Lipid–Protein Interactions in Monolayers: Egg Yolk Phosphatidic Acid and β-Lactoglobulin

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The interaction of β-lactoglobulin (β-lg) with phospholipids in monolayers was studied with a Langmuir film balance. Pressure–area curves of monolayers of β-lg mixed with either egg yolk phosphatidic acid (EYPA) or egg yolk phosphatidylcholine (EYPC) on pH 1.3 to 6 subphases were compared to curves calculated from the results obtained with the individual components. EYPA and β-lg exhibited electrostatic attraction for each other in mixed monolayers at the air–water interface when the pH of the subphase was 4 or 1.3. No interaction was observed on pH 6 subphase. Monolayers of egg yolk phosphatidylcholine–β-lg mixtures exhibited no interaction over the pH range studied. Calcium at 1 m*M* concentration in the subphase removed the evidence for interaction between EYPA and β-lg at pH 4, but not at pH 1.3. Circular dichroism spectroscopy suggested an increased amount of α helix and β sheet in the films as compared to solutions of β-lg.

INTRODUCTION

The importance of studying mixtures of lipids and proteins in films is obvious when one considers the currently accepted fluid mosaic model of membranes (1). The transport of a wide variety of solute molecules and ions is one property of biological membranes, yet there has been little success in the isolation of ionophoric species that could begin to account for the broad spectrum of biological transport capabilities (2). Phospholipids (2), especially phosphatidic acids (2, 3), have been suggested as possible transport species. These studies employed oil–water partitioning of complexes of lecithin and phosphatidic acid with calcium and a variety of solute molecules (2, 3). Phospholipids have received considerable attention as model components of biological membranes in studies of vesicles (4), bilayer lipid membranes (5), and monolayers at the air–water interface (6–9). The interaction of proteins with lipids at the air–water interface has been studied by many workers (10–14) usually by the technique of injecting protein under preformed lipid monolayers as pioneered by Schulman and co-workers (13, 14). Most of this work was concerned with the so called "hydrophobic" or nonpolar interactions, but electrostatic effects were also noted (10, 12).

With the increased interest in membrane-related areas of research such as ion and solute transport (2, 3, 15), vesicle fusion (16), and the increased availability of purified and thoroughly characterized proteins and polar lipids, it seemed desirable to investigate electrostatic interactions between lipids and proteins at the air–water interface.

Both β-lactoglobulin (β-lg) and egg yolk phosphatidic acid (EYPA) have been well characterized chemically and physically (7, 17–27). The amino acid sequence of β-lg is known (17) and the titration curves of the genetic variants, both native and denatured, have been determined (18, 19). The UV absorption spectrum of β-lg has been determined (20) as well as its optical activity under a variety of solvent conditions (21–23). The three-dimensional structure recently has been determined at 6-Å resolution by X-ray

crystallography (24). The surface chemical properties of β-lg also have been studied (25). The acid–base and ion-binding properties of phosphatidic acid have been determined (26, 27) and the behavior at the air–water interface has been studied (7). It was thus judged that EYPA and β-lg would be well suited to the study of lipid–protein interactions at the air–water interface.

The injection technique, used in most previous studies on lipid–protein interactions, has serious limitations in that the surface concentration of protein is not known. There have been attempts to overcome this through the use of radioactive labeling (10, 28, 29). Also the interpretation of these experiments has been difficult because there is uncertainty regarding the structure of protein molecules in adsorbed films (30). For the present work, it was decided to study the interaction of lipids with proteins at the air–water interface by spreading the components from a common solvent, and to study circular dichroism (CD) spectra of the films in an effort to surmount these difficulties.

