy a greater area at the interface, it should possess an optimum ratio of ordered to disordered structure; for bovine serum albumin this ratio apparently seems to be 50% α helix and 50% random coil.

The apparent disagreement between the conclusions of this study and those of the other studies (Graham and Phillips, 1979a,b) may be attributed to basic differences in the approach. In the earlier approaches, in order to elucidate the influence of conformation on adsorption, the surface adsorptivity and the behavior in the adsorbed state of various structurally different proteins were studied, and the differences were related to conformational differences among those proteins (Graham and Phillips, 1979a,b). However, the major limitation of this approach is that the differences in surface adsorptivities of various proteins may not solely be attributable to conformational differences alone, because variations in amino acid composition and sequence will also influence their surface behavior. However, in the present study, because only the conformation of a single protein is altered, the observed differences in the surface/interfacial adsorption behavior of the intermediates can solely be attributed to conformational differences alone.

With regard to the relevance of these studies, we point out that the major obstacle limiting utilization of seed storage proteins (e.g., soy proteins) as protein ingredients in formulated foods is the lack of desirable functional properties. In order to expand the utilization of these proteins, it is imperative that novel strategies and approaches to improve the functional properties of plant proteins be developed. One of the promising approaches in this regard is the genetic engineering of plant seed storage proteins. With the current and future advances in biotechnology, techniques will soon be available to successfully engineer the seed storage proteins of plants. Appropriate genetic alteration of amino acid residues, sequence, and/or conformation will improve the functional properties of these underutilized proteins. However, the major impasse in developing such protein engineering strategies is the lack of fundamental information on the structure–function relationship of food proteins. In other words, in order to facilitate developments in genetic engineering of plant proteins, it is urgently needed to have available the knowledge and fundamental database to relate functional properties to specific molecular confor-

mations. Although bovine serum albumin has been used as a model protein in this study, the approach and the results presented here may help in the study of the structure–function relationship of more complex seed proteins.

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A Gas–Liquid Chromatographic Method for Analysis of Pyruvic Acid and Lower Molecular Weight Fatty Acids in Plant Materials

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A gas–liquid chromatography (GLC) method has been developed for quantitative analysis of pyruvic acid and lower molecular weight fatty acids in plant materials. Plant extracts containing pyruvic acid and other low molecular weight fatty acids were treated with sodium borohydride, converting the pyruvic acid to lactic acid. Heating in butanol saturated with dry hydrogen chloride subsequently converted the acid components into the corresponding butyl esters, which were analyzed by GLC. GLC–mass spectrometry was used to confirm the identity of the GLC peaks. The efficiency of recovery of added pyruvic acid from Coastal Bermuda grass silage was 94%. Analyses were carried out on onion, apple, banana, and wheat kernels.

Since the elucidation of the mechanism of protein synthesis in plants is of major importance, considerable attention has been devoted to this topic (Bryan, 1976). Kretovich and Kasprek (1962) demonstrated the biosynthesis of proteins from pyruvic acid in rice and sunflower seeds. Therefore, because of the role played by pyruvic acid in protein synthesis and various biochemical cycles, interest in a method of quantitating pyruvic acid in biological materials has been investigated extensively. Quantitation of α-keto acids (pyruvate) in biological materials has always presented a serious challenge because of difficulties in their isolation, separation, and identification. Keto acids were found to be difficult to extract and esterify because of their instability. Luke et al. (1963) and other investigators experienced decomposition on chromatography (Ackerman et al., 1960; Rumsey et al., 1964). Several publications have appeared dealing with differing derivatization techniques for GLC analyses of this type of compound. Silylation of α-keto acids with hexamethyldisilazane and trimethylchlorosilane in pyridine reportedly resulted in multiple products as revealed by GLC analyses (Horii et al., 1965). According to this study Me_3Si–oxime derivatives of keto acids were stable on GLC analyses and gave single peaks. We were unable to reproduce their procedure for pyruvic acid; however, this could be due to minute quantities of water present in our samples. Presence of water in the reaction mixture would interfere with formation of both oxime and the subsequent Me_3Si derivative (Pierce, 1977). Other researchers have reported GLC analysis of pyruvic acid as its methyl ester; however, they reported that two peaks were produced (Rumsey et al., 1964).

Several recent publications have reported the separation of Krebs cycle acids, dairy product acids, and acids formed by metabolism of anaerobic microorganisms on various growth media by high-precision liquid chromatography (HPLC) (Guerrant et al., 1982; Marsill et al., 1981). However, Guerrant et al. have shown that oxalacetic acid decomposes to form pyruvic acid in either acidic or basic solutions at ambient temperature. Thus, accurate quantitative analysis for pyruvic acid and oxalacetic acids in an acid fraction would require very rapid isolation of the sample followed by HPLC analyses. Even though these investigators maintain that oxalacetic acid alone decomposes in acidic or basic solution, it is well-known [cf. Merck Index (1983)] that pyruvic acid also polymerizes and decomposes on standing if impurities are present or aerobic conditions exist in storage.

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