MATERIALS

The film balance was fully automated and provided with continuous recording of surface pressure versus film area. Inside dimensions of the Teflon[1] trough were 50 × 10 × 1 cm. Surface pressures were determined by a Wilhelmy plate (Cahn RG Electrobalance, Cahn Instrument Comp., Paramount, Calif.) mounted in the center of the balance. The films were compressed by Teflon barriers starting at each end of the trough and driven toward the center by counterrotating lead screws. Compression speeds and constant pressure control for film transfer were provided by an electronic control module (Klinetics, Inc., Media, Pa.). The Wilhelmy plate and the quartz plates used for CD spectroscopy have been described (31). Egg yolk phosphatidic acid (EYPA), from Avanti Polar Lipids, Inc. (Birmingham, Ala.), gave a single spot on thin-layer chromatograms (TLC) (chloroform:methanol:ammonia, 65:25:4, vol., and chloroform:methanol:water, 65:25:4) and was used without further purification. β-Lactoglobulin A was prepared by the method of Aschaffenburg and Drewry (32). Chloroform and methanol were ACS grade reagents and were passed through a column of activated coconut charcoal/acid alumina, Brockman activity I, to remove traces of film-forming impurities. Drummond dialamatic microdispensers (Drummond Scientific Comp., Broomall, Pa.) were used for monolayer spreading. Water for the subphase of the film balance was treated by ion-exchange followed by double distillation from an all quartz still. The pH was adjusted with hydrochloric acid, 0.1 *N* (pH 1.3), Walpoles acetate (pH 4), or McIlvanes buffer for pH 6 (33). The pH 4 and 6 subphases were made to 0.1 ionic strength with sodium chloride. Films for CD spectroscopy were transferred from subphases containing only 10^{-4} *M* sulfuric acid, pH 4. All other subphases except those with added calcium contained 10^{-5} *M* sodium EDTA. Sodium chloride was roasted at 500°C before use. Concentrated stock solutions of the buffer reagents, except citric acid, were passed through a charcoal/alumina column to remove surface-active impurities and stored at 4°C.

METHODS

Stock solutions of 1 to 2% β-lactoglobulin in pH 6.8 buffer were made up every 1 to 2 weeks and stored at 4°C. Concentration was determined from UV absorption at 278 nm using $E^{1\%}_{1cm}$ 9.6 (34). Stock solutions of about 6 mg/ml EYPA in chloroform were prepared fresh weekly, analyzed for phosphate by a standard procedure (35), and stored at −15°C. The spreading solvent was chloroform–methanol, 2:1 vol., containing 1-ml concentrated hydrochloric acid per 250

[1] Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

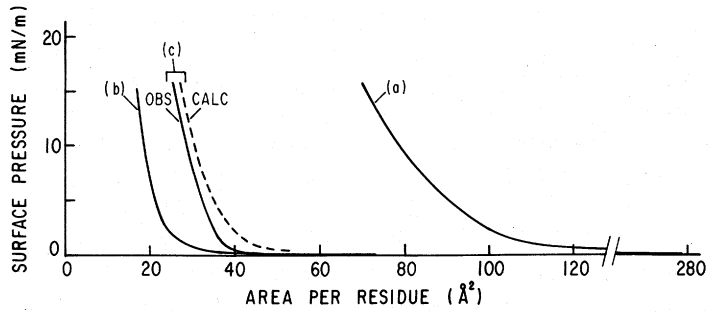

FIG. 1. Π/A curves of EYPA, β-lg, and a mixture of the two on pH 4.0 subphase, $T = 20 \pm 0.5°C$. (a) EYPA, (b) β-lg, (c) EYPA–β-lg, 80 mole (residue) percent β-lg; ——, experimental; - - -, calculated from Eq. [1].

ml. Spreading solutions were made up so as to require 30 to 50 microliters for film formation. For mixed films, EYPA and β-lg were mixed together in the same spreading solution before application.

TLC of EYPA stored in acidic chloroform methanol for periods up to 1 hr showed a single spot with the same mobility as EYPA spotted from neutral chloroform. Monolayers were prepared within 15 min of makeup of the spreading solution.

For monolayer runs, the surface of the subphase was cleaned, the Wilhelmy plate and compression barriers installed, the reference surface tension established, and isolation barriers lowered across the trough 5 cm from each side of the plate. The surface of the trough was thus divided into three areas: the Wilhelmy plate in the center compartment with an area of about 100 cm^2, and two end compartments of 170 cm^2 each. Monolayer spreading was by the direct application of numerous small (3 to 5 μl) drops of spreading solution to the surface of each end compartment. The solvent was allowed to evaporate for 15 min, the isolation barriers were raised, and compression started after an additional 5-min equilibration time. The closure rate of the two end barriers was 2 cm/min giving a compression time of about 15 min for each run. For Langmuir–Blodgett transfers, the quartz plates were lowered into the subphase between the isolation barriers prior to spreading of the film. Additional details of film transfer have been given (31).

RESULTS

Typical pressure–area curves for EYPA, β-lg, and a mixture of the two on pH 4.0 substrate are shown in Fig. 1. The initial pressures at high area were less than 0.3 mN/m for all runs. When the films containing lipid were spread without isolation barriers in place, the initial pressures were frequently unrealistically high, more than 1 mN/m. Presumably this was due to contact angle problems caused by the spreading solvent reaching the Wilhelmy plate. The film of pure β-lg shown in Fig. 1 gave an area per residue of 23.1 and 21.0 $Å^2$ at 3 and 5 mN/m pressure, respectively. The film of EYPA gave areas of 97.0 $Å^2$/residue and 90.5 $Å^2$/residue at 3 and 5 mN/m, respectively. The composition of the mixed film is presented in terms of molecules of EYPA per amino acid unit (residue) of β-lg. The mixed monolayer of EYPA and β-lg containing 80 mole (residue) percent β-lg shown in Fig. 1 gave surface areas of 34.2 $Å^2$/residue and 32.2 $Å^2$/residue at 3 and 5 mN/m pressure, respectively. These are smaller than the corresponding areas of 37.9 $Å^2$/residue and 34.9 $Å^2$/residue calculated from the areas of the pure components according to the relation

$$A_{\Pi,1,p} = A_{\Pi,1}N_1 + A_{\Pi,p}N_p\,, \qquad [1]$$

TABLE I

Areas per Molecule of Lipid or Residue of Protein Obtained from Pressure–Area Curves of the Pure Components

Component	pH	Ca^{+2} concn.	Pressure (mN/m) 3	5	17
			Area (Å²/residue)		
EYPA			104.8 (0.2)[a]	97.1 (0.1)	73.2 (0.3)
EYPC	6	0	109.9 (3.3)	103.1 (3.3)	—
β-lg			23.0 (0.6)	21.0 (0.6)	—
EYPA			96.6 (0.7)	89.9 (0.4)	68.5 (0.4)
EYPC	4	0	103.1 (0.5)	96.3 (0.4)	—
β-lg			22.7 (0.5)	20.6 (0.5)	—
EYPA			84.4 (0.2)	78.6 (0.6)	—
	4	1 m*M*			
β-lg			21.9 (0.0)	19.8 (0.1)	
EYPA			91.3 (2.2)	85.4 (1.9)	66.3 (0.9)
EYPC	1.3	0	107.6 (0.6)	100.6 (0.7)	—
β-lg			25.2 (0.7)	22.6 (0.5)	—
EYPA			90.5 (0.7)	84.2 (0.6)	—
	1.3	1 m*M*			
β-lg			24.4 (0.1)	21.8 (0.1)	—

[a] ( ) Mean deviations of 2 to 7 determinations.

where A is the area per molecule of lipid, 1, or residue of protein, p, at pressure, Π, and N is the mole (residue) fraction of the corresponding component. Equation [1] assumes that the components are either immiscible or mix ideally (36).

Table I lists the average value of the area per residue of protein or molecule of lipid at selected pressures determined from pressure–area curves of the pure component. The averages and mean deviations are pooled data from individual runs with two or more stock solutions except for EYPC, for which a single stock solution was made up for each pH. The subphases were selected to minimize the change in area of EYPA with pH (7) for maximum reproducibility and to include pH's above and below the isoionic point of β-lg. Very alkaline subphases were avoided since β-lg undergoes irreversible polymerization above pH 9 (37).

The surface areas for EYPA listed in Table I are in good agreement with previous results (7). The surface areas for β-lg are consistently about 10% larger than those given in an earlier report (25) which were obtained with a manual balance and long equilibration times between compression cycles. A specific surface area of 1.00 m^2/mg or 18.8 Å²/residue, at 5 mN/m and pH 7 was obtained with β-lg spread from aqueous solvent by the rod technique (25). In preliminary experiments with a previously described manual balance (31) where short pauses between compression cycles were employed, an average surface area of 1.06 m^2/mg (19.9Å²/residue) was obtained with β-lg spread by the rod technique from aqueous buffer onto pH 7 subphase (D. Cornell, unpublished observation). Slow manual compression would be expected to give lower specific areas than continuous compression but the results suggest that the difference between runs taking hours (25) and a few minutes (this work) is relatively small. Usually 12 to 15 μg of protein were spread from

a solution containing about 0.25 mg protein per ml, although two-fold variations in the amount of protein spread and the concentration of the spreading solution did not significantly alter the pressure–area curves. Negligible pressure rise was observed upon 10–1 compression of clean subphases, either before or after application of charcoal/alumina treated spreading solvent, hence, contaminations did not appear to be contributing significantly to the observed areas.

Although the deviation from ideal in the area of a mixed film can be seen by direct inspection of Fig. 1, a more convenient way of presenting the results of a number of runs on films of different compositions is shown in Fig. 2. Here the ratio—area observed/area calculated from Eq. [1]—is plotted as a function of the mole (residue) fraction N_β of β-lg in the film. The results can give one some idea of the stoichiometry of any suspected lipid–protein interaction products. Clearly, mixtures of EYPA and β-lg interact little if at all at pH 6 but exhibit nonideal behavior at pH 4 and pH 1.3 on subphases containing no calcium, while egg yolk phosphatidylcholine (EYPC)–β-lg mixtures show no interaction over the pH range studied. Mixed films of EYPA and β-lg on subphases containing 1 m*M* calcium exhibited behavior predicted by Eq. [1] when the subphase was at pH 4, but nonideal behavior at pH 1.3 virtually identical to that observed on calcium-free substrates.

The CD spectra of collapsed monolayers and cast films of β-lg and EYPA–β-lg are given in Fig. 3. The films were prepared and mounted as previously reported (31) to minimize linear dichroism. Mixed films of EYPA–β-lg for CD analysis contained 90 mole (residue) percent β-lg (58 wt.%) which gave maximum deviation from ideality as shown in Fig. 2b. Dilute sulfuric acid was used for the pH 4 subphase to minimize the light scattering observed with films transferred from buffer solutions. The contribution of light scattering to absorption over the 190 to 250 nm region was estimated by ex-

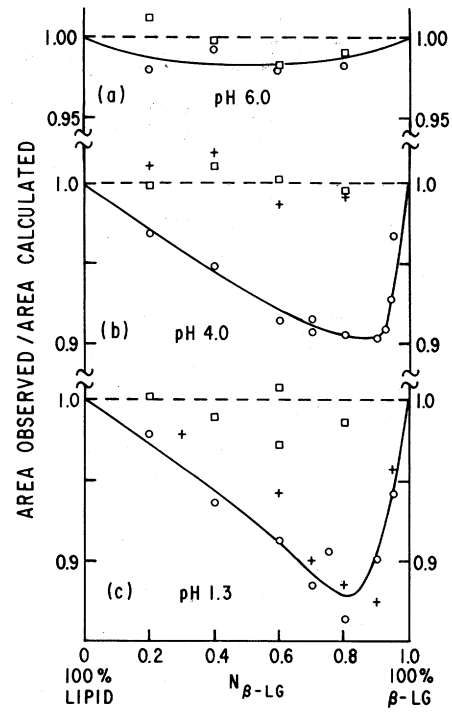

FIG. 2. Mixed monolayers of lipid and β-lg. Comparison of area observed to area calculated from Eq. [1] at 3 mN/m film pressure and $T = 20 \pm 0.5$°C. ○, EYPA–β-lg no calcium; +, EYPA–β-lg 1 m*M* calcium in subphase; □, EYPC–β-lg no calcium.

trapolation of a log–log plot of absorption versus wavelength from above 320 nm, where the chromophoric bands of the protein exhibit no absorption (38). This procedure, although frequently used, should be viewed with some caution (39). The average light scattering was estimated to be 0.09 absorbance units or 26% of the total signal at 193 nm for films containing EYPA and 0.06 absorbance units or less than 15% of the total signal for pure protein films. Assuming that these estimates of light scattering are reasonable, the concentration flattening quotient of Duysens (Fig. 1 of Ref. (40)) should approach unity, and, consequently, the correction for scattering artifacts (Eq. [1] of Ref. (41)) should be less than 15%. The differences among curves in Fig. 3 should thus not be due to scattering artifacts. The film concentrations were determined from the

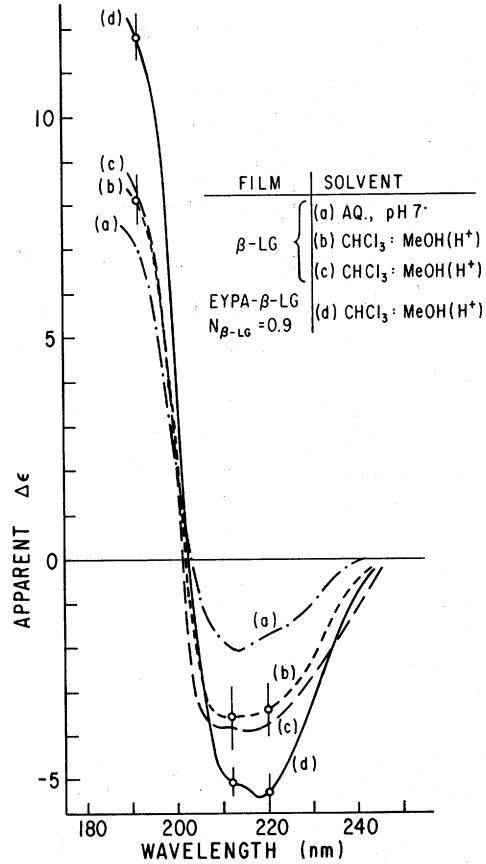

FIG. 3. Circular dichroism of protein and lipid–protein films. Monolayers (b, d) transferred from pH 4 subphases at pressures of 25 to 30 mN/m. Monolayer curves are averages of three runs. Vertical lines are ±1 standard deviation. Curves for cast films (a, c) are the results of single runs.

corrected UV spectra assuming a molar (residue) absorptivity of 9600 L/mole cm for β-lg as calculated from the results of McDiarmid obtained with aqueous solutions (20) and confirmed in the author's laboratory. This procedure ignores solvent and orientation effects, hence, the results are presented as apparent Δε versus wavelength to indicate that the strengths of the CD transitions are approximate.

DISCUSSION

A. Pressure–Area Curves

Preliminary work with a manual film balance indicated that EYPA interacted with β-lg on pH 4 but not pH 6 subphases in experiments where the components were mixed together in acidic chloroform–methanol prior to spreading. Results similar to those shown in Figs. 1 to 2 were obtained with about 10% deviation from ideality noted for a film containing 80 to 90% (residue) β-lg on pH 4 subphase (D. Cornell, unpublished observation). Payens observed a similar pH-dependent interaction between the phosphatides of milk and β-lg, where the components were spread separately, then mixed and compressed on a manual balance (12). These experiments, taken together with the results of the current work, suggest that the observations are best explained by some form of lipid–protein interaction rather than spreading or compression artifacts.

The isoionic point of β-lg is 5.3 at 0.1 ionic strength (18), and pK_1 for EYPA monolayers was determined to be 3.5 at high film pressures (7). Thus, at pH 4, the β-lg would carry a positive charge and EYPA a negative charge, and electrostatic attraction between the lipid and protein components of a mixed monolayer would be expected. The area occupied by each individual molecule or residue would be less in such a mixed film than in films of the pure components where the like charges would cause mutual repulsion of the molecules. The mixed film would exhibit a total area smaller than that expected based on the area of the pure components calculated from relation [1], as was shown in Figs. 1 and 2.

Calcium binds to both PA (26) and β-lg (42, 43) although the results reported suggest that at acid pH and 1 m*M* concentration the amount of Ca^{2+} bound to the protein is probably negligible (42, 43). The ideal behavior shown in Fig. 2b for EYPA–β-lg films on pH 4 subphases containing 1 m*M* calcium could thus be postulated as binding between calcium and phosphatidic acid with the resultant neutral moiety exhibiting no attraction for β-lg.

As noted in Fig. 2, EYPA–β-lg films exhibited nonideal behavior on pH 1.3 sub-

phases. This requires some explanation since the apparent pK_1 of phosphatidic acid was found to be 3.5 in films (7) and aqueous dispersions (27) and one would expect EYPA to be unionized and not exhibit attraction to β-lg at pH 1.3. The acid–base behavior was determined on films and dispersions of pure lipid where the head groups were close together (7, 27), and it is known that under such circumstances the apparent p*K*'s can be shifted by as much as 4 to 5 units toward weakness, i.e., higher in the case of acids (44, 45). Films of palmitic acid at pressures above 9 mN/m were found to have apparent p*K*'s of 8.6 to 9.9 compared to 4.7 in dilute aqueous solution, for example (45). In lipid–protein films which exhibit nonideal behavior, the components must be at least partially miscible (36) and the lipid molecules should be at least partially surrounded by protein residues. The positively charged protein residues would shield the lipid molecules from one another and the acid–base behavior of the individual phosphatidic acid units might be more nearly that of a relatively dilute solution rather than a pure film where the head groups are in intimate contact. For the case of miscible lipid–protein films, the soluble compound glycerol 2-phosphoric acid with pK_1 of 1.3 (46) in solution is probably a better model for the acid–base behavior of the lipid moiety rather than systems containing pure acidic phospholipids. If this is so, then one would expect partial ionization of EYPA in a mixed film and electrostatic attraction for the positively charged β-lg residues, giving rise to the nonideal behavior exhibited in Fig. 2c.

The above discussion illustrates one possible difficulty in the use of the additivity law, Eq. [1], in determining interactions in mixed films, namely, the assumption that the states of the molecules in a mixed film are the same as the states found in pure films of the individual components. This may not always be so. Clearly, however, the results presented in Figs. 2b and c represent something other than ideal mixing.

Mixed films of EYPA and β-lg on pH 1.3 subphases containing 1 m*M* calcium also exhibited nonideal behavior, in contrast to the ideal behavior observed at pH 4 with calcium present. Apparently, under very acid conditions, calcium does not bind strongly enough to EYPA to interfere with the lipid–protein interaction.

The question arises: How much of the observed interactions can be attributed solely to the ionized groups of the lipid and protein, how much to the hydrocarbon tails and side groups, and how much to other factors such as geometric perturbations in the mixed films? The ideal behavior noted for EYPA–β-lg films on pH 6 subphase and pH 4 subphase containing calcium would appear to argue for only electrostatic effects, but an additional test seemed desirable. Phosphatidylcholines are zwitterionic species isoelectric at pH 5.5 (8), and would be expected to exhibit very little net charge over a large portion of the pH scale. If only electrostatic effects are responsible for deviations from Eq. [1], then films of EYPC and β-lg should exhibit ideal behavior on subphases with pH above and below 5.3 where β-lg is isoionic. This is in fact what is observed as shown in Figs. 2a to c. This suggests that EYPC and β-lg were either ideally miscible or that any interaction between the two was too weak to prevent phase separation. It is not possible to distinguish between ideal mixing and phase separation in EYPC–β-lg monolayers based on the information in Fig. 2, however, the deviation from ideality for EYPA–β-lg films shown in Figs. 2b and c means that these two components were at least partially miscible. The interaction between EYPA and β-lg at pH 4 and 1.3 was thus strong enough to overcome any attractive forces among the hydrocarbon tails of the EYPA molecules. All the observations reported here also argue against geometrical considerations such as the molecular cavity model (47) as explanations of the nonideal behavior.

The results reported here suggest that EYPA and β-lg interact electrostatically in monolayer films on low pH subphases with

little, if any, contribution from the hydrocarbon tails of the lipid. This conclusion is supported by results obtained with mixed films of EYPA and β-lg on subphases with pH both above and below the isoelectric point of β-lg, and by results obtained with mixed films of EYPC and β-lg. Calcium ions at 1 m*M* in the subphase interfere with the electrostatic interactions at pH 4, but have no discernible effect on EYPA–β-lg films on very acid subphases.

Payens observed an interaction between β-lg and the phosphatides of milk at the air–water interface and attributed part of the nonideal behavior to other than electrostatic factors (12). The arguments were based on a comparison between experiment and the results of calculations using the Guoy–Chapman equation. This procedure has been questioned for low area, i.e., high pressure, films at the air–water interface since the Guoy–Chapman equation neglects nonpolar intermolecular forces (44). In this connection, it is interesting to note that good agreement between experiment and theory was obtained with phospholipids at an oil–water interface where intermolecular forces within the film are much reduced (48). Thus, electrostatic factors alone were sufficient to account for the results obtained (48). It does not seem necessary to invoke anything other than electrostatic phenomena to explain the observations in the current work.

B. Circular Dichroism of Films

The strength of the CD transitions found in the spectra of films are not necessarily the same as those occurring in the spectra of solutions, although the differences do not appear to be large for the α helical and β structures (31, 51). The differences may be due in part to mounting of the sample in a plane and to quadrupole terms which can contribute to CD in oriented systems (52). The strong similarity between the CD of films and solutions (31, 51), however, permits a qualitative discussion of the spectra in Fig. 3 in terms of familiar structures.

The main conclusion to be drawn from the CD spectra of β-lg films shown in Fig. 3 is that monolayers of the protein have more regular structure, α helix and β sheet, and less of the unordered conformation than the native molecule in aqueous solution. Circular dichroism (22) and optical rotatory dispersion (23) measurements indicate that native β-lg in aqueous solution consists of a small amount of α helix, almost 50% unordered structure, and the remainder β sheet. The CD spectrum of a film of β-lg cast from aqueous solution as shown in Fig. 3a is almost identical above 200 nm to the spectra of solutions of genetic variant A (22). This suggests that casting from aqueous solution causes little change in the conformation of native β-lg.

The spectra of monolayers and films cast from acidic chloroform–methanol solvent shown in Figs. 3b to d exhibit positive CD transitions below 200 nm and negative transitions above 200 nm, which are more intense than is the case with the spectra of aqueous solutions of β-lg (22). This suggests that these films have a higher content of the more ordered structures, either α helix or β sheet or both, than the native molecule. Previous work with β-lg has shown that acidic methanol solvent can cause a dramatic increase in α-helical content in solution (23). It has also been suggested that the α-helical structure is not affected by the spreading process in the formation of monolayer films (49). Evidence for an increase in the β structure in β-lg monolayers was obtained by infrared spectroscopy (50). One might speculate from this that the acidic chloroform–methanol solvent caused an increase in the α-helical content of β-lg, which was stable toward the spreading process with β sheet formed in the monolayer. The spectra in Fig. 3 are consistent with this picture. The protein in an EYPA–β-lg mixed film showed an additional increase in the β structure over that found in the pure β-lg films as indicated by the stronger negative transition at 218 nm in Fig. 3d. This is consistent with a picture of the nonhelical portion of the protein stretched

out as might occur upon interaction with the lipid.

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